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RuBisCO: a potential protein ingredient to enrich wheat-based food?
Understanding the impact of RuBisCO on wheat dough structure,
protein-protein interactions and protein *in vitro* digestibility

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La RuBisCO : un ingrédient protéique pour enrichir les aliments à base de blé ?

Etude de la structure, des interactions protéine-protéine et de la digestibilité in vitro des protéines de la pâte de blé enrichie en RuBisCO

Maude Ducrocq

*« Je suis de ceux qui pensent que la science est d'une grande beauté.
Un scientifique dans son laboratoire est non seulement un technicien :
il est aussi un enfant placé devant des phénomènes naturels qui
l'impressionnent comme des contes de fées. »*

Marie Skłodowska-Curie

Abstract

Dietary proteins from plants often display an unbalanced amino acid profile in comparison to animal proteins. A way to overcome this problem is to combine plant proteins from different sources with complementary amino acid compositions. RuBisCO is the main protein of leaf protein concentrates. Because of its high content in lysine, RuBisCO could improve the amino acid profile of wheat-based products. However, the introduction of non-wheat proteins in wheat-based foods often dilutes the gluten network or even impede its proper formation. This leads to poorer textural and culinary properties of the food. In this context, we have studied the possible consequences of RuBisCO enrichment on the quality of wheat-based products. The study was performed on a model system, a wheat dough. For comparison, enrichments with gluten and pea proteins were also studied. The main output of this work is that RuBisCO behaviour differs from that of legume proteins when introduced in dough. RuBisCO subunits establish weak bonds and form disulphide bridges when introduced in wheat dough. In addition, RuBisCO enrichment enhances the formation of disulphide bonded large size polymers during the dough thermal treatment. This may be related to its high content of cysteines and free thiols. This could prevent the negative consequences of gluten network weakening in wheat-based food. The enrichment of dough with RuBisCO modifies the kinetics of proteolysis during *in vitro* digestion. However, the global *in vitro* digestibility of proteins remains unchanged. These promising results confirm the potential of RuBisCO to be used for enriching wheat-based foods.

Résumé

Les protéines végétales ont généralement un profil d'acides aminés déséquilibré par rapport aux protéines animales. Une façon de pallier ce problème est de combiner des protéines végétales avec des compositions en acides aminés complémentaires. La RuBisCO est la principale protéine des concentrés de protéines de feuilles. En raison de sa teneur élevée en lysine, la RuBisCO pourrait améliorer le profil en acides aminés des produits à base de blé. Cependant, l'introduction de nouvelles protéines dans les aliments à base de blé dilue le réseau de gluten et peut empêcher son développement. Cela provoque la détérioration des propriétés texturales et culinaires de l'aliment. Dans ce contexte, nous avons étudié les conséquences de l'enrichissement en RuBisCO sur la qualité des produits à base de blé. L'étude a été réalisée sur un système modèle, une pâte à base de blé. À titre de comparaison, l'enrichissement de la pâte avec du gluten et des protéines de pois a également été étudié. Nous avons montré que le comportement de la RuBisCO diffère de celui des protéines de légumineuses lorsqu'elle est introduite dans la pâte. Les sous-unités de la RuBisCO forment des liaisons faibles et des ponts disulfures lorsqu'elles sont introduites dans la pâte de blé. De plus, la RuBisCO favorise la formation de polymères de grande taille liés par des ponts disulfures lors du traitement thermique de la pâte. Cela pourrait limiter les conséquences négatives d'un affaiblissement du réseau de gluten dans les véritables aliments à base de blé. L'enrichissement de la pâte avec de la RuBisCO modifie la cinétique de la protéolyse pendant la digestion *in vitro*. Cependant, la digestibilité globale des protéines *in vitro* reste inchangée. Ces résultats prometteurs confirment le potentiel de la RuBisCO à être utilisée pour enrichir les aliments à base de blé.

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Introduction

The word "protein" comes from the Greek word *proteios*, which means "primary" or "essential" because of their abundance in animal and plant tissues. The term was first used in 1839 by Gerardus Johannes Mulder, who suggested the idea that animals get most of their proteins from plants. Nowadays in most European countries, the main sources of dietary proteins for adults are meat and meat products, followed by grains and grain-based products, and milk/dairy products (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). These three food groups contribute to about 75% of our protein intake and altogether, more than 60% of the proteins we eat comes from animal sources. In addition, the average protein intake of adults in Europe often meets or exceeds the mean reference intake of 0.83 g per kg body weight per day (average intake of 0.8 to 1.25 g/kg) (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). This overconsumption of animal products raises environmental and public health issues (Aiking, 2014; Friel et al., 2009; John Reynolds, David Buckley, Weinstein, & Boland, 2014). Indeed, food of animal origin were shown to be the major source of greenhouse gases in the food system, and their relative importance is likely to increase in the future (Springmann, Godfray, Rayner, & Scarborough, 2016). Moreover, it has been shown that people following a vegan, vegetarian, pescatarian or semi-vegetarian diet had a lower global mortality risk than omnivores (Orlich et al., 2013). All of these concerns have led a group of experts to emphasize the urgent need for collective efforts to transform our diets and our food systems. They overall underline the need to shift to a diet richer in plant proteins (Willett et al., 2019).

In addition, Europe has been facing an important protein deficit for several years. The total EU protein crop production occupied only 3% of the EU arable land and supplies only 30% of the protein crops consumed as animal feed in the EU (European Parliament, 2011). This protein dependency originates in part from the trade agreements negotiated within the framework of the GATT (General Agreement on Tariffs and Trade) in the 1960s. In this context, American countries specialized in the production of plant proteins, while Europeans focused on cereal crops (Ministère de l'Agriculture, de l'agroalimentaire et de la forêt, 2014). Today, reducing the European protein deficit could have major economic benefits, leading European institutions to stress out the importance of increasing the production of plant proteins in Europe and diversifying their sources.

In this context, the GreenProtein project has received funding from the European Union's Horizon 2020 research and innovation program (grant agreement no. 720728). It aims at revalorizing agro-industrial green waste into functional protein ingredients with high added value intended to human consumption. Green agro-industrial waste represents an important amount of raw material, whose valorisation would result in an increased commercial value. The major objective of the GreenProtein project is to establish a DEMO plant for the extraction and purification of food grade fully functional leaf protein concentrate (LPC). It is expected to process 1500 kg of plant wastes per hour, leading to the extraction of 12 kg of protein per hour.

Leaf proteins constitute between 15 to 35% of the leaf dry mass (Aramrueang, Zicari, & Zhang, 2017; Yeoh & Wee, 1994) and are often classified according to their affinity with water. Water-insoluble proteins are mainly located in the membrane of plant organelles. They are commonly considered unsuitable for human consumption because of their green colour and strong astringency

due to their association with lipophilic pigments. In contrast, water-soluble proteins are referred to as "white proteins" and are composed of up to 50% of RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Fiorentini & Galoppini, 1983). RuBisCO is an enzyme of photosynthesis that converts inorganic CO₂ from the atmosphere into organic carbon used in the plant's metabolism (Weissbach, Horecker, & Hurwitz, 1956). RuBisCO is an enzyme with low efficiency that is balanced by its high level of expression in the growing leaf, which makes RuBisCO the most abundant protein on Earth (Kung, 1976). Besides its abundance, the interest of RuBisCO also aroused from its balanced amino acid profile. From a nutritional point of view, RuBisCO presents an excellent amino acid profile with a quantity of essential amino acids often higher than that of the ideal reference profiles (Barbeau & Kinsella, 1988; Gerloff, Lima, & Stahmann, 1965). In addition, *in vivo* and *in vitro* studies reveal that white LPC digestibility is usually higher than 95% (Fiorentini & Galoppini, 1983). RuBisCO also has interesting functional properties. The overall solubility of RuBisCO was estimated to be higher than that of other plant protein isolates and comparable to animal protein solubility, such as whey proteins (Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019). RuBisCO is also interesting for its gelling properties, with a low critical gel concentration (Martin, Nieuwland, & De Jong, 2014). The functionality of proteins in food matrices and their nutritional value are two necessary parameters to estimate their interest for human food. RuBisCO may be a good protein ingredient for human consumption. Although several extraction processes have been described (Edwards et al., 1975; Knuckles, Bickoff, & Kohler, 1972), RuBisCO or LPC are up to now not used as an ingredient in human food. This is related to the difficulty of extracting a functional white concentrate that meets the demands of industrials and consumers. However, advances in extraction process technology and recent studies on functionality are encouraging research on the use of LPC as an ingredient in human food (Firdaous et al., 2017; Hadidi, Khaksar, Pagan, & Ibarz, 2020; Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016).

The food processing industry has developed several strategies for the diversification of processed foods offering an alternative to animal proteins in Western countries. In recent years, there has been an increase in the variety of meat analogues and dairy substitutes on offer. Today, they are mainly soy or gluten based, although pea products are emerging (Schreuders et al., 2019). Another strategy is the partial replacement of animal proteins by plant proteins in animal protein-based products (Mession, Roustel, & Saurel, 2017; Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, 2019). However, the nutritional quality of plant proteins is not as good as that of animal proteins. They have, in particular, deficiencies in certain essential amino acids. A strategy to overcome this problem is to associate different plant proteins with complementary amino acid profiles. Grain-based products, and particularly wheat, are often used for these purposes because they are widely consumed and after meat, they represent the second source of protein intake for adults in Europe. Their deficiency in lysine can be counterbalanced by the addition of proteins with high lysine content, such as legume proteins. Similarly, RuBisCO has higher lysine content than wheat proteins (Gerloff et al., 1965), so its incorporation into wheat-based products should improve the amino acid profile in addition to increasing the protein content.

A major issue of protein enrichment of wheat-based products is the impairment of their organoleptic qualities. Enriching durum wheat pasta with legume flours generally increases cooking loss and modifies the textural properties of pasta (Laleg et al., 2017; Monnet et al., 2019). Similarly,

the addition of legume proteins to bread dough can affect bread volume by reducing its gas retention capacity (Boukid, Zannini, Carini, & Vittadini, 2019). These changes in quality usually result from modifications of wheat protein structure. Under the effect of hydration and energy input, wheat storage proteins are able to form a viscoelastic protein network, the gluten network. Under the effect of temperature, some of these proteins further polymerize via thiol/disulphide exchange, resulting in the formation of large polymers. The unique viscoelastic properties of the gluten network are responsible for the textural properties of wheat-based foods and are essential for their quality. The introduction of non-wheat protein often dilutes the gluten network or impedes its formation, leading to a deterioration of textural and culinary properties of the food. It is thus challenging to develop protein-enriched wheat-based foods with minimized or even no gluten impairment.

In view of using RuBisCO to fortify wheat-based foods, we have studied the possible consequences of RuBisCO enrichment on the quality of wheat-based products. For comparison, we also studied the effect of an enrichment with gluten and pea proteins. The low quantities of LPC available at the beginning of the PhD study did not allow the production of real food products. The study was performed on a model system, a wheat dough composed of semolina, water and protein concentrate. The objectives of this PhD were to assess the impact of RuBisCO introduction on the mechanical properties and protein structures of dough and on the *in vitro* digestibility of proteins. A detailed characterisation of the LPC was first carried out. Then, the characterisation of the mechanical properties of dough and of its protein structures was performed with a particular focus on the study of protein-protein interactions. Finally, the protein *in vitro* digestibility of the protein-enriched doughs was evaluated.

This manuscript will be divided into 6 chapters.

Chapter 1 is devoted to a state of the art. It describes the characteristics of LPC and RuBisCO, the mechanism of the gluten network formation during wheat-based food processing and the influence of protein enrichment on the techno-functional and nutritional properties of wheat-based foods.

Chapter 2 presents the scientific strategy adopted throughout this thesis and presents experimental details concerning the choice of the raw materials and the dough fabrication.

Chapters 3 to 5 presents the experimental results and are written in the form of scientific publications. Chapter 3 provides a detailed characterisation of the LPC and its biochemical and physical-chemical properties. Chapter 4 deals with the structural modifications resulting from RuBisCO enrichment of doughs. Chapter 5 presents the modifications related to the *in vitro* digestibility of proteins.

Finally, Chapter 6 consists of a general discussion of the results. The manuscript will be concluded with a general conclusion and the prospects for future work.

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Chapter 1. State of the art

This literature review is divided into three parts, which purposes are to explain:

- * What are the nutritional and technological properties of RuBisCO?
- * How wheat grain components, and especially wheat storage proteins, allow the structuring of wheat-based foods during their processing ?
- * How protein enrichment of wheat-based foods can modify their protein structure and what are the consequences on their quality ?

This bibliographic chapter will also report a non-exhaustive list of methods used for the study of protein structures in food matrices and for the evaluation of their protein *in vitro* digestibility.

Part 1. RuBisCO, a potential food ingredient for human nutrition

1 Discovery and characteristics of the most abundant protein on Earth: RuBisCO

1.1 *The discoveries of Ribulose-1,5-bisphosphate carboxylase/oxygenase*

Numerous reviews relate the history of RuBisCO discoveries (Erb & Zarzycki, 2018b; Ogren, 2003; Portis & Parry, 2007; Wildman, 2002). They also tell of the enthusiasm of researchers who have studied it, often dedicating their entire careers to it. RuBisCO was first isolated from spinach leaves in 1947 by Sam Wildman and James Bonner while they were working on auxin, a type of plant hormones. The protein fraction containing RuBisCO precipitated at 35% saturation in ammonium sulphate. At that time, they did not know the biological role of RuBisCO, they, therefore, called it "fraction I protein" as opposed to "fraction II" which does not precipitate under these conditions (Wildman, 2002). In the 1950s, Melvin Calvin and Andrew A. Benson identified a carboxylation enzyme involved in photosynthesis mechanisms that they called the "carboxydismutase". Shortly afterward, Horecker's group purified the same enzyme, which they rather called "carboxylation enzyme" (Portis & Parry, 2007). The first demonstration that the Fraction I protein and the photosynthesis carboxylation enzyme were the same protein only occurred in 1965 (Trown, 1965). The multiple discoveries of RuBisCO and the progress of research on its biological functions have led to controversy over the nomination of the protein. The full name of Ribulose-1,5-bisphosphate carboxylase/oxygenase was not used until 1971, the year of the discovery of its oxygenase activity (Bowes, Ogren, & Hageman, 1971). The term "RuBisCO" was first used in 1979 by Eisenberg at a seminar in honour of the career of Sam Wildman. "RuBisCO" is now widely employed. The International Union of Biochemistry reports more than 14 different names for RuBisCO today (<https://www.enzyme-database.org/index.php>).

1.2 *RuBisCO, a poorly effective but very abundant catalyst*

RuBisCO is an enzyme of the Calvin-Benson-Bassham pathway that acts in the first step of photosynthesis. **Figure 1** shows, in a simplified manner, the two enzymatic activities associated with RuBisCO. Its biological role is to sequester inorganic CO₂ of the atmosphere into organic carbon of the biosphere. It catalyses the carboxylation of ribulose-1,5-diphosphate (RuBP) and yields to two molecules of 3-phosphoglyceric acid (3PG) using atmospheric CO₂ and water (Weissbach, Horecker, & Hurwitz, 1956). RuBisCO also catalyses an oxidation side-reaction of RuBP with atmospheric O₂ into two molecules of 2-phosphoglycerate (2PG) and one molecule of 3PG (Bowes et al., 1971). This oxygenase activity is considered as a non-productive reaction because the 2PG is a toxic compound for the plant and is recycled during an energy-demanding process called respiration. Oxygenase activity can account for 20 to 40% of its total activity according to the environmental conditions (reviewed in Erb & Zarzycki, 2018).

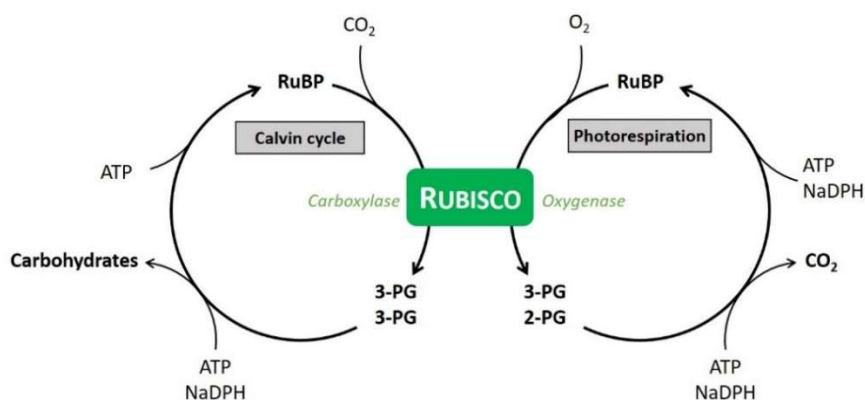


Figure 1. Simplified diagram of the two enzymatic activities associated with RuBisCO: Carboxylase and oxygenase activity in the Calvin cycle and photorespiration process respectively.

The efficiency of RuBisCO may be assessed through its turnover number for its carboxylase activity. It corresponds to the maximum number of chemical conversions of substrate molecules per second for a single catalytic site. It has been reported to be about 4 s^{-1} for a RuBisCO from spinach (Tcherkez, Farquhar, & Andrews, 2006). By comparison, it is 292 s^{-1} for phosphoglycolate phosphatase, a chloroplastic enzyme involved in photorespiration (Y. Kim et al., 2009). Research efforts are dedicated to the improvement of RuBisCO efficiency through genetic approaches (Martin-Avila et al., 2020; Sharwood, 2017). However, simulations suggested that RuBisCO catalytic rate is negatively correlated with its CO_2/O_2 specificity ratio (Tcherkez et al., 2006). Increasing its catalytic rate would thus decrease its affinity for CO_2 and vice-versa. Some researchers compare the evolutionary history of RuBisCO to the concept of Pareto optimality. This concept is used to describe a state of society in which one cannot improve the well-being of one individual without deteriorating that of another (Erb & Zarzycki, 2018a; Shoval et al., 2012). In other words, genetic manipulations of RuBisCO would not increase its specificity without reducing its carboxylation rate. The balance of RuBisCO, between specificity and velocity, may be optimal as it is.

The low specificity for CO_2 as well as the low activity of RuBisCO is compensated by its high level of expression in the growing leaf. RuBisCO concentration in the stromal compartment of chloroplasts can reach 300 mg.mL^{-1} . The enzyme represents up to 30-50% of the soluble proteins in leaves and estimations report that there would be constantly of 5kg of RuBisCO per human (Phillips & Milo, 2009). Altogether, this makes RuBisCO being claimed to be the most abundant protein on Earth (Ellis, 1979; Kung, 1976).

The important role of RuBisCO in the development of biomass makes it an active field of research in Biology. One of the objectives of modern biology is to overcome the limitations of RuBisCO by a transgenic approach to increase photosynthesis (Parry, Madgwick, Carvalho, & Andralojc, 2007) and biomass production.

1.3 Three-dimensional structure of native RuBisCO

There are several forms of RuBisCO depending on the organism (Spreitzer & Salvucci, 2002). The following sections only deal with form I RuBisCO since it is the form found in all higher plants.

Form I RuBisCO is one of the world largest enzymes with a molecular weight of about 560 kDa. It is composed of two different types of subunits, a large and a small one (Rutner & Lane, 1967). The large

chain (LC) has a molecular weight of about 55 kDa and the smaller chain (SC) has a molecular weight of about 15 kDa. RuBisCO is composed of 8 large chains forming 4 dimers (LC dimer) (Baker, Eisenberg, Eiserling, & Weissman, 1975). The two large chains of each dimer have a head-to-tail conformation and they are capped at each end by two small chains (**Figure 2A**). These groups composed by two large chains and two small chain (L2S2 unit) are arranged around a four-fold axis (**Figure 2B**), forming the hexadecameric structure of RuBisCO (reviewed in Schneider et al., 1990).

Electrophoresis patterns in denaturing conditions of alfalfa RuBisCO in presence or absence of a reducing agent were shown to be similar, suggesting that no intermolecular disulphide bridges are involved in its quaternary structure (Hood, Cheng, Koch, & Brunner, 1981). However, the structure of RuBisCO is sensitive to *in vivo* oxidative stress, resulting in intermolecular cross-links of LC by disulphide bonds (Mehta, Fawcett, Porath, & Mattoo, 1992). *In vitro*, the LC also tends to form LC dimers cross-linked with disulphide bridges after disruption of the protein (Rintamaki, 1989). The authors hypothesized that this susceptibility to cross-link by disulphide bridges may be due to the redox state of the well-conserved Cys-247 residue of the LC.

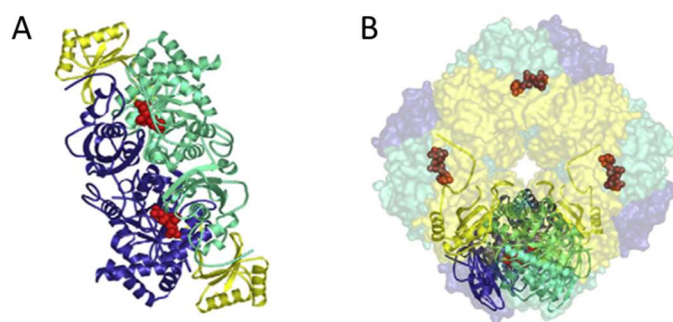


Figure 2. Spinach RuBisCO's quaternary structure. **A:** L2S2 unit of spinach RuBisCO. **B:** The hexadecamer viewed along the 4-fold axis. Large subunits are blue and green, small subunits are yellow and active sites are red. Extracted from Andersson & Backlund (2008).

The functional unit of RuBisCO is the LC dimer that contains two active sites (Andersson et al., 1989), as shown on **Figure 2A**. The folding of one LC forms the active site and residues from the adjacent LC complete this active site. The position of SC at each end of the LC dimers is expected to be involved in the assembly of the catalytic units (Schneider et al., 1990). It was also assumed that it would have a role in the specificity of the LC catalytic action (Schneider et al., 1990). More recently, the creation of chimeric SC resulted in the alteration of the catalytic properties of the enzyme without affecting the structure of the active site (Karkehabadi et al., 2005). These data have led to numerous studies on the dynamics of the interfaces between the different subunits, their role in the enzyme activity and the potential of these interfaces for genetic improvement of RuBisCO (Van Lun, Van Der Spoel, & Andersson, 2011).

1.4 The primary structure of RuBisCO and its related physical-chemical properties

Primary structure of RuBisCO and its subunits

Apart from scientific articles, several sequences of RuBisCO are reported in public deposits such as UniprotKB. The sequences of RuBisCO subunits from lettuce (*lactuca sativa*; accession numbers of LC and SC: P48706 and Q40250), tobacco leaf (*nicotiana tabacum*; accession numbers of LC and SC:

P00876 and P69249) and spinach (*spinacia oleracea*; accession numbers of LC and SC: P00875 and P00870) were extracted and analysed for the purpose of this chapter. UniProtKB Align software has been used to compare the primary structures of RuBisCO subunits. The LC of lettuce, tobacco and spinach have 475, 475 and 473 amino acids respectively with 91.4% homology in their sequences. In comparison, the SC have 124, 123 and 123 amino acids (excluding the sequence of chloroplastic precursors) with 62.6% sequence homology. The primary structure of the LC is, therefore, more preserved than that of the SC. The secondary structure of the LC was also shown to be well preserved (reviewed in Andersson & Backlund, 2008). This may be related to the preservation of its catalytic activity along evolutionary time.

Despite differences in protein sequence, a study performed on 9 plant species highlighted that the overall amino acid composition of RuBisCO extracted from leaves were only slightly affected by the plant species and cultivation methods (Gerloff, Lima, & Stahmann, 1965). **Table 1** gives the amino acid profiles of lettuce, tobacco, and spinach RuBisCO as compared to pea, lentil, soybean, and durum wheat protein profiles. The whole RuBisCO sequences of lettuce, spinach and tabacco were obtained by adding 8 large chain sequences and 8 small chain sequences, excluding the precursors. The amino acid profiles were computed using the ProtParam Tool of the ExPASy server (<https://web.expasy.org/protparam>). The sequence homologies between species result in very similar amino acid profiles between the three RuBisCO. RuBisCO has a high content in sulphur amino acids (cysteine and methionine), aromatic amino acids (tyrosine and tryptophan), and aliphatic amino acids (alanine, leucine, isoleucine and valine) in comparison to others proteins. In contrast, RuBisCO has lower amounts of glutamic acid and serine. Its content in lysine is similar to those of pea, lentil and soybean proteins and much higher than that of wheat proteins.

Table 1. Amino acid composition in mg.g⁻¹ of protein for several RuBisCO as compared to other plant proteins. The amino acid profiles were obtained from: ¹ Protein sequences of UniProtKB; ² (FAO, 1970); ³(Abdel-Aal & Hucl, 2002). Note that the amino acid composition of plant proteins other than RuBisCO is not exactly for 1g of protein, probably due to tryptophan that was not assayed and/or standard deviations.

			RuBisCO ¹			Other plant proteins			
			Lettuce RuBisCO (Lactuca sativa)	Spinach RuBisCO (Spinacia oleracea)	Common tobacco RuBisCO (Nicotiana tabacum)	Pea (<i>Pisum</i>) ²	Lentil (<i>Lens culinaris</i>) ²	Soybean (<i>Glycine max</i>) ²	Durum wheat ³
A	Ala	Alanine	50	52	54	41	43	47	32
R	Arg	arginine	77	77	79	95	87	79	43
D	Asp	Aspartic acid	84	88	87	110	116	128	46
C	Cys	Cystein	18	15	18	11	9	15	21
E	Glu	Glutamic acid	117	112	123	161	166	205	337
G	Gly	Glycine	47	46	45	40	42	46	36
H	His	Histidine	37	33	31	23	27	28	23
I	Ile	Isoleucine	54	39	45	43	43	50	26
L	Leu	Leucine	84	88	86	68	76	85	70
K	Lys	Lysine	65	63	65	75	72	70	24
M	Met	Methionine	25	24	20	9	8	14	15
F	Phe	Phenylalanine	61	62	57	46	52	54	44
P	Pro	Proline	46	49	42	39	43	60	87
S	Ser	Serine	30	26	27	43	53	56	43
T	Thr	Threonine	54	59	51	41	40	42	30
W	Trp	Tryptophane	33	33	36	n.d.	n.d.	14	13
Y	Tyr	Tyrosine	61	66	68	27	33	34	29
V	Val	Valine	58	67	66	47	50	53	44

Isoelectric point, hydrophobicity and thermo-stability

The sequence homologies observed between RuBisCO from different sources may suggest similar physical-chemical properties. **Table 2** gives some physical-chemical parameters of lettuce, tobacco, and spinach RuBisCO calculated from the whole RuBisCO sequence using the ProtParam Tool of the ExPASy server (<https://web.expasy.org/protparam>). Their molecular weights are very close and range from 534.2 to 537.7 kg.mol⁻¹. The isoelectric point (pI) was calculated to be between 6 and 6.2 for the three RuBisCO. However, the isoelectric point of RuBisCO from spinach was measured to be between 5 and 5.5 and that of lettuce RuBisCO has been reported to be around 7 (reviewed in Douillard & de Mathan, 1994). These discrepancies between theoretical and experimental values may arise from charged residues buried in the quaternary structure of protein. Moreover, the isoelectric point of RuBisCO is not constant among different species. Using isoelectric focusing, the isoelectric point of maize RuBisCO was reported to be around 4.6 (Reger, Ku, Pottert, & Evans, 1983) while alfalfa one was reported to be close to 6 (Iwanij, Chua, & Siekevitz, 1974). Care should therefore be taken when comparing different RuBisCO for properties that depend on the protein charge.

Table 2. Physical-chemical parameters of RuBisCO from lettuce, tobacco and spinach calculated from their protein sequence using the ExPASy server.

	Lettuce (<i>lactuca sativa</i>)	Tobacco (<i>nicotiana tabacum</i>)	Spinach (<i>spinacia oleracea</i>)
Molecular weight (kg.mol⁻¹)	537.736	537.647	534.163
Isoelectric point	6.10	6.16	6.03
Mass extinction coefficient (L.g⁻¹.cm⁻¹)	1.547	1.696	1.600

RuBisCO has been reported to have a relatively high hydrophobicity of 1275 cal/residue, close to the β -casein hydrophobicity of 1298 cal/residue (Bigelow, 1967; Hood et al., 1981). Non-polar hydrophobic side chains are thought to be buried inside the protein, leaving polar residues exposed on the protein surface. Still, RuBisCO remains highly soluble at neutral pH (Bahr, Bourque, & Smith, 1977; Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019).

Extinction coefficient

RuBisCO from lettuce, tobacco and spinach have calculated extinction coefficients of 1.547, 1.696 and 1.600 L.g⁻¹.cm⁻¹, respectively. These values are similar to previously reported experimental data (Douillard & de Mathan, 1994).

Due to its important role in photosynthesis, RuBisCO is crucial for biomass production. It has a low specificity and a low activity compensated by a high rate of synthesis. It is the most abundant protein on earth.

RuBisCO is an enzyme of ~560 kg.mol⁻¹, formed of eight large chains and eight small chains linked by weak bonds. Its physical-chemical properties can vary among species but its amino acid composition is well preserved. Its amino acid profile is of nutritional interest compared to other plant proteins.

2 Isolation of RuBisCO / leaf protein concentrate

2.1 *Leaf protein extraction: discovery and history*

Leaf proteins were first described by Rouelle in 1773. Proteins constitute between 15 to 20% of the leaf dry mass and most of them (75%) are located in the chloroplast (reviewed in Syrine Kerfai, 2011). Leaf proteins are often classified according to their affinity with water. Water-insoluble proteins are mainly originated from the membrane of plant organelles and often associated with lipophilic pigments such as chlorophyll. This is why they are also called "green proteins" or "coloured proteins" (Thornber, 1975). Water-soluble proteins are mainly composed of chloroplastic and cytoplasmic enzymes and are made up about 65% RuBisCO. As opposed to water-insoluble proteins, they are often referred to as "white proteins".

Research on leaf proteins was stimulated in the early 1940s, when Norman Pirie discussed the potential of leaf protein concentrates (LPC) as a source of proteins for human consumption to overcome wartime food shortages (Pirie, 1942). The technology he developed aimed at extracting the maximum amount of protein from the leaves using heat precipitation, resulting in a green concentrate whose odour and taste made it unacceptable for human consumption. The first commercial LPC production was developed in 1967 on alfalfa leaves (reviewed in U.S. Congress, 1983) using a highly mechanized process, called the Pro-Xan process. The initial aim was to separate fibre from leaf proteins to obtain a fibre-rich press cake for ruminant feed and a green concentrate rich in protein and xanthophyll for monogastric and poultry feed (Knuckles, Spencer, Lazar, Bickoff, & Kohler, 1970). Once improved, the process allowed the fractionation of water-soluble and water-insoluble proteins, resulting in a green LPC with lower protein content and in a white protein concentrate suitable for human consumption (de Fremery et al., 1973; R. H. Edwards et al., 1975). Few years later, Leaf protein Inc. developed and patented a pilot plant scale process for extracting green and white LPC from the leaves of tobacco. The identification of RuBisCO, or "Fraction-I protein", as the major component of white LPC will be made in late 1970s.

Despite the broad literature existing on the extraction of leaf proteins, there are still not used in human nutrition at the opposite of other plant protein concentrates, such as soybean or pea proteins. A lot of studies attempted to improve the quality of products for animal feed (Tamayo Tenorio, Boom, & van der Goot, 2017; Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016), but we will further focus on the studies and advances that have been made on the extraction of the white protein concentrate. Although quantitative measurements of the RuBisCO content of white LPC are rarely made, qualitative testing (e.g. SDS-PAGE) validates the predominance of RuBisCO in these concentrates.

2.2 *Classical methodology for white protein concentrate production*

The objective of these processes is to separate the coloured and astringent pigments from the colourless and tasteless proteins. Also, several conditions must be satisfied: the LPC must be functional to be used in food formulation and the industrial-scale process must be economically viable. Conventional processes applicable only at the laboratory scale for leaf protein extraction, such as ammonium sulphate precipitation (Bahr et al., 1977; Paulsen & Lane, 1964), will, therefore, not be detailed in this section.

Separation of fibres and green juice by pressing

Most leaf protein fractionation processes are based on the same main steps, represented in **Figure 3**. First, a green juice is extracted from the leaves by pressing: under the effect of mechanical pressure, a high protein raw juice and a high fiber pressed cake (green residues) are obtained. The green juice contains about 10% dry matter and its protein content is around 17% in dry basis (Tamayo Tenorio et al., 2016). Techniques such as disintegration or preliminary crushing of leaves have also been used (Chayen, Smith, Tristram, Thirkell, & Webb, 1961; Knuckles et al., 1970). However, the use of a twin-screw press was found to be the most efficient mechanical technique for a low cost (Knuckles, Bickoff, & Kohler, 1972) and is still used in most articles dealing with LPC extraction (la Cour, Schjoerring, & Jørgensen, 2019; Santamaria-Fernandez, Ambye-Jensen, Damborg, & Lübeck, 2019; Tamayo Tenorio et al., 2017). Incorporation of sodium metabisulfite before leaves pressing is systematic because its antioxidant action prevents the binding of quinones to the sulfhydryl groups of proteins, thus preventing the formation of pigments/protein complexes that subsequently give a dark colour to the protein isolate (Edwards et al., 1975; reviewed in Knuckles, De Fremery, Bickoff, & Kohler, 1975). More recently, a thermally-assisted mechanical dewatering (TAMD) process was developed based on heat supply through the walls of the press to increase the yield and save energy (S. Kerfai, Fernández, Mathé, Alfenore, & Arlabosse, 2011).

Heat precipitation of the coloured protein material and recovery of the brown juice

The separation of the coloured proteins from the white proteins of the green juice is often based on their differential solubilities. A heating around 60°C leads to the precipitation of membrane proteins associated with chlorophyll, carotenoids and lipids. During this treatment, temperature is below the denaturation temperature of RuBisCO, thus preserving the solubility of the white protein fraction (de Fremery et al., 1973) and avoiding co-precipitation of the soluble proteins (Huang et al., 1971). Also, pH adjusting to 6 before heat treatment may be necessary to optimize the sedimentation level during centrifugal separation and reduce the foaming of the high-protein juice (R. H. Edwards et al., 1975). This stage of heat precipitation can be supplemented by filtration, centrifugation or additional heating/cooling cycles as in Pro-Xan process. Also, microbial coagulation methods have been investigated such as low-cost alternatives to thermic and chemical coagulation (Godessart, Pares, & Juarez, 1987).

The proteins, pigments and lipids that have been precipitated are then removed after centrifugation or membrane filtration. The use of cationic flocculants may improve the separation of chloroplastic material from alfalfa juice, using either centrifugation or membrane filtration, and even enhanced the soluble protein concentration in the permeate (Knuckles, Edwards, Kohler, & Whitney, 1980). More recently, lignosulfonates have been proved to improve the separation of pellet and supernatant in the juice (la Cour et al., 2019). The precipitated green fraction is rich in pigments, lipids and proteins and can be used for ruminant or monogastric feed (R. H. Edwards et al., 1975). The composition of the supernatant, called the brown juice, can vary according to the process. The term "brown juice" used in many papers (Firdaous et al., 2017; Knuckles et al., 1972; Miller, de Fremery, Bickoff, & Kohler, 1975; Prevot-D'Alvise et al., 2004; U.S. Congress, 1983) may be controversial since the colour of the juice depends on its oxidation rate. Using a plant-scale continuous centrifugation, Firdaous et al. (2017) obtained a brown juice with 6% dry matter and about 20% proteins (dry basis). Despite this interesting

protein composition, the product still had undesired organoleptic properties such as brown colour probably related to the presence of phenolic compounds (6.1%).

The use of polar solvents has been efficient to directly obtain white and tasteless protein isolates (Chayen et al., 1961; Huang et al., 1971) from the untreated green juice. However, in addition to be costly and hard to transpose to plant scale, this technique led to an insoluble and non-functional white protein concentrate.

Recovery of RuBisCO protein concentrate

The separation and concentration of white proteins from the brown juice can be achieved in several ways (Zhang et al., 2017). The classical methods of protein technology include thermal and acid precipitation. Thermal precipitation is achieved by heating the brown juice at 80°C, resulting in a light coloured isolate of about 88% of proteins (R. H. Edwards et al., 1975). The white LPC prepared by heat precipitation also shows good nutritional properties but it has very limited solubility and functional properties (reviewed in Knuckles & Kohler, 1982). Miller, de Fremery, Bickoff, & Kohler (1975) obtained a soluble protein fraction by precipitation at pH 3.5 and low temperature (2°C). The isolate contained about 70% protein and could be further dissolved at pH7 after freeze-drying (Miller et al., 1975). More recently, an enzymatic pathway was proven to be efficient in solubilise acid precipitated white proteins (Prevot-D'Alvise et al., 2004). Several processes, including the LPI inc. process, used crystallisation to recover crystals of RuBisCO (reviewed in Douillard & de Mathan, 1994). However, this process was developed only on tobacco leaf probably due to the specific properties of tobacco RuBisCO for crystallisation. Although very widely used, these purification techniques based on protein precipitation have a major drawback since the denaturation of the proteins often leads to the loss of functional properties.

Membrane technologies use the differences in molecular weight to separate components, avoiding a protein denaturation. Several studies used ultrafiltration (UF) to concentrate and purify white leaf proteins (Fernandez, Menendez, Mucciarelli, & Padilla, 2007; Koschuh et al., 2004), but they faced with the difficulty of the membrane fouling. Recent advances proposed several improvements to control flux decline during UF, yet tested at lab-scale (Zhang, Grimi, Jaffrin, & Ding, 2015). Also, an additional step of diafiltration was shown to be efficient at lab-scale in removing residual pigments after ultrafiltration (Knuckles et al., 1975) but this process was difficult to transpose to plant scale (Knuckles, Edwards, Miller, & Kohler, 1980) in the 80s. Techniques such as gel filtration or freeze-drying have also been shown to be effective on a laboratory scale but too costly to transpose to an industrial scale (Fishman & Burdick, 1977).

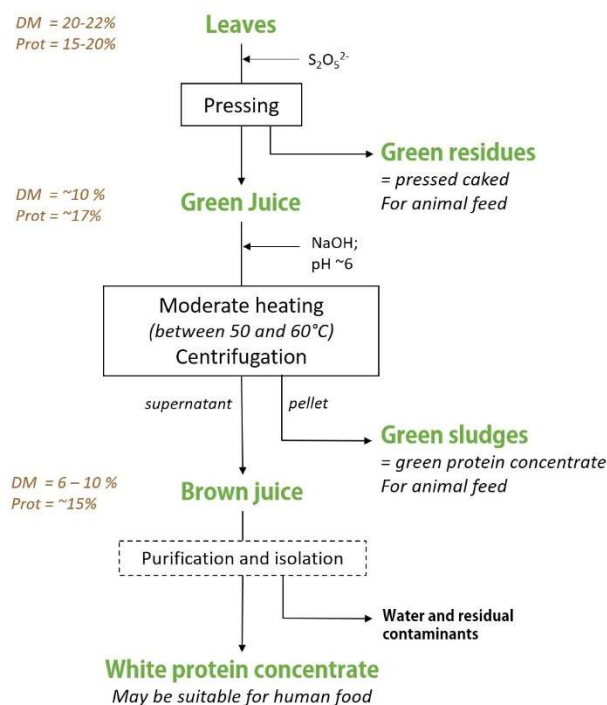


Figure 3. Classical steps of the fractionation of leaf proteins with associated dry matter (DM) and protein contents (Prot) found in the literature.

2.3 Phenolic compounds removal during LPC extraction

Phenolic compounds are secondary metabolites found in plants and foods of plant origin (Cheynier, 2012). Phenolic compounds include many molecules with different structures, such as phenolic acids, polyphenols or flavonoids. The green leaves are made up to 5% of phenolic compounds in dry mass (Andersen, Tso, & Chaplin, 1972) and phenolic compounds can be found in LPC. Depending on the process and the plant specie, chlorogenic acid (Lahiry, Satterlee, Hsu, & Wallace, 1977), soluble polyphenols, coumestrol (Rambourg, Luzerne, & Grignon, 1983) or flavonoids (Hernández, Hernández, & Martinez, 1991) can be co-extracted with leaves proteins. Phenolic compounds in LPC can be free or bound to proteins. The formation of protein-phenolic complexes is a major limiting factor for its use in food due to their poor organoleptic quality (brown colour, bitterness and astringency) and their potential anti-nutritional effects (D'alvise, Lesueur-Lambert, Fertin, Dhulster, & Guillochon, 2007; Hernández, Hernández, & Martinez, 1991). Several mechanisms of protein-phenolics interactions have been described (Le Bourvellec & Renard, 2012).

In neutral or alkaline environment, the oxidation of phenolic compounds by the polyphenol oxydase (PPO) results in the formation of ortho-quinones. These quinones are electrophilic and can react with nucleophiles. In this way, phenolic compounds can irreversibly bind to the primary and secondary amine groups and to the free thiols groups of proteins. Moreover, quinones can react with each other, resulting in the formation of high molecular weight brown polyphenols. The first stage of leaf grinding brings the proteins, phenolics and the PPO into contact in the green juice. From this step, oxidation reactions between proteins and phenolics can be avoided by the use of metabisulfite (Knuckles, Edwards, Miller, et al., 1980; Loomis, 1974; Valero, Varon, & Garcia-Carmona, 1992). Also, steam blanching may be used to inactivate polyphenol oxydase (PPO) and peroxidase (POD) both partly responsible for browning reactions (Lee, Tweed, & Sullivan, 2013).

In addition to covalent interactions, weak interactions may occur between phenolic compounds and amino acids. The stages of brown juice filtration (microfiltration, ultrafiltration, diafiltration) remove part of the free polyphenols. However, the LPC still contains phenolics compounds (Firdaous et al., 2017). Several studies have compared the effects of different resins, highlighting the importance of the compromise between polyphenol removal and protein loss on resins (D'alvise et al., 2007; Firdaous et al., 2017). The difficulties encountered for the elimination of phenolic compounds from LPC is related to the diversity of mechanisms involved in leaves protein-phenolics interactions. Similar to what has been done on canola proteins (Xu & Diosady, 2000, 2002), the search for effective means for the elimination of phenolic compounds would require an in-depth study of the interactions between LPC proteins and phenolic compounds.

Depending on the technique used for recovering soluble protein, the coagulum or the protein powder can have different compositions and properties. White LPC is still not used as an ingredient in human food because no extraction process has yet produced an ingredient of satisfactory quality with an economically viable process. The critical point of extraction is to separate the proteins from pigments such as chlorophyll or phenolic compounds (Johal, 1980) while keeping a functional concentrate.

The fractionation of water-soluble and water-insoluble proteins results in a green fraction with lower protein content and a white protein concentrate suitable for human consumption. RuBisCO is the major component of white LPC. The fractionation of leaf proteins consists first in a pressing of leaves to remove fibers. Then, a heat coagulation of membrane proteins linked with pigments allows recovering a clarified juice. Finally, the concentration of proteins can be performed by precipitation or by using ultrafiltration to avoid protein denaturation. The critical point of extraction is to separate the proteins from pigments such as chlorophyll or phenolic compounds, while keeping a concentrate with good functional and nutritional properties.

3 Nutritional and functional properties of leaf protein concentrates

3.1. Nutritional properties of leaf protein concentrates

Chemical score of essential amino acids of rubisco

Dietary proteins are composed of amino acids that are involved in the turnover of body proteins. Among them, the Essential Amino Acids (EAA) are not produced by the body and have to be provided by the diet. The composition of diet proteins in EAA is therefore an important criterion of their nutritional quality. The approaches recommended by FAO/WHO and ANSES (French Agency for Food, Environmental and Occupational Health & Safety) for the evaluation of the nutritional quality of a protein are partly based on the comparison of the composition in EAA of the dietary proteins with an ideal amino acid profile (AFSSA, 2007). The chemical score (CS) of an amino acid corresponds to its content in the protein as a percentage of its content in the reference protein. When its CS is below 100, the AA is deficient in the protein.

Early investigations of the amino acid profile of leaf protein concentrates suggested that these concentrates could be of high biological value ; this criterion has been a major driving force in the research related to their extraction and characterisation (Pirie, 1942). **Table 3** shows the chemical

scores of EAA from several RuBisCO and other dietary proteins using the AFSSA ideal amino acid profile as a reference. Lettuce, spinach and tobacco RuBisCO have chemical scores higher than 100 for all EAA, meaning that this protein has no deficiency in EAA. The amino acid profile of RuBisCO seems not to be affected by the species (reviewed in Gerloff et al., 1965), RuBisCO would thus have a balanced amino acid profile, regardless of the species from which it is extracted. Animal proteins such as milk casein or egg proteins and soybean proteins also present a balanced amino acid profile with every chemical scores higher than 100. In contrast, durum wheat proteins show a high deficiency in Lysine with a chemical score of 53. Pea and lentils proteins display deficiencies in sulphur amino acids, methionine and cysteine, with chemical scores of 88 and 75 respectively. Wheat and legume proteins are often used in combination in wheat-based foods to balance the amino acid profile of the food (Bahnassey, Khan, & Harrold, 1986; Boukid, Zannini, Carini, & Vittadini, 2019; Carini, Curti, Spotti, & Vittadini, 2012; Monnet, Laleg, Michon, & Micard, 2019). RuBisCO chemical scores suggest that it could be used to enrich wheat-based foods to compensate for their lack of lysine, in the same way as other animal and plant proteins.

Table 3. Chemical scores of several RuBisCO as compared to other plant and animal proteins. Chemical scores were calculated using the ideal amino acid profile recommended by AFSSA (2007) for adult people. The amino acid profiles used for calculation were obtained from ¹: Protein sequences of UniProtKB, accession numbers are given in table 1; ²: (FAO, 1970); ³: (Abdel-Aal & Hucl, 2002).

	RuBisCO ¹			Other plant proteins				Animal proteins ²	
	Lettuce RuBisCO (<i>Lactuca sativa</i>)	Spinach RuBisCO (<i>Spinacia oleracea</i>)	Common tobacco RuBisCO (<i>Nicotiana tabacum</i>)	Pea (<i>Pisum</i>) ²	Lentil (<i>Lens culinaris</i>) ²	Soybean (<i>Glycine max</i>) ²	Durum wheat ³	Milk Casein	Whole egg
Methionine +Cysteine	190	170	165	88	75	123	157	137	251
Histidine	216	193	180	134	161	163	135	171	143
Isoleucine	200	144	168	158	160	184	96	200	232
Leucine	143	149	146	115	129	144	119	161	149
Lysine	144	141	144	167	160	155	53	180	155
Phenylalanine + tyrosine	298	311	305	179	207	216	178	269	241
Threonine	217	236	205	162	159	169	120	186	205
Tryptophane	554	558	600	n.d	n.d	233	217	n.d	n.d
Valine	230	267	266	188	200	210	176	269	273

Digestive and metabolic bioavailability of RuBisCO

Besides its EAA composition, the nutritional value of a protein also depends on how it is hydrolysed, assimilated and used by the body. RuBisCO proteolysis rate was compared *in vitro* with casein and bovine serum albumin (BSA) using sheep rumen fluid (Nugent & Mangan, 1981). It was reported that casein had the highest hydrolysis rate, followed by RuBisCO. Using a pH-stat method, Barbeau & Kinsella (1985) showed that pancreatin hydrolysis of RuBisCO was lower than that of casein and BSA but higher than that of soy protein.

A biological evaluation of the nutritive value of crystalline RuBisCO was performed *in vivo* on rats by Ershoff, Wildman & Kwanyuen (1978). They used the protein efficiency ratio corresponding to the ratio between the average weight gain of the rat on the amount of protein ingested by the rat over 28 days. They found that the average weight increment and the PER of rats fed with diet containing RuBisCO

were greater than that of rats fed with diet containing casein. Also, RuBisCO displayed a protein efficiency ratio (3.0) slightly higher than that of casein (2.8). Other plant proteins were shown to have lower PER values than RuBisCO (soybean 2.57 and wheat 1.59) (Di Stefano, Agyei, Njoku, & Udenigwe, 2018). The efficiency of the use of RuBisCO in relation to a growth parameter seems to be good, even better than that of other proteins. However, care must be taken when comparing these values as reproducibility of such experiments is limited between laboratories (AFSSA, 2007). Indeed, the results obtained depend on the quantities of protein consumed by the rats ; the smell and taste of the food are sources of variations that are difficult to control.

The biological value of a protein relates to its metabolic bioavailability. The metabolic bioavailability of proteins and amino acids refers to their ability to be used in the oxidative and anabolic metabolic pathways. Carlsson & Hanczakowski, (1989) measured the biological value of leaf protein concentrates mixed with soya bean meal, wheat gluten, bone-meat meal, fish meal, skim milk and whey. They showed that the biological value of the mixture was higher than that of each component, except for fish meal.

Biological activities of RuBisCO and of its derived peptides

Biological activities of RuBisCO and of peptides derived from its hydrolysis by digestive enzymes have been widely reviewed in Udenigwe et al. (2017). In early 2000s, several studies highlighted the antihypertensive properties of several peptides from alfalfa and spinach RuBisCO. These peptides inhibits an enzyme (angiotensin I-converting enzyme) which catalyse the synthesis of angiotensin II, responsible for an increase in blood pressure. This peptide is formed during RuBisCO hydrolysis with protease, and could therefore be released during RuBisCO containing food digestion.

Impact of phenolic-protein interactions on the nutritional properties of LPC

The co-extraction of polyphenols with RuBisCO may affect its nutritional properties. The free amine groups of the lysine, the methionine residues, the free thiols of the cysteine residues and the indol rings of the tryptophan residues are the nucleophilic side chains involved in the covalent protein-phenolics interactions (Le Bourvellec & Renard, 2012). The decrease in the bioavailability of these EAAs has been reported on several dietary proteins such as soy proteins (Rawel, Czajka, Rohn, & Kroll, 2002) or casein (Hurrell, Finot, & Cuq, 1982) following their interaction with phenolics. In addition, several studies has highlighted that phenolics could bind to digestives enzymes, inhibiting their activities (reviewed in Ozdal, Capanoglu, & Altay, 2013). A model system study has shown that chlorogenic acid binds to RuBisCO via both weak and covalent bonds (Barbeau & Kinsella, 1983). They showed that non-covalent interactions induces a change in the conformation of RuBisCO. This leads to an increase in the rate of protein hydrolysis by trypsin. In contrast, covalent interactions decrease the rate of hydrolysis of RuBisCO by trypsin. Moreover, this decrease of digestibility was positively correlated with the decrease of available lysine content. Trypsin prefentially splits peptides bonds that contains lysine or arginine (Olsen, Ong, & Mann, 2004). It has therefore been suggested that the decrease in the rate of intestinal hydrolysis was linked to the decrease in the availability of lysine (Barbeau & Kinsella, 1985).

3.2. Functional Properties of leaf protein concentrates

Although proteins provide EAA in the diet, dietary proteins are also widely used for their functional properties such as foaming, gelling, or emulsifying properties (Foegeding & Davis, 2011).

Solubility

Protein solubility and hydration properties of a protein powder are fundamental for other functionalities of food protein such as foaming, emulsification, and gelation. Unless otherwise specified, solubility refers here to the amount of protein or protein nitrogen solubilised in regards to the total protein content of the powder used.

The solubility of RuBisCO has been shown to depend on plant species. RuBisCO extracted from spinach, cotton, and maize has higher solubility than tobacco RuBisCO in the same buffering conditions (Bahr et al., 1977). Spinach RuBisCO was found 10 times more soluble than other species in the range of temperature and pH tested (pH 5.6 to 8.4, at 4°C and 37°C). The solubility of sugar-beet leaves RuBisCO is represented on **Figure 4** as a function of pH and compare to soy proteins (SP) and whey protein isolate (WP). The drop of solubility around the isoelectric point is much more pronounced for soybean and RuBisCO as compared to whey protein isolate (WPI). The ionic strength effect on RuBisCO solubility depends on the type of ions used since some cations such as Mg^{2+} , Fe^{2+} and Ca^{2+} are ligands of RuBisCO and their binding can thus induce conformational changes of the protein. For instance, tobacco RuBisCO crystallize at low ionic strength only when treated with $MgCl_2$ (Bahr et al., 1977).

Knuckles & Kohler (1982) studied the influence of the alfalfa RuBisCO extraction process on protein solubility profiles. They showed that an increase in the spray drying outlet temperature from 85°C to 95°C decreased nitrogen solubility at pH 3 by more than 75%. In addition, heat coagulation of RuBisCO during extraction highly decreases its solubility as compared to a purification using membrane technologies (ultrafiltration and diafiltration) and freeze drying. Similarly, it has been shown that acetone or acid precipitations of RuBisCO decrease its solubility in major pH range as compared to ultrafiltrated LPC (Lamsal, Koegel, & Gunasekaran, 2007; Zhong et al., 2012). This decrease in solubility is explained by the denaturation of RuBisCO during extraction. Despite the effect of extraction process, the solubility of tobacco RuBisCO prepared in three different ways has been proved to be higher than the solubility of three commercial soy protein isolate (SPI) in most pH ranges (Sheen & Sheen, 1985).

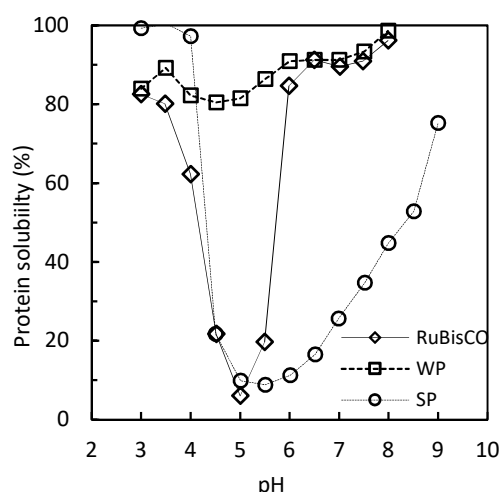


Figure 4. RuBisCO (diamonds), whey protein (squares) and soy protein (circles) solubility at 10 g.kg⁻¹ protein dispersion as a function of pH at 25°C. Adapted from (Martin et al., 2019).

Gelling & thermal denaturation

The gelling properties of a protein depend on its ability to form a gel and on the physical properties of the gel. The functionality of proteins for gelation relies on the conditions to form a gel, such as concentration and physical chemical conditions; and on the ability of the protein to form protein-protein interaction and to interact with other components of the system (Foegeding & Davis, 2011). Thermal gelation is the most studied technique of gel formation for RuBisCO. RuBisCO isolated from different sources displays slight different denaturation temperatures (T_d) at pH above 7, with alfalfa RuBisCO showing denaturation temperature of 66.5°C and 67.5°C (Béghin et al., 1993; Libouga, Aguié-Béghin, & Douillard, 1996) and spinach RuBisCO showing a T_d of 64.9°C (Martin, Nieuwland, & De Jong, 2014). These denaturation temperatures are relatively low as compared to other plant proteins. For instance, soy proteins have two major peaks around 73°C and 90°C (Renkema, Lakemond, De Jongh, Gruppen, & Van Vliet, 2000), pea proteins have an endothermic peak at 82°C (Mession, Sok, Assifaoui, & Saurel, 2013).

The critical gel concentration (C_{gel}) corresponds to the minimal protein concentration for the gel formation. It has been shown that RuBisCO C_{gel} also varies according to RuBisCO species. At pH 7, Libouga et al. (1996) measured a C_{gel} at 2 g.L⁻¹ for a alfalfa RuBisCO and Martin et al. (2019) measured a C_{gel} of 1 g.L⁻¹ for sugar beet leaves RuBisCO. At same pH, spinach RuBisCO displays a much higher C_{gel} of about 25 g.L⁻¹ (Martin et al., 2014). The authors associated these discrepancies to the differences in isoelectric points of these three species. Isoelectric point of RuBisCO from spinach is around 5 whereas isoelectric point of sugar beet leaves or alfalfa were reported around 6. Therefore, at neutral pH, spinach RuBisCO has a higher net charge than alfalfa RuBisCO, resulting in enhanced electrostatic repulsion and a higher C_{gel} . The increase of RuBisCO net charge with the pH increase leads to the strengthening of repulsions between molecules. Besides these variations, the values of C_{gel} are generally lower than other animal or plant food proteins (Martin et al., 2014).

Figure 5 shows the evolution of the storage modulus of protein gel as a function of protein concentration for spinach RuBisCO, whey, egg white, soy, lupin and pea protein isolates (Martin et al., 2019, 2014). The gelation of spinach RuBisCO as a function of time was followed during a heating-cooling cycle using small deformation rheology (Martin et al., 2014). As shown in **Figure 5**, the 2.5%

RuBisCO gel has final G' values similar to those obtained with a gel made up of 10% egg white proteins (EWP) and 12% whey proteins. Alfalfa RuBisCO at 5% also showed high gel strength, more than twice that a soy protein gel at 15% (Knuckles & Kohler, 1982). Spinach RuBisCO gels were shown to be more brittle than gels made from whey or egg white proteins and were therefore more susceptible to breakdown both at small and large deformations. The authors explained this behaviour by the dominant role of hydrophobic and hydrogen bonds during the network formation. The same results were obtained on sugar beet leaves RuBisCO gel (Martin et al., 2019).

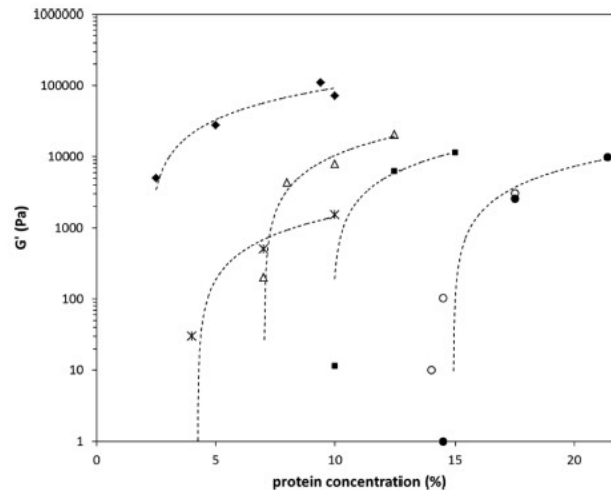


Figure 5. Evolution of the storage modulus G' of protein gels as a function of protein concentration for RuBisCO (diamonds), whey protein isolate (squares), egg white proteins (triangles), soy protein isolate (crosses), lupin protein isolate (full circles) and pea protein isolate (empty circles). Measurements were performed at pH 7. Copied from (Martin et al., 2014).

The amino acid profile of RuBisCO is equilibrated and its relatively high lysine content make it a good candidate to fortified wheat-based food. RuBisCO shows promising *in vitro* and *in vivo* digestibility and good functional properties as compared to other plant and animal proteins. It has remarkable gelling properties with a low denaturation temperature ($\sim 65^{\circ}\text{C}$) and a low critical gel concentration (down to 0.1%).

Keypoints: RuBisCO has been studied since long because of its crucial role in biomass production. Its potential use in human food appears as an evidence due to its abundance, its balanced amino acid profile and its promising functional properties. However, technological issues related to its purification have limited studies on its functionality as a food ingredient. Technological advances on its extraction highlight once again the need to deepen our knowledge on the possible use of RuBisCO in food matrices.

Part 2. The gluten network: structure and role in wheat based food quality

Typical cereal grain is composed of three parts: the peripheral layers, the endosperm and the germ. The endosperm represents 80 to 85% of the grain mass and mainly contains starch and proteins. The outer and germs layers are composed of fibres, vitamins and minerals (Izydorczyk & Biliaderis, 1995). The purpose of grain milling is to separate the endosperm from the peripheral parts and the germ and to reduce it in flour (fine particles) or semolina (coarse particles). Wheat is the fourth cereal commodities produced worldwide (<http://www.fao.org/faostat/en/#home>). *Triticum aestivum*, referred to as common or bread wheat, represents most of the world wheat crop (>90%). Common wheat is divided into soft and hard cultivars, based on the hardness of the kernel endosperm. Hard wheat flour is mainly used for bread making whereas soft wheat flour is used for applications such as cakes, cookies and crackers. Durum wheat (*Triticum turgidum*) is the second cultivated wheat species. Durum (meaning “hard” in Latin) wheat takes its name from the vitreousness and hardness of its starchy endosperm, which by far exceed those of all hard common cultivars. The hardness of its endosperm makes durum wheat favourable for coarse particles (semolina) production through milling. Semolina and flour are essentially composed of starch (70-80% db), proteins (8-18% db), non-starchy polysaccharides (2-8% db), lipids (1-3% db) and minerals (0.4-0.8% db) (MacRitchie, 1984; Sissons, 2008). Whether in bakery or in the pasta industry, the wheat proteins are of major technological importance. They can be separated into two groups according to their biological function. Albumins and globulins are metabolic proteins of low molecular weights (10-60 kDa) that represent from 15 to 20% of total proteins. The other proteins, the storage proteins, composed mainly of prolamins, form a second group with various molecular weights and properties. Prolamins form a viscoelastic network, the gluten network, under the effect of hydration and energy supply. The formation of the gluten network gives the wheat-based foods their textural properties and is essential for the quality of the food.

This state of the art first describes the composition and primary structure of wheat storage proteins. Then the structural models of the gluten network are presented. Finally, the mechanism of the gluten network formation during food processing and its role in product quality are explained.

1 Wheat storage proteins

Several classifications of wheat proteins have been proposed since the 20th century. Among them, Osborne's classification describes wheat storage proteins according to their solubility in different solvents (Osborne, 1907). Later, the classification of Shewry's group proposed an additional degree of classification and separated the storage proteins according to their degree of polymerisation and their content in sulphur amino acids (Shewry, Tatham, Forde, Kreis, & Mifflin, 1986). **Figure 6** shows the classification of wheat storage proteins according to these two latter classifications.

1.1 Gliadins

Gliadins are the monomeric wheat proteins soluble in aqueous ethanol at 70%. They represent from 40% to 50% of the total wheat storage proteins. Gliadins can be classified according to their decreasing mobility on electrophoresis at acidic pH: α -, β -, γ - and ω - gliadins. Alpha-, β - and γ - gliadins have

molecular weights comprised between 30 and 45 kDa. The molecular weights of ω -gliadins are slightly higher (up to 75 kDa) (Herbert Wieser, 2007). **Figure 7** represents the schematic primary structure of wheat storage proteins. The primary sequences of the α - and β -gliadins are very similar, therefore they are often grouped together under the term of α -type gliadins or α/β -gliadins. Gliadins are particularly rich in glutamine and proline residues due to the highly repetitive sequence of their central domain (Khan & Bushuk, 1979). However, they contain few charged amino acids such as arginine or lysine. Omega-gliadins do not contain any cysteine residue but α/β - and γ -Gliadins respectively contain 6 and 8 cysteines residues. They are thus separated into sulphur-poor and sulphur-rich prolamins respectively (**Figure 6**). All cysteines residues are located in the C terminal domain of gliadins and they are all supposed to be involved in intrachain disulphide bonds (Shewry & Halford, 2002). Several authors report the existence of gliadin-like proteins that present an odd number of cysteines and are capable of forming interchain disulphide bridges (Schmid, Wieser, & Koehler, 2016). As a result, they are involved in polymers of glutenins (Masci et al., 1999; Masci, Rovelli, Kasarda, Vensel, & Lafiandra, 2002).

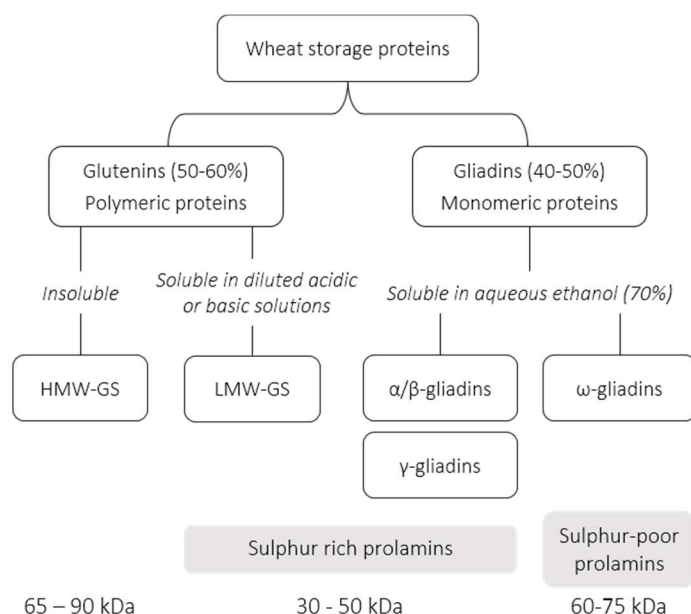


Figure 6. Wheat storage proteins classification based on Osborne and Shewry classification (Osborne, 1907; Shewry et al., 1986).

1.2 Glutenins

Glutenins are a group of polymeric proteins stabilised by intermolecular disulphide bonds. Glutenin polymers may be larger than 10^6 g.mol⁻¹. After reduction of the interchain disulphide bonds, two groups of glutenin subunits can be identified according to their molecular weight: the low and high molecular weight glutenin subunits (LMW- and HMW-GS).

Low molecular weight glutenin subunits (LMW-GS, from 30 to 50 kDa) are soluble in diluted acid or basic solutions. LMW-GS are similar to α/β - and γ -Gliadins in molecular weight and amino acids compositions (Tatham, Field, Smith, & Shewry, 1987). They can be divided into 3 groups (B, C and D) according to their SDS-PAGE patterns. The LMW-GS of group B are the most abundant; they have a primary sequence similar to α -, β - and γ -gliadins except that they contain two additional cysteine (Grosch & Wieser, 1999). They are expected to form interchain disulphide bonds and to play a chain-

extender role in the glutenin polymers formation. In contrast, most of the D and C-types LMW-GS only contain one free-sulfhydryl groups and can only act as a chain-terminator (D'Ovidio & Masci, 2004; Kasarda, 1989).

High molecular weight glutenin subunits (HMW-GS, from 65 to 90 kDa) are insoluble in non-denaturing buffers and can be divided according to their molecular weights: x type (83 to 88 kDa) and y type (67 to 74 kDa). The different HMG-GS are similar in sequence and present, in their central domain, repeated sequence pattern rich in proline, glutamine and glycine. As for gliadins, they contain few ionised amino acids. However, they differ from LMW-GS and gliadins compositions in their high content of glycine (Shewry et al., 1986). The N-terminal and C-terminal lateral domains contain most, if not all, of the cysteines residues. X-type HMW-GS contain 4 cysteines residues (except Dx5 that contain 5 cysteines) two of which are supposed to form an intrachain bond. Y-type HMW-GS contain 7 cysteines residues. The polymeric structure of glutenins is not in a stable state and undergoes multiple changes from the maturation of the grain to the final product (Herbert Wieser, 2007). In addition to their high molecular weight, the relatively low content of charged groups (aspartic and glutamic acids, histidine, lysine and arginine) in gluten proteins is a limiting factor to their solubility in water (H. Singh & MacRitchie, 2001). It has been hard accurately determining the glutenin subunits native tri-dimensional structure because the use of denaturing and/or reducing agents is necessary for their solubilisation but leads to the loss of information on their structure.

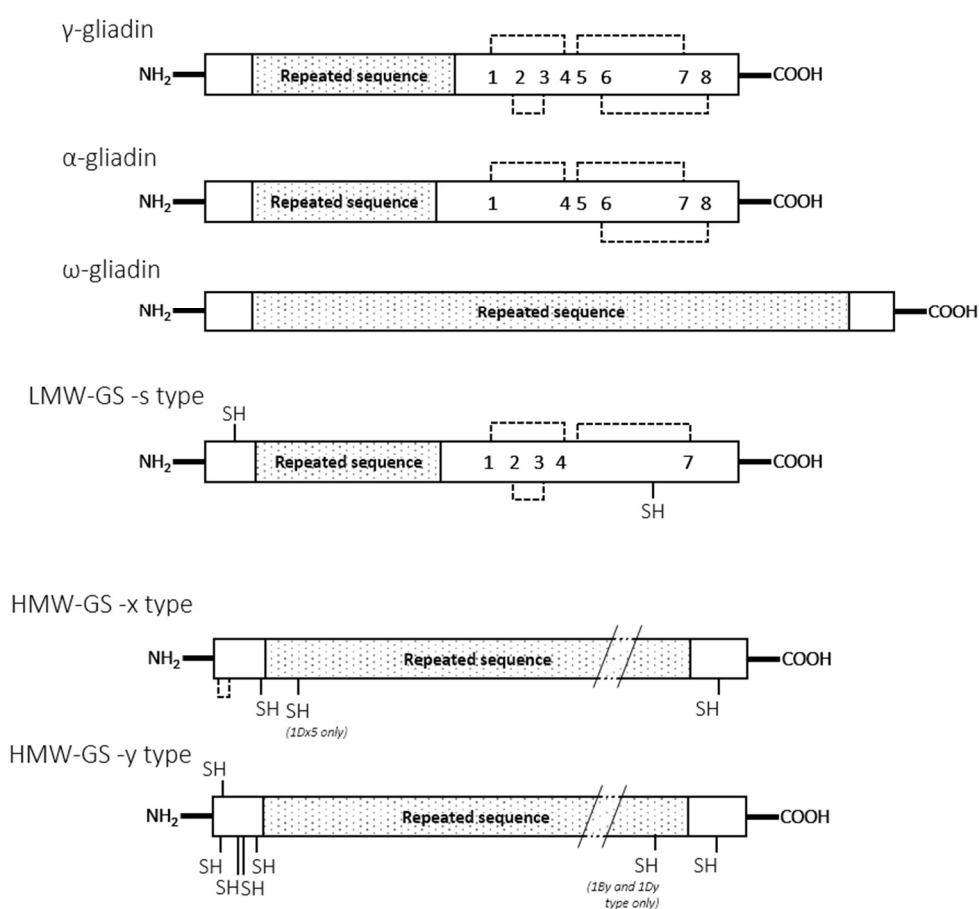


Figure 7. Schematic representation of the primary structures of wheat gluten proteins. Conserved cysteines are represented with numbers for gliadins and LMW-GS. Dashed lines represent intermolecular disulphide bonds. Scheme is adapted from (D'Ovidio & Masci, 2004; Lindsay & Skerritt, 1999; Shewry & Halford, 2002).

Gluten proteins include monomeric proteins, gliadins, and polymeric proteins, glutenins. Gliadins and glutenins both have a low content of charged amino acids. On the contrary, they are rich in glutamine residues that may promote the establishment of hydrogen interactions. Gliadins and glutenins differ in their content of cysteine residues involved in intrachain disulphide bridges. Only glutenins possess free thiols groups capable of forming intermolecular bridges.

2 The gluten network: models of structure

2.1. Structural organisation of glutenin polymers

Although the study of glutenin polymers structure is very difficult due to their insolubility in non-denaturing and non-reducing buffer, several models have been proposed for their structure. The oldest models proposed a linear structure of glutenin polymers with a low level of branching. As glutenin subunits represent only 3% of the dough mass, a linear structure would be the most efficient arrangement to explain their important role in the rheology of the dough (Ewart, 1972, 1979). Later, it has been proposed that the linear glutenin polymers formed aggregates through non-covalent bonds (Kasarda, 1989; Khan & Bushuk, 1979). Several authors then proposed branched models for glutenin polymers. Graveland et al. (Graveland et al., 1985) proposed a branched model in which HMW-GS would be linked by disulphide bonds and form a linear backbone. LMW-GS would be branched on this skeleton by disulphide bonds with γ -type HMW-GS. Gao et al. (Gao, Ng, & Bushuk, 1992) proposed a block model with oligomeric units that differed according to whether they continued as HMW-GS or as a mixture of HMW-GS and LMW-GS. Lindsay and Skeritt (Lindsay & Skeritt, 1999) proposed a linear backbone consisting of either HMW-GS only or both HMG and LMW-GS. Other glutenin subunits would branch out from this GS skeleton, which are capable of forming at least 3 interchain disulphide bridges.

The structural organisation models of glutenin polymers differ mainly on the composition of the glutenin backbone, comprising HMW-GS alone or with LMW-GS; on the position of the branches of the glutenin subunits; and on the nature of the bonds involved in the polymer structure.

2.2. Supramolecular organisation of the gluten network

The formation of the gluten network is therefore partly based on the polymeric nature of glutenins. In the dough, the glutenins would be responsible for the elastic properties, while the gliadins would be responsible for the viscous properties of the gluten (R. J. Hamer, Weegels, & Marseille, 1992; Pritchard & Brock, 1994). The elasticity of a material is based on its ability to return to its original dimensions after stress. Disulphide cross-links alone cannot explain the elastic behaviour of glutenin polymers (Belton, 1999). Several structural models were therefore proposed in an attempt to explain the viscoelastic properties of gluten. They are defined by the interactions within the glutenin network and the interactions between glutenins and gliadins. Two major theories exist concerning the structure of gluten: the polymer and the particles based theories.

i. The polymer based theory

The polymer-based theory is taken from the polymer science and consider the gluten as a continuous network of glutenin polymers stabilised by covalent, non-covalent and/or entanglement zones. Two major models were proposed depending on the type of involved interactions.

Belton (Belton, 1999) postulates a « loop and train » mechanisms relying on a high number of hydrogen bonds between glutenin subunits. Glutenins contain repeated sequences rich in glutamine and are therefore highly hydrophilic and able to form numerous intra- and interchain hydrogen bonds. Residues involved in hydrogen bonds interactions with water molecules form “loop” region, whereas residues involved in polymer-polymer hydrogen bonds interactions form “train” region. The regions are in dynamic equilibrium, a loop regions being able to turn into a train and vice versa. The train regions are assumed to adopt β -sheet structures while the hydrated regions are mobile and would adopt β -turns. The balance between loops and trains would be modified depending on the hydration level and shear rate of the dough, as depicted on **Figure 8**. With increasing hydration, loop regions increase at the expense of trains regions. Despite a very high hydration of polymers and the formation of a large number of loop regions, the protein would not dissolve in the water because it is unlikely to break up all the train regions at the same time, regarding the large number of glutamines. The authors point out that it is energetically simpler to deform loops regions than train regions and that may explain why the addition of water has a plasticising effect on the dough and makes it easier to deform. When stretched, the loops would first be deformed until they are flattened and as the interchain hydrogen bonds broken, the protein chains may slip. The elastic restoring force would be ensured by restoring the balance of the loop and trains structure.

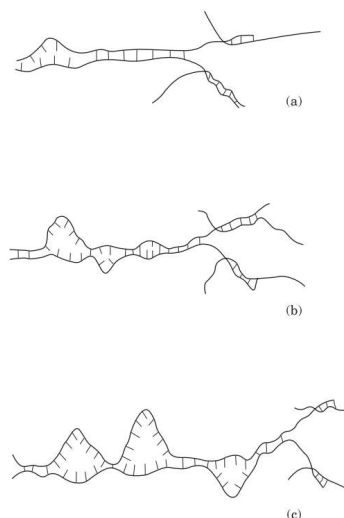


Figure 8. Effect of hydration on the hydrogen interactions between two chains of HMW-GS as proposed by Belton (1999): (a) Low hydration level (a), medium hydration level (b) and high hydration level (c). The increase in hydration leads to an increase in "loop" regions, corresponding to zones of hydrogen interactions between residues and water. The train regions correspond to the hydrogen interactions between glutenin subunits. Copied from (Belton, 1999).

Shewry et al. (Shewry, Popineau, Lafiandra, & Belton, 2000) proposed an enriched model assuming that the loop and train model could also be applied to LMW-GS and that gliadins could also interact with glutenins via non-covalent interaction. These interactions would not explain the elasticity of the gluten but its viscosity.

Singh and MacRitchie (H. Singh & MacRitchie, 2001) took a more physical approach to describe the structure of gluten and use knowledge in polymer science to explain the properties of the dough as measured by empirical tests. The model uses the correlations found between the content of high molecular weight glutenin polymers and the maximum strength of the dough. In entanglement theory,

this observation can be related to the threshold molecular weight below which entanglement does not occur. Therefore, they proposed the gluten proteins to form a continuous network of flexible random coiled polymers with widely spaced junction points along the chain, referred to as entanglements. Entanglement would be held through weak bonds and dough strength and extensibility would be explained by the presence of these entanglement points. As for polymers under stress, dough mixing would lead to disentanglements, chain orientation and bond rupture. The slippage of chain through entanglement points would act as transient cross-links responsible for the viscoelastic behaviour of dough.

These two models mainly differ on one assumption: Belton assumes the presence of a pre-existing gluten network in the flour, while MacRitchie estimates that before the dough is kneaded, glutenins exist in the flour in the form of "discrete molecules" and that they reach a threshold molecular weight during kneading (MacRitchie, 2014). In addition for the McRitchie model the gluten elasticity would rely only on entropy why Belton model implies strong enthalpy contributions. Although these two models are different, they agree on the principles of elasticity and resistance to extinction. Elasticity results from the extension of polymer chains between cross-links or between points of entanglement (consider as transient cross-links). Resistance to extension and extensibility depend on the density of cross-links or entanglement points.

ii. The particle based theory and the hyper-aggregation model

The particle based theory for gluten structure was proposed by Hamer and van Vliet (Rob J. Hamer, Vliet, & Van Vliet, 2000) and Lefebvre (J. Lefebvre, Pruska-Kedzior, Kedzior, & Lavenant, 2003). They consider the gluten as a network of insoluble colloidal particles in close interactions rather than as a network of polymers. This model implies physical interactions at the mesoscopic levels (0.1-100 μm) that affect dough properties. Hamer and van Vliet proposed a hyper-aggregation model describing three levels of organisation. The first level corresponds to the polymers formed by glutenin subunits linked by covalent bridges. At the second level, these polymers form entangled aggregates stabilised by hydrogen bonds and additional disulphide bridges. This level of aggregation is that found in the wheat grain or during the rest of the dough after mixing. At the third level, further aggregates would be stabilised by weak bonds and their formation would be greatly influenced by the process. The numerous inter-particles interactions would be the basis of the viscoelasticity. The authors point out that this particle structure is found in other viscoelastic materials such as mayonnaise, yoghurt and tomato sauce. Moreover, this model can easily integrate the effect of other dough components.

Although starch is the major constituent of wheat flour or semolina, the structure of the gluten network is mainly responsible for the viscoelastic properties of the dough. The dough is therefore considered as consisting of a continuous network phase, the gluten network, in which starch granules are dispersed as filler particles. In an attempt to clarify the role of starch in dough rheology, number of study have been done on reconstituted doughs (Eliasson, 1983). The extrapolation of these models to the complex dough system is sometimes controversial, especially concerning the distribution of water between starch and gluten, which is of great importance in their thermal modification (Faridi & Faubion, 1989). Several authors agree that the surface properties of starch influence gluten/starch interactions (N. M. Edwards, Dexter, & Scanlon, 2002; Hibberd, 1970; Lindahl & Eliasson, 1986; Schiedt, Baumann, Conde-Petit, & Vilgis, 2013). However, the effect of these interactions on the viscoelasticity of the system remains unclear (Brandner, Becker, & Jekle, 2019).

The scientific community has not found a consensus on the structure of gluten. The defenders of the different theories broadly agree on the experimental results and partially agree on their interpretation. They differ, however, in the scale and pattern of structure most likely to explain the observed behaviours. However, all models highlight the role of both covalent and non-covalent bonds in stabilising the three-dimensional structure of the protein network.

3 Structuration of the gluten network during processing

The gluten is defined as the rubbery mass that remains when a dough, made from the mixing of flour and water, is washed in an excess of water. Dough washing leads to the removal of most of starch and soluble components. The remaining gluten contains about 75-80 % of proteins (dry basis), mostly gliadins and glutenins, and residual components are mainly starch (7-12 %) and lipids (5-10 %). Gluten is a viscoelastic material, it has both viscous and elastic characteristics. The viscoelasticity of the gluten strongly influences the quality parameters of the products, such as the bread loaf volume or the consistency of the dough. The elastic property of gluten is related to the glutenin fraction and especially to the polymers molecular size distribution; and so strongly linked to the ability of glutenin subunits to associate each other by disulphide bridges (Ewart, 1968). During wheat-based food processing, three main steps affect the gluten network structure: hydration of wheat constituents, input of mechanical energy and application of thermal treatment. In this section, changes in dough texture along the process will be described and related to biochemical and physical changes in wheat protein structure.

3.1 Physical changes related to the hydration of wheat constituents

Whether in the bakery or pasta industry, the first step of processing is the hydration of flour or semolina constituent. Knowledge of the starch and gluten phase transition according to temperature and water content helps to understand the impact of the process conditions of dough structuring. **Figure 9** represents the phase diagrams of starch and gluten proteins according to temperatures and water content (Cuq, Abecassis, & Guilbert, 2003). The glass transition temperature of biopolymers, like gluten and starch is lowered by the addition of water which act as a plasticizer (**Figure 9**). In polymer science, the glass transition temperature corresponds to the temperature below which kinetic motion practically ceases and the polymer behaves as a glass. Gluten proteins and starch are in the rubbery state at ambient temperature (20-25°C) and at hydration above 15-20% (gluten) and 20-25% (starch). Glass transition concerns all types of gluten proteins and the amorphous zone of semi-crystalline starch in wheat dough. The rubbery state provides an increased molecular mobility of the protein chains that is needed for the formation of gluten network.

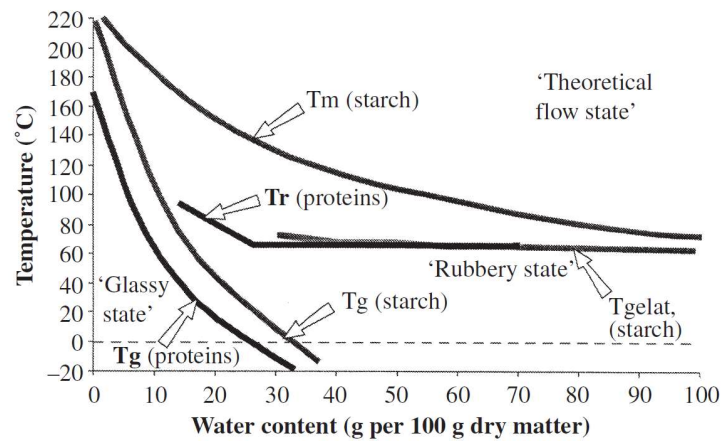


Figure 9. Phase transitions of starch and gluten proteins according to water content and temperature (copied from (Cuq et al., 2003)).

3.2 Dough kneading and gluten network development

In standard industrial practice, hydration always occurs in the same time than the mixing of the ingredients, rendering difficult the study of sole hydration impact on the structure of the dough (Gras, Carpenter, & Anderssen, 2000). The mechanical energy input to the flour/semolina and water mixture allows the mixing of the ingredients and promotes the hydration of the constituents. In pasta industry, the mixing leads to the formation of hydrated lumps with diameter between 2 and 3 cm but some particles remain non hydrated (Petitot, Abecassis, & Micard, 2009). Starch granules do not undergo significant structure modifications (Vansteelandt & Delcour, 1999) and the gluten network does not seem to develop (Matsuo, Dexter, & Dronzek, 1978) due to the heterogeneous and low hydration rate and the limited energy supply (Icard-Vernière & Feillet, 1999). In bakery industries, the kneading result in the formation of a continuous gluten network that imparts viscoelastic properties to the dough. During bread production, the yeast fermentation produces CO_2 that leads to the growing of the air bubbles initially incorporated into the dough during mixing. The viscosity of the dough must be high enough to hinder bubble migration and merging. However, the dough must also be sufficiently extensible to allow for bubbles expansion without breaking the inter-cell walls. In the following part, we further focus on the gluten network development in hydrated dough systems that undergo kneading.

i. Change in dough consistency during kneading

Dough development during kneading can be monitored by measuring the dough's resistance to the mixing blade motion. This resistance is expressed by the torque (in Nm) exerted on the rotor shaft by the blades or by a direct measure of the mixing energy consumption (Watt), depending on the type of machine. In any case, mixing is performed at a constant mixing speed. On a laboratory scale, two mixers are classically used: Farinograph® and Mixograph®. These two mixers differ mainly in their blades geometry and modes of motions, as shown on **Figure 10**. The Farinograph® imparts extensional deformation and shear stress, with its two counter-rotating sigma-blades. The Mixograph® would mainly result in dough stretching by soliciting the dough with pins (Connelly, 2008; Gras et al., 2000). Another major difference between the two devices is the quantity of flour required, which varies from

50 g to 300 g for the Farinograph and from 2 g to 50 g for the Mixograph. These devices are used to evaluate the technological qualities of flours and semolina.



Figure 10. Geometry of the mixing system in Farinograph (left) and Mixograph (right). Copied from (Frédéric Auger, 2008)

Figure 11 represents two torque mixing curves obtained with Farinograph® (left) and Mixograph® (right). The torque value (vertical axis) is representative of the dough consistency and the horizontal axis is the mixing time. Both curves exhibit the same profile. First, the dough consistency increases until reaching a maximum. The time needed to reach this peak is called the optimum mixing time. The peak coincides with the mixing time giving the optimum development of gluten network. If the kneading is continued beyond, the dough consistency declines, the dough is over-kneaded. The amount of energy supplied to the dough determines the good development of the dough during mixing and it depends on the composition of the flour, the amount of water added, the geometry of the mixer and the mixing speed (Frédéric Auger, 2008). The Farinograph is commonly used to determine the optimal hydration of a flour for bread making, called the water absorption. The water absorption of wheat flour is described as the amount of water required to reach a torque value equivalent to 500 Brabender Units. The measurement of water absorption is generally done according to two similar methods: the ICC No. 115/1 method and AACC No. 54-21.02 method. The strength of a wheat variety can be estimated from the stability time of the dough, which roughly corresponds to the Farinograph mixing time during which the consistency of the dough remains around 500 BU. The Farinograph enables to determine other empirical parameters that define the quality of a flour such as the development time, the stability time or the mixing tolerance. Although some methods have been transposed to the Mixograph (Reiko, Aya, Hideki, & Masaharu, 2016; Suchy, Lukow, & Ingelin, 2000), the Farinograph remains the most commonly used device in the field.

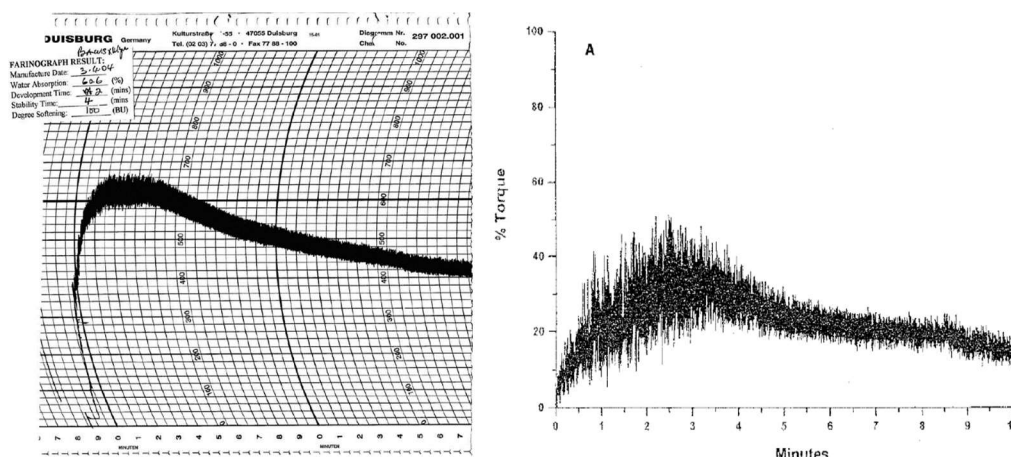


Figure 11. Typical Farinograph (left) and Mixograph (right) curves for common wheat flour dough (copied from (Bejosano & Corke, 1998; Stojceska & Butler, 2008)).

Numerous studies underline a correlation between the dough physical parameters measured by empirical methods, the bread-making quality and the glutenin polymers content and composition (Gupta, Batey, & MacRitchie, 1992; Weegels, Hamer, & Schofield, 1997; Weegels, van de Pijpekamp, Graveland, Hamer, & Schofield, 1996; H. Wieser & Kieffer, 2001). This highlights the predominant role of gluten proteins in increasing the consistency of the dough during kneading.

ii. Rheological behaviour of dough

The empirical tests are practical because they allow evaluating the baking qualities of raw materials. However, they do not give information on the intrinsic mechanical properties of materials because the strain imposed is complex and non-uniform. In contrast, dynamic oscillatory measurements in the material linear regime involve small and reversible deformations. Dynamic rheological tests performed on dough have been adapted from polymer science and are widely used to measure the fundamental properties of the dough. They allow to relate the stress with the strain rate (or the inverse) and thus highlight specific rheological behaviours. The resulting data are used to calculate the energy stored and dissipated during deformation, respectively by the dynamic storage modulus (G') and loss modulus (G''). The loss factor ($\tan \delta$) corresponds to the ratio of viscous and elastic response. These tests are only valid in the linear domain where stress and strain are linked by a linear relationship regardless of time. When it exists, the linear domain of a wheat dough is located at low strain rate inferior to 0.1% (Jacques Lefebvre, 2006). **Figure 12** represents the frequency dependence of the elastic modulus (G'), loss modulus (G'') and loss factor ($\tan(\delta)$) for a dough (left) and for isolated gluten (B). The viscoelastic behaviour of the dough is easily evidenced by the higher G' values than G'' , also characterised by $\tan(\delta)$ values lower than 1 (S. Singh & Singh, 2013).

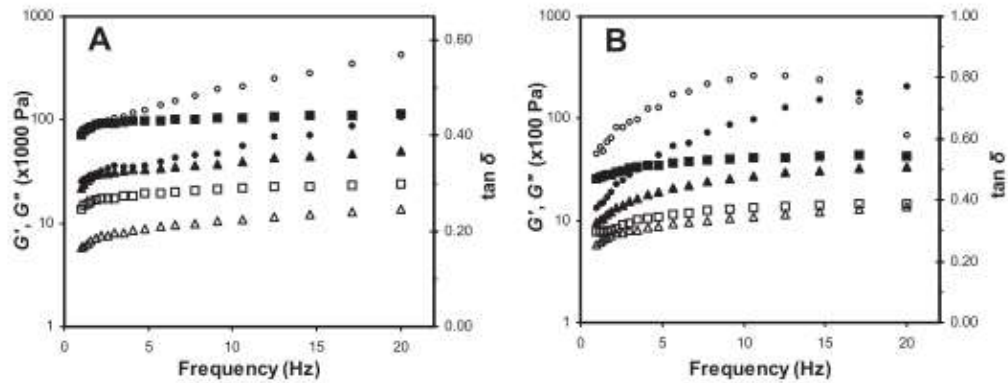


Figure 12. Frequency dependence of the elastic modulus (G' , squares), the loss modulus (G'' , triangles) and the loss factor ($\tan(\delta)$, circles) for dough (A) and gluten (B) made from a strong-weak (full symbols) and a weak wheat (empty symbols). Copied from (S. Singh & Singh, 2013).

By carrying out dynamic analyses on optimally-, under- and over-mixed doughs, it is possible to establish relationships between the empirically measured dough consistency and its rheological parameters. The evolution of G' , G'' and $\tan(\delta)$ until the optimum mixing time depends, in part, on the strength of the wheat variety. Strong wheat display maximal G' and G'' at the optimum mixing time. On the contrary, weak wheat varieties display maximal G' and G'' when under-mixed. Authors attributed the under-mixed doughs behaviour to the insufficient mixing and hydration resulting in the presence of rigid flour particles (S. Singh & Singh, 2013). Whatever the dough stability, dough over-mixing leads to a decrease in the elastic modulus and an increase in the loss factor, representative of a dough with a more pronounced liquid-like behaviour and therefore a weakening of the network (S. Singh & Singh, 2013). These data allow the rheological behaviour to be related to the empirical parameters measured during dough mixing, however there is no clear consensus on the relation between the results obtained in dynamic rheology and the baking qualities of dough (Janssen, Van Vliet, & Vereijken, 1996).

Dough rheological parameters are also highly related with the flour protein composition. The Glutenin MacroPolymer (GMP) corresponds to the fraction of glutenin polymers that is insoluble in sodium dodecyl sulphate (SDS) solution. It is also called the SDS-unextractable polymeric protein (UPP), although some authors make a difference between both terms according the SDS concentration of buffers (Clyde Don, Lichtendonk, Plijter, Van Vliet, & Hamer, 2005). Negative correlation was found between $\tan(\delta)$ value of optimally mixed dough and its content in UPP (S. Singh & Singh, 2013), confirming the role of high molecular weight glutenin polymers in the viscoelastic behaviour of dough. Empirical parameters such as dough maximum resistance to extension were correlated with the GMP content of both flour and dough, suggesting the importance of GMP modification during kneading for dough resistance (Weegels, van de Pijpekamp, et al., 1996). It has been shown that the HMW-GS have an important role in the extractability of the glutenin polymers (Popineau, Cornec, Lefebvre, & Marchylo, 1994). In particular, both their quantity and their composition influence the levels of unextractable glutenin polymers and gluten viscoelasticity. Therefore, it is widely supposed that the structure and rheology of glutenins are mainly responsible for dough viscoelasticity and baking performances. However, studying the rheological behaviour of reconstituted gluten fractions, it has been shown that the glutenins/gliadins ratio also highly influences the rheological properties of gluten

(Janssen et al., 1996). The higher the ratio, the more elastic the system was, suggesting that glutenin polymers confer elasticity to the dough while gliadins confer viscous properties (N. M. Edwards, Mulvaney, Scanlon, & Dexter, 2003; Macritchie, 1987). Gliadins were also supposed to influence dough extensibility acting as plasticizers and promoters of the extensible network (Song & Zheng, 2008).

iii. Molecular mechanism associated with the development of the dough

In an optimally mixed wheat flour dough, the gluten network development has been observed to result in an alignment of gluten polymers forming strands (Frederic Auger, Morel, Lefebvre, Dewilde, & Redl, 2008; Boitte, Hayert, & Michon, 2013; Peighambardoust, Van Der Goot, Van Vliet, Hamer, & Boom, 2006). The strands would form an extensive network in which other particles such as starch granules are incorporated, leading to an increase in the stiffness and elasticity of the dough.

Figure 13 shows the evolution of the Glutenin macropolymer (GMP) content in doughs made from the flours of two wheat varieties according to mixing times. It is clear that the GMP content decreases during dough mixing (Skerritt, Hac, & Bekes, 1999; Weegels, Hamer, & Schofield, 1996). The global molecular weight of GMP also decreased and mixing was associated with an increase in the LMW-GS content of GMP (Weegels et al., 1997). The GMP breakdown would be due to thiols/disulphide interchanges occurring during mixing (Frater & Hird, 1963; Morel, Redl, & Guilbert, 2002). The reverse reaction spontaneously occurs when the mixing is stopped, resulting in re-polymerisation of the gluten (Clyde Don et al., 2005; Weegels et al., 1997). Moreover, both the decrease in global size of glutenin polymers and their reassembly were shown to depend on the mixing energy level (Frederic Auger, Morel, Dewilde, & Redl, 2009; Peressini, Peighambardoust, Hamer, Sensidoni, & van der Goot, 2008). This can suggest that thiols/disulphide interchanges are favoured by the mechanical stretching of glutenin disulphide bonds (Baudouin et al., 2020).

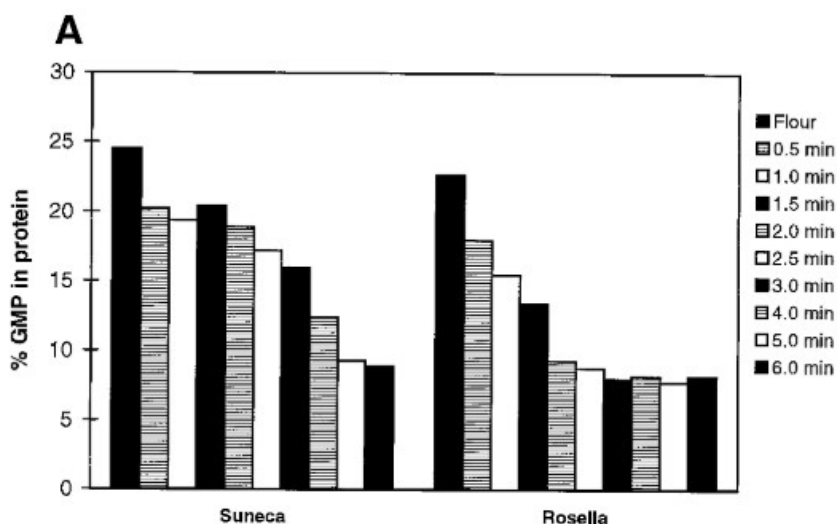


Figure 13. Evolution of the GMP (Glutenin MacroPolymer) content during mixing of two types of flour (copied from (Skerritt et al., 1999)).

Specifically, the tripeptide glutathione naturally present in the flour (GSH) could catalyse these interchanges reactions. Glutathione addition before dough mixing results in a decrease of the elastic modulus G' and an increase of $\tan(\delta)$ of dough after mixing, representative of a less elastic dough with a higher liquid-like behaviour (Guo et al., 2020). The interchain disulphide bridges of the gluten

proteins (and more specifically LMW-GS) were shown to be cleaved by GSH (Grosch & Wieser, 1999; Hüttner & Wieser, 2001). GSH contains only one free thiol group, by forming a disulphide bridge with a gluten-free thiol, it interrupts the formation of the network. The addition of oxidizing agent such as ascorbic acid in the bread-making process allow the GSH to be oxidised and prevents their interaction with the gluten proteins, thus strengthening the characteristics of the dough. These thiols/disulphide exchange reactions determine the rheological properties of dough (Frater, Hird, Moss, & Yates, 1960). However, the relationship between the development of the dough and the viscoelastic network and the depolymerisation of glutenin polymers remains unclear (C. Don, 2005). For other authors, the transient depolymerisation would be due to an increase in the solubility of glutenin polymers due to kinetic effects rather than thermodynamic (Belton, 2005). Hence, mixing and hydration would at first promote the breakage of the intermolecular hydrogen bonds that stabilize the glutenin polymers supramolecular assemblies in a ground relaxed state. The latter is characterized by the presence of β -sheets, while mixing allow for β -turns folding by inducing the stretching of the polymers chains. Belton proposed that this change in the chain folding would decrease the intermolecular interactions and increase the glutenin polymer solubility in SDS buffer (Belton, 2012; Georget, Underwood-Toscano, Powers, Shewry, & Belton, 2008; Jazaeri et al., 2015). In addition, the addition of thiol blockers resulted in a decrease in β -sheet in favour of β -turn (Jazaeri et al., 2015), suggesting that intermolecular disulphide linkages are integral to the formation of sheet structures.

3.3 Mechanical energy input through pasta extrusion

The crumbly dough resulting from mixing the semolina with water is extruded or laminated to form the pasta into the desired shape. These two forming processes differ according to the type of deformation imposed on the pasta dough. Lamination mainly causes extensional deformation with little shear while shear prevails in extrusion. In addition, the amount of mechanical energy applied to the dough is generally higher for extrusion. The influence of extrusion on the structure of the protein fraction is unclear. Some authors only report a decrease in the globulin solubility (Dexter & Matsuo, 1977) without modification of their molecular weights. Other studies highlight an increase in the SDS solubility of glutenin polymers after extrusion or lamination, representative of the depolymerisation of glutenin polymers (Joubert, Lullien-Pellerin, & Morel, 2018; E. H. J. Kim et al., 2008). As observed for mixed bread dough, glutenin polymers insolubility in SDS would be restored by resting after pasta extrusion. The structural changes associated to extrusion cannot be solely related to mechanical energy. Local temperature increase due to energy dissipation could induce protein polymerisation despite low extrusion temperature ($< 50^{\circ}\text{C}$). The structural changes would therefore be the result of the balance between the mechanical and thermal energies.

3.4 Thermal energy input in bread dough

An increase in temperature in the presence of sufficient water causes hydrothermal transformations of starch and gluten proteins. According to the phase transition diagram (**Figure 9**), at temperatures above 60°C the proteins and starch that are in rubbery states, can undergo reticulation and gelatinisation respectively.

i. Dough mechanical properties during heating

During the bread baking process, two distinct temperature-humidity changes can be observed. The dough surface is exposed to high temperatures and undergoes dehydration to reach a water content

of less than 10%. Due to dehydration, starch gelatinisation is limited in the crust (Fessas et al., 2008; Vanin, Michon, Trystram, & Lucas, 2010) and starch and gluten proteins undergo a glass transition (Cuq et al., 2003). The formation of the crust prevents a significant loss of water from the inside of the dough and allows the formation of the crumb. The bread crumb then shows a higher water content between 35 and 40%. The increase in temperature (up to $\sim 100^{\circ}\text{C}$) allows the gelatinisation and melting of starch, corresponding to the irreversible swelling of the starch granules followed by the solubilisation of its molecules, mainly amylose. This transition is usually followed by differential scanning calorimetry (DSC). Two endotherms are observed in excess of water, one at 65°C corresponding to starch gelatinisation and the second at 95°C corresponds to the fusion of amylose-lipids complex (Biliaderis, Maurice, & Vose, 1980).

During the increase in the dough core temperature, the mechanical properties of the dough change significantly, representative of the change from a viscous liquid state to an elastic solid state. The evolution of dough viscosity during bread baking has been well described. **Figure 14** shows the evolution of G' and $\tan(\delta)$ of a flour-water dough during heating (Dreese, Faubion, & Hosene, 1988a). The apparent viscosity follows the same evolution as the G' modulus. With an increase in temperature from 25 to 40°C , the viscosity decreases. In these temperature ranges, viscosity can be described by an Arrhenius law and its decay is commonly associated with an increase in the mobility of water and polymeric chains (Bloksma, 1975, 1990). Between 45 and 60°C the viscosity increases rapidly until it reaches a peak. Re-heating of the dough after cooling does not change the modulus value, indicating that changes between 55°C and 75°C are irreversible. The marked increase in elastic modulus between 50°C and 75°C corresponds to the cumulative effect of starch gelatinisation and protein aggregation. However, there is no clear scientific consensus on which of the two phenomena dominates the elastic modulus response in these temperature ranges. It has been shown that the maximum value of the elastic modulus is positively correlated with the starch content of starch-gluten mixtures, whereas gluten is assumed to have little influence on G' values (Dreese, Faubion, & Hosene, 1988b; Jekle, Mühlberger, & Becker, 2016; Zanoletti et al., 2017). Conversely, some studies on wheat dough have reported that changes in E' with temperature are mainly due to polymerisation of gluten proteins (Rouillé, Chiron, Colonna, Della Valle, & Lourdin, 2010). Starch gelatinisation occurs in the same temperature range as gluten cross-linking, leading to difficult separation of their respective effects on dough thermo-mechanical behaviour.

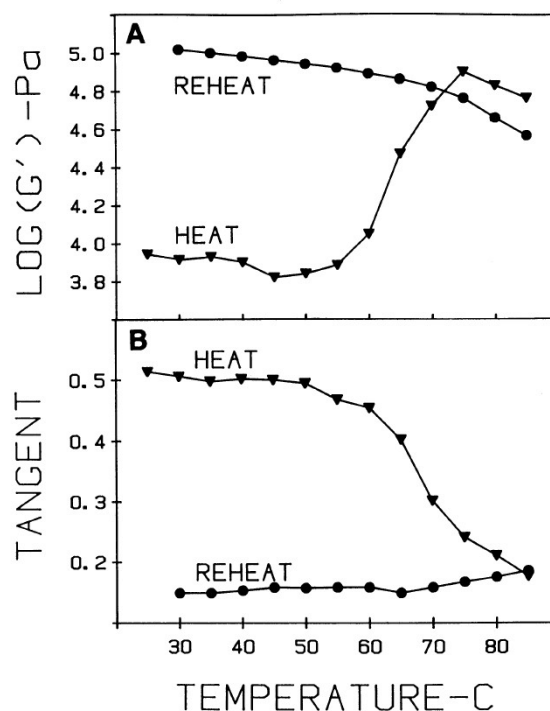


Figure 14. Temperature dependence of the elastic modulus (A) and loss factor (B) of a flour-water dough (“heat”, triangles) and of a dough that was previously heated to 90°C and cooled (“Reheat”, circles). Copied from (Dreese et al., 1988a).

ii. *Gluten thermosetting mechanisms*

The formation of the gluten heat-set gel involves the covalent cross-linking of gluten proteins through disulphide bonds (Bloksma, 1990; Dreese et al., 1988a; Kasarda, 1989; Schofield, Bottomley, Timms, & Booth, 1983). This polymerisation results in the decrease of the SDS-soluble protein content. It has been shown that up to 75°C the solubility of glutenins is particularly affected (Schofield et al., 1983). The total thiol content remaining the same, these experiments highlighted the phenomenon of thiol/disulphide interchange enhanced by temperature. Glutenins and gliadins (above 75°C) are both involved in these mechanisms, except ω -gliadins which do not contain cysteine (Lagrain, Thewissen, Brijs, & Delcour, 2008; H. Singh & MacRitchie, 2004).

3.5 *Pasta drying and cooking*

After extrusion or lamination, pasta thermal treatments occur during both the drying and cooking steps. The temperature-moisture conditions differ from than that encountered during bread baking. Pasta drying can be performed at several temperatures from 40°C (low-temperature) to more than 84°C (very high temperature) and lead to a decrease of water content until less than 12.5% (Cimini, Cibelli, Messina, Marconi, & Moresi, 2019; Petitot, Boyer, Minier, & Micard, 2010). **Figure 15** shows the effect of drying temperature-moisture conditions on the protein solubility in pasta. As for bread baking, increasing the drying temperature from 55°C to 90°C leads to a decrease of SDS soluble protein content. This is related to an increase in the molecular size of the large polymeric proteins (Lamacchia et al., 2007) and an increase in the content of SDS insoluble while the content in SDS-DTE soluble proteins remains unchanged, indicating the involvement of disulphide linkages in the solubility loss of large polymeric proteins. Drying the pasta at 90°C increases the quantity of non-extractable proteins

(even in SDS-DTE buffers), suggesting the formation of non-disulphide covalent bridges such as iso-peptide bonds. The effect of drying on starch gelatinisation remains unclear (Petitot, Abecassis, et al., 2009).

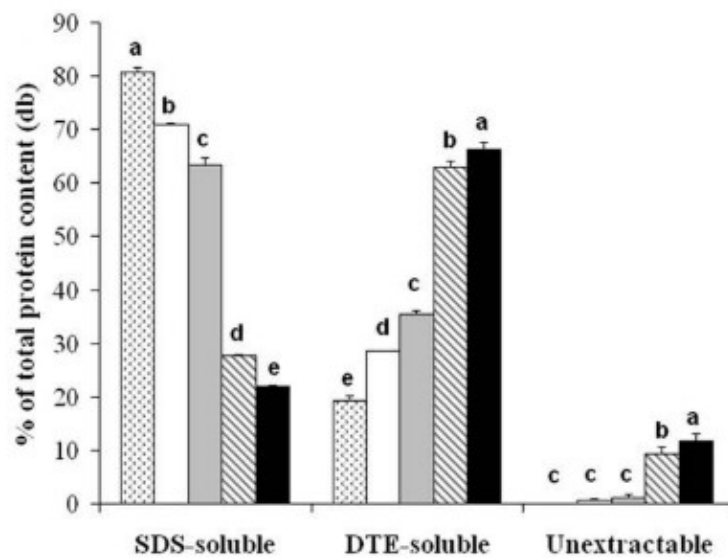


Figure 15. Peaks area of SDS soluble, SDS insoluble but SDS-DTE soluble and unextractable protein fractions of semolina (dotted area) and dried pasta as measured by SE-HPLC. Pasta drying profiles are represented as followed: pasta dried at low temperature (55°C; white area), high temperature (70°C; grey area), very high temperature applied at the end of drying profile (90°C; dashed area) and very high temperature all along the drying (90°C, ; black area) as measured by SE-HPLC. Copied from (Petitot, Brossard, et al., 2009).

Both gluten and starch recover a glassy state after pasta cooling. During cooking, the water diffuses concentrically into the pasta, leading the starch and protein to rehydrate and recover the rubbery state. Then the starch gelatinizes and the proteins polymerise and both phenomenon competing for water (Pagani, Gallant, Bouchet, & Resmini, 1986). The rate of proteins polymerisation during cooking also depends on the drying profile. Indeed, the cooking step reduces the differences in protein aggregation observed on dried pasta after different drying profiles (Petitot, Brossard, et al., 2009). The starch completely gelatinizes during cooking, as confirmed by the absence of gelatinisation peak on thermogram obtained by DSC on cooked pasta (Fardet et al., 1999). However, a physical competition occurs between the polymerisation of proteins into a continuous network and the starch swelling, which thus remains limited. Pasta cooking quality is determined by these antagonistic phenomenon (Resmini & Pagani, 1983). The degree of polymerisation of the gluten thus determines the textural qualities of the dough (Bruneel, Pareyt, Brijs, & Delcour, 2010). Drying and cooking are therefore key steps in achieving the optimum degree of gluten polymerisation to guarantee the quality of the pasta.

During the process of wheat-based foods, the gluten network develops mainly through hydration and mixing of the constituents as well as during heat treatment. The formation of the gluten network is based on the ability of its constituent proteins to associate via both disulphide bridges and weak bonds. The visco-elastic properties of gluten are indispensable for the development of bread volume or pasta texture.

Keypoints: During the process, the wheat storage proteins form a viscoelastic protein network, the gluten network, which is essential for the quality of wheat-based foods. Although there is no consensus on the gluten network structure, it is well recognized that its formation and its viscoelastic properties are based on the ability of the glutenins and gliadins to associate via disulphide bridges and weak bonds. The formation of the gluten network is mainly studied by rheological analysis of the dough and by sequential extraction of the proteins into denaturing and reducing buffers. The gluten network begins to form due to hydration and mechanical energy input and is further enhanced by temperature through thiol/disulphide interchanges, leading to the increase of large-size disulphide linked polymers content.

Part 3. Influence of a protein enrichment on wheat-based food culinary, textural and nutritional properties

Enriching wheat based product with extrinsic proteins provides a way to improve their protein and amino acid contents. In this section, we will focus on the effect of a protein enrichment on the quality of the end-products in regards to their structure. Culinary, textural and nutritional properties will be tackled.

1 Culinary and textural properties of protein-enriched wheat-based foods

1.1 Protein enriched bread quality

The effects of partial substitution of wheat flour in bread are usually evaluated from dough farinographic characteristics. **Figure 16** represents the evolution of dough stability and loaf volume according to the enrichment rate of wheat flour with lupin seed flour or lupin seed protein isolates (Mubarak, 2001). The dough stability decreases with an increase of the lupin enrichment rate, indicating a weakening of the dough. This weakening results in the collapse of the loaf volume after baking. The decrease in dough stability has also been highlighted, in *chlorella vulgaris* enriched doughs above 3% enrichment (Graça, Fradinho, Sousa, & Raymundo, 2018) and in whey protein enriched bread doughs until 10% (Zhou, Liu, & Tang, 2018). The lowered loaf volume was suggested to result from a decrease in dough gas retention capacity. It has also been hypothesized that during baking, lupin proteins could reduce the amount of steam generated, because of their high water absorption capacity, leading to changes in crumb structure (Ribotta, Arnulphi, León, & Añón, 2005). It may result in smaller gas cells due to a decrease in the elasticity of the crumb (Paraskevopoulou, Provatidou, Tsotsiou, & Kiosseoglou, 2010) or in larger cells due to coalescence (Graça et al., 2018). However, a protein enrichment may lead, in some cases, to an increase in dough stability as demonstrated for lupin protein isolate until 10% bread enrichment (Paraskevopoulou et al., 2010) or in soy proteins enriched breads until 30% enrichment (Zhou et al., 2018). In these cases, there was nevertheless a weakening of the network as illustrated by the decrease in elasticity measured with the farinograph (Paraskevopoulou et al., 2010) or by the impaired textural properties of the bread after baking (Zhou et al., 2018). The authors hypothesised that, in these latter cases, the added proteins might be hydrated but not fully dispersed particles, so they may act as gluten network fillers, leading to the greater dough stability. As shown in **Figure 16**, the use of lupin flour reduces the volume of bread more than the use of lupin protein isolate for the same flour substitution level until 12.5%. This could be related to the much higher total protein content of isolate. Contrary to the use of protein isolates, the incorporation of flours also results in an enrichment of other co-products such as fibres or sugars that may affect bread properties. It has also been shown there was a threshold above which the decrease in loaf volume becomes significant (12%) (Mubarak, 2001). On the contrary, some proteins have a positive effect on the bread quality above a certain level of enrichment. This has been shown on bread enriched with whey protein at 20% substitution level (Zhou et al., 2018). It is regularly highlighted that protein enriched bread doughs have higher water absorption than regular dough, due to the higher hydration capacity of the added protein as compared to wheat flour components (Graça et al., 2018; Mizrahi, Zimmermann, Berk, & Cogan, 1967; Paraskevopoulou et al., 2010). As for dough stability, water absorption changes depend on the type of proteins used for enrichment. For instance, the

addition of whey proteins from 5% enrichment can lead to a decrease in water absorption (Tang & Liu, 2017).

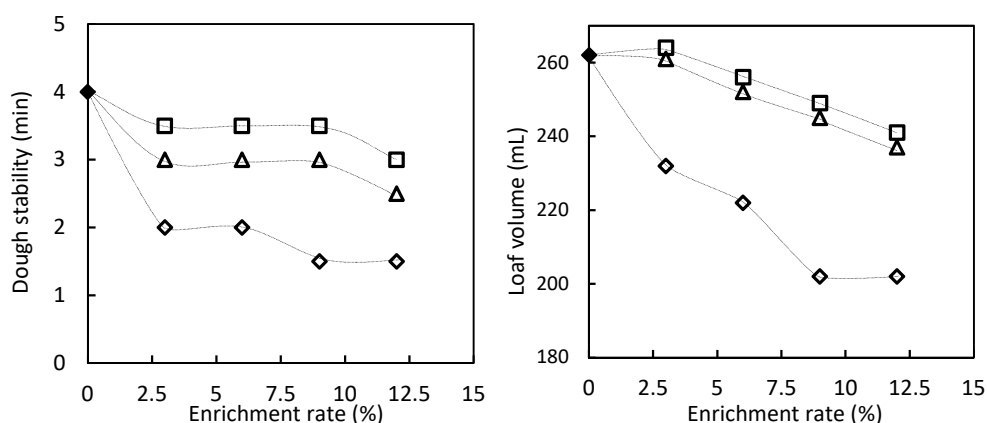


Figure 16. Dough stability (left) and loaf volume (right) evolution according to the enrichment rate of bread dough with sweet lupin seed flour (diamonds) or with two different types of sweet lupin protein isolates (triangles and squares). Dotted lines are included to guide the eye. Data are from (Mubarak, 2001).

1.2 Protein enriched pasta quality

As for breads, pasta quality is affected by the partial substitution of wheat semolina with non-wheat flours or protein isolates. Pasta quality can be evaluated from the measurement of the water absorption during cooking, cooking losses and the texture of cooked pasta. Cooking losses correspond to the leaching of particles into the cooking water. Low cooking loss are significant of pasta resistance to disintegration during cooking. The principal leached component is amylose (Matsuo, Malcolmson, Edwards, & Dexter, 1992). Numerous studies have been made on legume enriched pasta showing that above 10% of legume incorporation rate, cooking losses generally increase. The enrichment of pasta with 35% faba flour leads to an increase of 13% of cooking losses (Laleg, Barron, Santé-Lhoutellier, Walrand, & Micard, 2016). Similarly, the enrichment of pasta from 5% to 30% with lupin flour leads to an increase of up to 35% of the cooking losses (Rayas-Duarte, Mock, & D., 1996). Similar results were obtained on pasta enriched either with pea, split pea, lentil or chickpea (Petitot et al., 2010; Zhao, Manthey, Chang, Hou, & H., 2005). On the opposite, a pasta enrichment with up to 20% gluten leads to a 22% reduction of its cooking loss (Fardet et al., 1999).

The firmness is also a frequently measured criterion for pasta quality assessment. However, the effect of protein enrichment on firmness is not clear. It has been shown that the addition of chickpea flour above 20% (Wood, 2009) and of wheat bran protein concentrate above 10% (Alzuwaid, Fleming, Fellows, & Sissons, 2021) decrease pasta firmness. On the opposite, the addition of up to 20% lupin protein isolate (Georgios Doxastakis et al., 2007), up to 30% chickpea flours (Zhao et al., 2005) and of 6% gluten and 5% egg powder (Laleg, Barron, et al., 2016) increased the pasta firmness. In addition, no significant effect of faba flour enrichment at 35% was observed on pasta firmness (Laleg et al., 2017). It is difficult to have consistent results between studies because the pasta firmness does not only depend on gluten. Pasta firmness may be affected by the amount of water absorbed during cooking, the total amount of protein in the pasta, the amylose content (Gianibelli, Sissons, & Batey, 2005) and the content of non-starchy polysaccharides that can be brought by the added flours. In addition, pasta firmness may vary depending on the method used to measure it. Finally, the sensory

properties are also regularly used to measure the organoleptic quality of the pasta. In addition to analytical textural measurements, sensory properties can provide information on the texture of cooked pasta. Petitot et al. (Petitot et al., 2010) showed that an enrichment in faba bean or split pea flours at 35% leads to an increase in pasta hardness, elasticity and fracturability as measured by sensory evaluation.

2 Modification of the gluten network structure as a result of protein enrichment

The assembly properties of gluten proteins during the food process are the basis for the quality of food products. A partial substitution of semolina or wheat flour by another ingredient will result in a modification of its technological properties by modifying the structure of the food.

2.1 *The gluten network dilution*

Most studies explain the deleterious effect of protein enrichments on the quality of wheat-based products by the proportional decrease in gluten proteins due to semolina partial substitution (G. Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002; Laleg et al., 2017; Paraskevopoulou et al., 2010; Rayas-Duarte et al., 1996). The gluten is diluted by the addition of a non-wheat ingredients; this is called the "dilution effect". Gluten dilution depreciates the strength of the protein network, resulting in a weakening of the overall structure of the food and a depreciation of its texture. For instance, the protein network in bread is not able to retain the gas cell expansion anymore. In pasta, the protein network cannot avoid solids to leach in cooking water anymore. The dilution effect can be illustrated by the evolution of SDS-solubility of protein according to the gluten substitution level in pasta. Sequential extraction of proteins in a denaturing buffer (usually containing SDS)¹ and then in a denaturing and thiol-reducing buffer (as SDS-DTE² buffer) allows the extraction of proteins and small polymers bound by weak bonds and larger disulphide-linked polymers, respectively. These sequential extraction provide an indication of the state of aggregation of proteins in the system. **Figure 17** displays the evolution of SDS and SDS/DTE soluble proteins for faba flour enriched pasta (Laleg et al., 2017). With an increasing level of faba enrichment from 0 to 100%, the SDS-soluble protein content increases linearly at the expense of SDS-DTE soluble proteins, meaning that the global aggregation state of the protein decreases. The linearity of the reduction results from the linear diminution of gluten content due to semolina substitution by faba flour.

In addition to this dilution effect, some studies performed on protein-enriched bread or pasta mention a "gluten disruption". This term refers to a mechanical disruption linked to the steric hindrance that can be caused by the presence of protein particles (Carini et al., 2012; Graça et al., 2018; Paraskevopoulou et al., 2010).

¹ SDS : Sodium dodecyl sulfate

² DTE : dithioerythritol

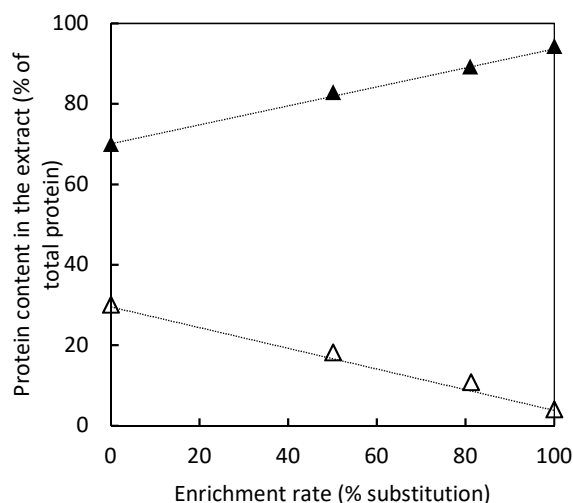


Figure 17. Evolution of SDS-soluble protein content (full symbols) and SDS insoluble but SDS-DTE soluble protein content (empty symbols) on pasta according to their enrichment rate with faba flour. Dotted lines are included to guide the eye. Data are from (Laleg et al., 2017).

2.2 Protein-protein interactions resulting in the gluten network modifications

In addition to dilute the gluten proteins, some proteins may also interact with gluten proteins. The addition of egg proteins contributes to the formation of pasta with a more compact structure and better cooking behaviour (Alamprese, 2017; Laleg, Barron, et al., 2016). The effects of the addition of whole egg, egg white or egg yolk has been studied on noodles. At flour to egg protein ratio of 3:2 and 2:1, proteins in whole egg were shown to polymerise to a larger extent than in control noodles (Lambrecht, Rombouts, Nivelle, & Delcour, 2017). This polymerisation is enhanced in noodles enriched with egg white. This was explained by the formation of intermolecular disulphide cross-links between ovalbumin and probably between gluten and ovalbumin, stimulating the polymerisation of gluten proteins (Wouters, Rombouts, Lagrain, & Delcour, 2016). However, whereas the addition of whole egg has no significant impact on cooking loss, the addition of egg white increases cooking loss (Lambrecht et al., 2017). Indeed, the extensive and rapid polymerisation of proteins in egg-white enriched noodles may alter the ability of the protein network to cope with starch swelling (Lambrecht et al., 2017). This highlighted the importance of the ratio of egg white to egg yolk.

It has been shown that soy proteins also interact with gluten proteins through covalent and non-covalent bonds during dough mixing (Ribotta, León, Pérez, & Añón, 2005). However, these interactions impairs the gluten network by reducing the overall size of the polymers, resulting in a more porous network (Pérez, Ribotta, Steffolani, & Le, 2008) and a poor bread quality (Ribotta, Arnulphi, et al., 2005). The deleterious effect of some proteins is also regularly associated with the competition for water with gluten and starch, leading to incomplete gluten network development or to the incomplete starch gelatinisation (Alzuwaid et al., 2021; Carini et al., 2012; Zhou et al., 2018).

2.3 Example of technological solutions to counter these deteriorations

i. The use of additives

A way to improve the quality of protein-enriched pasta is to add other hydrocolloids to mimic the viscoelasticity of gluten (Wang et al., 2018). The addition of carboxymethylcellulose (CMC) can increase the viscosity of the dough and improve the structure and volume of the bread, probably due to the

increased water absorption (Correa, Ferrer, Añón, & Ferrero, 2014). The addition of CMC and gluten in a dough enriched with legume flours (pea, soybean and chickpea) results in a bread that meets textural and sensory expectations (Angioloni & Collar, 2012). Another study has shown that adding 2% guar gum to pasta enriched with 15% pea flour reduces cooking losses (Padalino et al., 2014). Similarly, the addition of 2% guar seed flour in bread enriched with pea flour at 5%, led to softer crumbs and higher scores in sensory analysis (Mastromatteo et al., 2015). Incorporation of faba flour sourdough containing dextran produced by bacterial fermentation improved the dough viscoelastic properties, and increased the specific volume (~21%) of the final bread (Wang et al., 2018).

ii. *The modification of process parameters*

The use of high temperatures for pasta drying was shown to improve their culinary quality (Zweifel, Handschin, Escher, & Conde-Petit, 2003). A high drying temperature promotes protein aggregation and leads to a strong protein network that entraps the starch granules, which prevents the leaching of amylose into the cooking water and thus reduces stickiness (Petitot, Brossard, et al., 2009; Zweifel et al., 2003). Increasing the drying temperature from 55°C to 90°C also enhances the protein polymerisation in pasta fortified with faba flour from 0 to 100%, leading to increased resilience and reduced cooking loss, reflecting the strengthening of the protein network (Laleg et al., 2017; Petitot et al., 2010). However, Petitot et al. (2010) highlighted that Maillard reactions that are promoted during drying are enhanced with higher temperature leading to undesirable brownish colour. Although high drying temperature tended to decrease starch and protein *in vitro* enzymatic hydrolysis in durum wheat pasta (De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007; Petitot, Brossard, et al., 2009), the degree of protein hydrolysis in faba flour enriched pasta was not altered by heat treatment at any enrichment rate (Laleg et al., 2017).

3 Impact of a protein enrichment on wheat-based food nutritional value

3.1 *General description of the nutritional value of dietary proteins*

The metabolism of body proteins is a dynamic process in which proteins and amino acids are continuously synthesised (anabolism) and degraded (catabolism). The physiological role of protein digestion is to provide the amino acids necessary for the body protein maintenance and growth (Mariotti, Huneau, Mahe, & Tomé, 2000). Maintenance involves a neutral nitrogen balance between nitrogen input and output, while growth involves the deposition of protein in the tissues. Apart from their use as substrates for protein synthesis, amino acids are also used as precursors for the biosynthesis of compounds that play various physiological roles. Traditionally, the nutritional quality of dietary proteins corresponds to their capacity to meet, for a given intake, the nitrogen and amino acids needs to ensure growth and maintenance of tissues. This ability depends on the composition of proteins in essential amino acids (EAA), on their digestibility and on their metabolic use. Many methods are available to assess the quality of a protein intake. *In vivo* measurements of changes in growth performance according to the ingested protein provide a simple method of assessing the quality of protein intake. *In vitro* evaluation of the digestive bioaccessibility of proteins and amino acids can be performed by measuring the release of amino acids after exposure of the protein to the action of proteases under standardised conditions. Such methods allow the estimation of protein digestibility and/or the availability of certain amino acids.

Part of the amino acids assimilated by the intestine will enter into oxidative-type metabolic pathways and will therefore be unavailable for protein synthesis in the liver and other organs. These oxidative losses decrease the metabolic bioavailability of amino acids and are sometimes considered to be inversely correlated with protein quality. They are assessed *in vivo* using coefficient based on nitrogen balance measurements.

A final traditional approach is to analyse two parameters that govern the capacity of a protein to satisfy protein requirements: its composition in EAA and its digestive utilisation. The chemical index or chemical score is used to assess the quality of a protein based on its EAA composition. The chemical score of an amino acid corresponds to its content in the protein as a percentage of its content in a reference protein or in an ideal amino acid profiles determined based on the EAA requirement for human. When the chemical score of an amino acid is below 100, the AA is deficient in the protein. The chemical score of a protein or a product corresponds to the chemical score of the most deficient EAA. To take into account the differences in digestibility between proteins, an index weighting the chemical index by digestibility has been proposed: the PD-CAAS (Protein Digestibility Corrected Amino Acid Score). It is calculated as follow,:

$$PDCAAS (\%) = \frac{\text{Chemical score of 1g of dietary protein} \times \text{True faecal protein digestibility}}{100}$$

The true digestibility is assessed in growing rats and corresponds to the ratio between the quantity of nitrogen digested and the quantity of nitrogen ingested taking into account the endogenous nitrogen losses not specific to the feed produced during the digestive process. When the PDCAAS is below 100% for a dietary protein, it indicates that this dietary protein cannot fully meet the body's requirements for EAA.

3.2 *Benefits of a protein enrichment on the amino acid composition of wheat-based foods*

Wheat proteins are known to contain low levels of certain amino acids such as lysine and threonine (Abdel-Aal & Hucl, 2002). **Table 4** represents the chemical scores of durum wheat grain, durum wheat semolina and durum wheat pasta compared to the ideal amino acid profile proposed by AFSSA in 2007. In this case, the only limiting amino acid in semolina and dough is lysine with very low chemical scores of 49 and 47%. Threonine is not limiting when its chemical score is calculated according to the ideal amino acid profile proposed by AFSSA in 2007. Process steps can reduce the content of EAA. For example, the content of lysine, methionine, tryptophan, and histidine is reduced by 8, 16, 13 and 11%, respectively in pasta compared to semolina (Abdel-Aal & Hucl, 2002). A further reduction in lysine availability has been measured in high-temperature dried pasta (Dexter, Tkachuk, & Matsuo, 1984), which may be due to Maillard reactions involving proteins and reducing sugars and carbohydrates (Anese, Nicoli, Massini, & Lerici, 1999).

Table 4. Chemical scores of Essential Amino Acids (EAA) of durum wheat grain, durum wheat semolina and durum wheat pasta calculated using the ideal amino acid profile proposed for Adult by (AFSSA, 2007) as a reference profile. Data of amino acid profiles were taken from (Abdel-Aal & Hucl, 2002).

Essential Amino acids	Durum wheat grain	Durum wheat semolina	Durum wheat pasta
Methionine +Cysteine	157%	170%	161%
Histidine	135%	124%	112%
Isoleucine	96%	115%	111%
Leucine	119%	122%	119%
Lysine	53%	49%	47%
Phenylalanine + tyrosine	178%	193%	195%
Threonine	120%	124%	116%
Tryptophan	217%	183%	167%
Valine	176%	160%	152%

The protein enrichment of wheat-based foods not only increases the overall protein content but also improves the EAA profile. Legumes, egg proteins and milk proteins have much higher lysine contents than wheat grains. Their incorporation in wheat-based foods improves the overall chemical score of the food. For example, the addition of lupin at 30% substitution rate increases the lysine content of pasta by 126% (Rayas-Duarte et al., 1996). Similarly, the enrichment of pasta with faba flour at 35% leads to an increase in lysine content from 20.5 to 38.6 mg per gram of protein (Laleg et al., 2017). Enrichment with 65% faba flour allows obtaining pasta without lysine deficiency in comparison to the recommendations of WHO/FAO/UNU recommended levels (Berrazaga, Bourlieu-Lacanal, et al., 2020).

Despite the improvement of the nutritional profile, the addition of plant proteins to wheat-based foods may increase their content of certain bioactive compounds, considered as anti-nutritional factors. Legumes generally contains anti-nutritional factors such as protein inhibitors and phytic acid (Chouchene, Micard, & Lullien-Pellerin, 2018; Dijkstra, Linnemann, & Van Boekel, 2003). Protease inhibitors inhibit the action of various digestive enzymes such as trypsin, chymotrypsin or amylase, which reduces the digestibility of starch and protein. Trypsin inhibitors inactivate digestive enzymes and thus reduce protein availability. The trypsin inhibitor activity would be very sensitive to the processing and cooking stages of pasta. After cooking, pasta enriched with 20% lentil or pea flours no longer showed trypsin inhibitory activity (Zhao et al., 2005). Processing and cooking of 100% faba four pasta allow the decrease of trypsin inhibitor activity of 68% (Laleg, Cassan, Barron, Prabhasankar, & Micard, 2016). On the opposite, phytic acid which is recognised as an inhibitor of minerals absorption in human (Sandberg, 2002), is only slightly affected by the process (Laleg, Cassan, et al., 2016). Despite their potential anti-oxidant activity, phenolic compounds, often present in flours or concentrates of plant proteins, can reduce protein hydrolysis rate due to their enzyme inhibitor activity (Rohn, Rawel, Wollenberger, & Kroll, 2003). Moreover, it has been suggested that phenolic acids and flavonoids reacting with proteins could decrease the availability of EAA such as lysine and tryptophan (Rawel et al., 2002).

3.3 Modification of protein digestibility

The digestion process includes mechanical, chemical and enzymatic steps necessary to release the amino acids and facilitate their absorption. The introduction of non-wheat protein can affect the enzymatic degradation of the protein network. It has been shown that increasing the faba flour content of pasta from 0% to 100%, significantly increased the *in vitro* protein hydrolysis rate from 42% to 52% (Laleg et al., 2017). The authors related this increase of *in vitro* protein hydrolysis rate to the weakening of the protein network in enriched pasta. A slight increase of the *in vitro* protein hydrolysis was also reported in 30% lupin enriched pasta (Rayas-Duarte et al., 1996) and in wheat-based snacks enriched at 15% with chickpea, lentil and pea flours (Patil, Brennan, Mason, & Brennan, 2016). In contrast, the incorporation up to 20% of cowpea flour does not modify the *in vitro* protein digestibility in macaroni (Nur Herken, Ibanoglu, Öner, & Ibanoglu, 2006). The *in vitro* protein digestibility was decreased with an increasing content of spirulina protein concentrate in pasta up to 20% (Rodríguez De Marco, Steffolani, Martínez, & León, 2014). According to the authors, this drop in the rate of proteolysis could result from formation of a protein/phenolics complex that inhibits proteolytic activity. The impact of protein enrichment on the digestibility of proteins in cereal matrices has also been evaluated *in vivo*. The protein efficiency ratio, corresponding to the amount of weight gain per unit of protein consumed, were shown to be increased in bread and in maize tortillas with an increasing percentage of soybean proteins (Acevedo-Pacheco & Serna-Saldívar, 2016; Mizrahi et al., 1967). The nutritional values of legume-enriched pasta proteins were compared to those of casein-based diets. It has been shown that rat diets composed of legume-enriched pasta (62% faba-bean enriched pasta, 65% lentil enriched pasta and 79% split pea enriched pasta) had a 5% lower protein digestibility than casein-based diet but similar net protein utilisation or skeletal muscle protein synthesis (Berrazaga, Salles, et al., 2020). Laleg et al. shown that rat diets composed of pasta enriched at 35% of faba bean flour lead to similar growth rate and muscle weights than in casein-based diets.

Keypoints: The enrichment of wheat-based food with plant proteins is motivated by an improvement of their amino acid profile and an increase in their total protein content. The effects of these enrichments on culinary and textural properties depend on the type of protein used, the level of enrichment and the protein content of the added ingredient. In many cases, protein enrichments induce dilution or structural modifications of the gluten network, leading to the deterioration of the quality of the finished product. Protein proteolysis during gastrointestinal digestion may be modified but *in vivo* studies report a general tendency of improving the nutritional performance.

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Chapter 2. Objectives, experimental approach and methods of the study

Part 1. Objectives and experimental approach

The GreenProtein project aims at establishing a DEMO plant for the extraction and purification of a food grade and functional LPC, containing mostly RuBisCO. As reported in the previous section, RuBisCO has high level of lysine and is therefore an interesting protein for enriching wheat-based foods to improve their amino acid profile. However, a protein enrichment of wheat-based foods often leads to the alteration of the food textural, organoleptic and nutritional properties. For many systems, this results from the dilution of the gluten network by the non-wheat added proteins. To our knowledge, no study has been performed on the properties of LPC-enriched wheat-based matrices. When dispersed in water at neutral pH, RuBisCO has very good gelling properties, due to its solubility and its ability to form a network after thermal denaturation. Moreover, in the native state, RuBisCO contains several sulfhydryl groups buried in its quaternary structure (Hood, Cheng, Koch, & Brunner, 1981). If these thiols groups remain reduced after the purification of the LPC, RuBisCO could be chemically incorporated into the polymeric structure of glutenins through thiol/disulphide interchange reactions under the effect of heating. The consequences of such interactions could be beneficial for the gluten network structure and the product final quality.

The general objective of this thesis is to investigate the effect of rubisco enrichment on the quality of wheat-based products. To do so, micro wheat semolina dough was used as a model food system and the effects of gluten and pea proteins enrichments were also studied as a comparison. The experimental approach was divided into three parts, as represented in **Figure 1**.

- * The first part consists in the detailed characterisation of the biochemical and physical-chemical properties of the LPC (Chapter 3). The objective is to determine the composition of LPC and the major characteristics of RuBisCO using a combination of techniques based on biochemical assay, chromatography, and spectroscopy.
- * The second part consists in the study of mechanical and protein structural changes of wheat dough induced by the protein enrichment. The impact of RuBisCO, gluten and pea proteins on dough structuring during heating, on protein polymerisation both before and after dough thermal treatment and on the overall structure of dough was studied (Chapter 4).
- * The third part addresses the impact of RuBisCO enrichment on the *in vitro* digestion of proteins in enriched wheat doughs (Chapter 5). The sensitivity of proteins to hydrolysis was assessed by the degree of hydrolysis and the rate of nitrogen solubilisation during the digestion. The *in vitro* digestibility of proteins was estimated from the nitrogen content in the soluble fraction of digesta with molecular weight below 10 kDa.

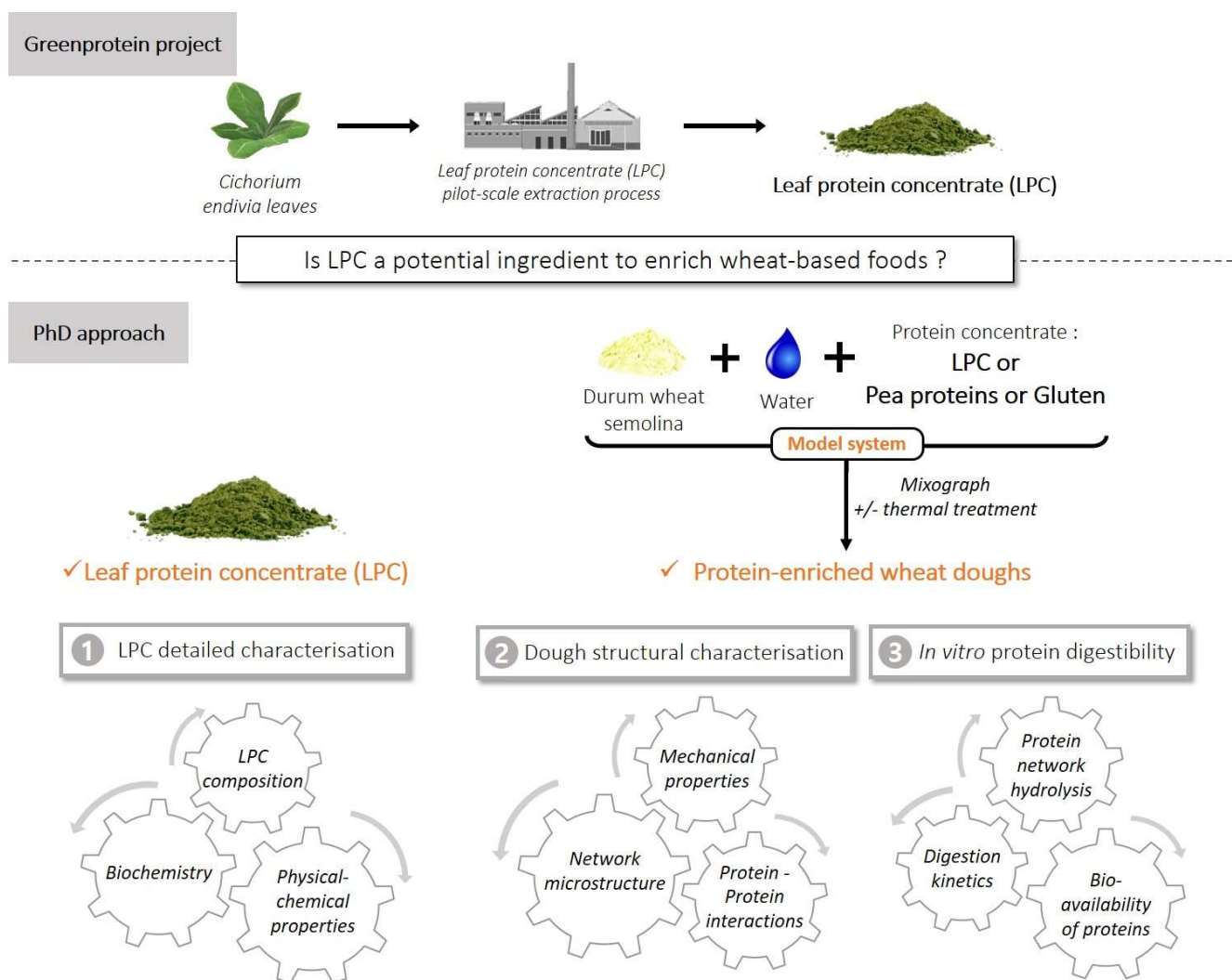


Figure 1. Scheme of the PhD experimental approach.

Part 2. Source of raw materials and production of model systems

The following section aims at providing experimental details about the raw materials and the method of micro-doughs fabrication. The chapters 3, 4 and 5 of this manuscript include a description of the material and methods specific to the results presented in each chapter. To avoid any redundancy, these methods are not described in the following section.

1 Source of raw materials

1.1 Choice and origin of raw materials

The dense wheat matrix model system used in this study is made from durum wheat semolina, as one of the work package of the Green protein project aims at producing RuBisCO-enriched *durum* wheat pasta. We decided to compare the behaviour of RuBisCO in wheat matrices with that of pea proteins, since pea protein concentrates are one of the most popular plant protein concentrates in the food industry in Europe, after gluten and soy protein. Gluten-enriched matrices have also been studied to have a 100% wheat matrix with a high protein content.

Wheat semolina was provided by La Semoulerie de Bellevue (Panzani, Marseille, France) and pea protein concentrate (Nutralys, F85F) was purchased from Roquette (Lestrem, France). The gluten was extracted from a durum wheat semolina dough based on Auger, Morel, Dewilde, & Redl (2009) (more details in chapter 4). RuBisCO was extracted from *Cichorium endivia* leaves by a partner of GreenProtein project, using the extraction conditions described below.

1.2 Pilot-scale extraction process of LPC

In the GreenProtein project, RuBisCO is extracted from green wastes using a purification process patented by the TNO (Netherlands Organisation for Applied Scientific Research) (WO 2014/104880). **Figure 2** illustrates the steps of the process, based on classical leaf protein concentrate extraction process presented in the Literature review of this manuscript. The juice is extracted from raw material by pressing using a twin-screw press with addition of a reducing agent (e.g. sodium metabisulphite) to avoid phenolic oxidation and cross-linking. Membrane proteins and pigments are then coagulated upon heating at 50°C for 20 minutes. After cooling and decantation, cross-flow microfiltration step on a 0.2 µm membrane allows the sterilisation of the supernatant. The juice is then concentrated by ultrafiltration using a 100 kDa molecular weight cut-off and subsequently diafiltered to remove salts, phenolics, and other impurities. In addition, the process also involves a hydrophobic chromatography of the diafiltered juice to separate and remove the residual phenolic compounds and chlorophyll. This step was not performed in the production of the LPC used in this study. The LPC used in the study was freeze-dried. The DEMO plant, optimised through the GreenProtein project, is expected to process 1500 kg/h of plant wastes, leading to the extraction of 12 kg of protein per hour.

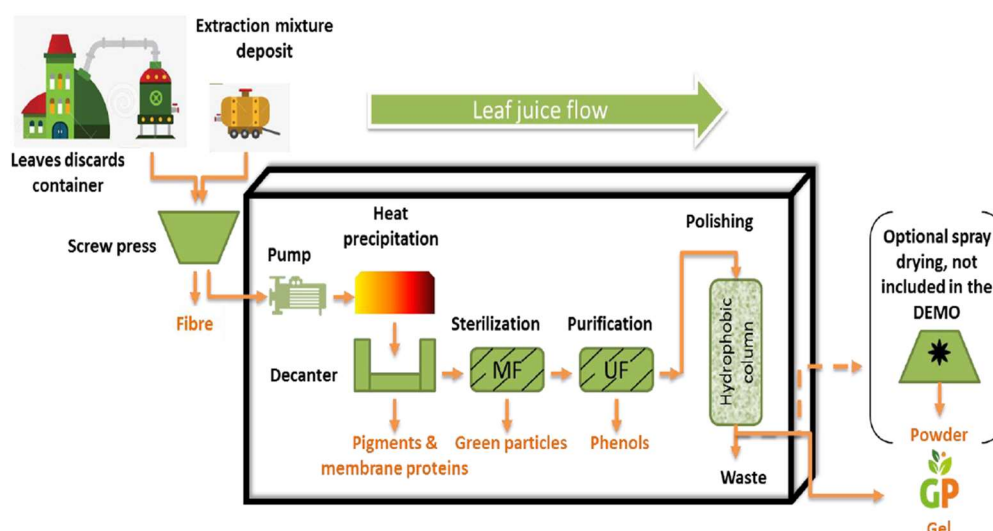


Figure 2. Successive steps of the DEMO-scale LPC extraction process based on the patent WO 2014/104880. Source: GreenProtein BBI proposal.

2 Composition and fabrication of the wheat doughs

The study of RuBisCO behaviour in wheat matrices was performed on model systems rather than in real food due to the low availability of LPC (~ 35 g) at the beginning of the project. The model system chosen is a micro wheat dough allowing to obtain homogeneous wheat matrices with a developed gluten network.

2.1 Protein composition of doughs

To produce homogeneous micro-dough, 200 µm sieved-semolina was used. The 100% semolina dough was considered as « control dough » in the whole study. **Table 1** sums up the dough composition according to the substitution level used for each protein concentrate. For the protein-enriched doughs fabrication, from 1.5% to 10% of the semolina weight was replaced with one of the protein concentrates. In this way, protein-enriched doughs have the same content of wheat proteins that come from semolina, also called intrinsic proteins, for a given substitution level regardless of the source of the extrinsic protein. However, this implies that the total protein content of enriched doughs slightly varies depending on the protein concentrate. Since we work with protein concentrates and at low substitution rates (10% w/w maximum), these variations are below 1.5%. In this manuscript, the composition of the enriched doughs will be expressed in terms of extrinsic protein content in percentage of total proteins.

Table 1. Total content and protein composition of the doughs according to the semolina substitution rate and the type of protein concentrate used. “Intrinsic proteins” corresponds to the semolina proteins, “extrinsic proteins” stands for the proteins coming from the added protein concentrate.

Dough composition	Semolina substitution (% wb)	Intrinsic proteins	Extrinsic proteins	Total protein content	Intrinsic proteins	Extrinsic proteins
		<i>g / 100g db</i>	<i>g / 100g db</i>	<i>g / 100g db</i>	% total protein	
Control	0.0	14.0	0.0	14.0	100.0	0.0
RuBisCO	1.5	13.8	1.1	14.9	92.4	7.6
	3.5	13.5	2.6	16.2	83.7	16.3
	6.0	13.2	4.5	17.7	74.4	25.6
	8.0	12.9	6.0	18.9	68.1	31.9
	10.0	12.6	7.5	20.1	62.6	37.4
Gluten	1.5	13.8	1.0	14.8	93.1	6.9
	3.5	13.5	2.4	15.9	85.1	14.9
	6.0	13.2	4.1	17.2	76.4	23.6
	8.0	12.9	5.4	18.3	70.4	29.6
	10.0	12.6	6.8	19.4	65.0	35.0
Pea proteins	1.5	13.8	1.2	15.0	92.0	8.0
	3.5	13.5	2.8	16.3	82.9	17.1
	6.0	13.2	4.8	17.9	73.4	26.6
	8.0	12.9	6.4	19.2	67.0	33.0
	10.0	12.6	7.9	20.5	61.3	38.7

2.2 Wheat dough fabrication

i. The 2 g mixograph

The protocol of dough production was built to yield to a homogenous, non-sticky dough with an optimum protein network development. We used a mixograph prototype that produces dough with only 2 g of semolina or semolina-protein concentrate mixture. This set-up was adapted to the small amount of RuBisCO available during the first part of the project. As shown in chapter 1, the mixograph is a recording dough mixer. It consists in mixing wheat flour with water and recording the torque generated by the dough resistance to mixing. The registered torque allows the calculation of many parameters, which reflect dough properties and protein network development (Walker & Walker, 1992).

Figure 3 represents the three main entities that composed the mixograph: the mixer, the control box and a computer. The mixograph has four moving spins on the upper part and three fix spins on the lower part. This provides both elongation and shear to the dough during mixing (Gras, Carpenter, & Anderssen, 2000). The control box provides the electric alimentation and the speed control. The computer is used for data acquisition and treatment. In addition, the mixograph was equipped with a

homemade aluminium double-walled jacket coupled with a temperature-controlled water bath to control the bowl temperature during the dough fabrication.

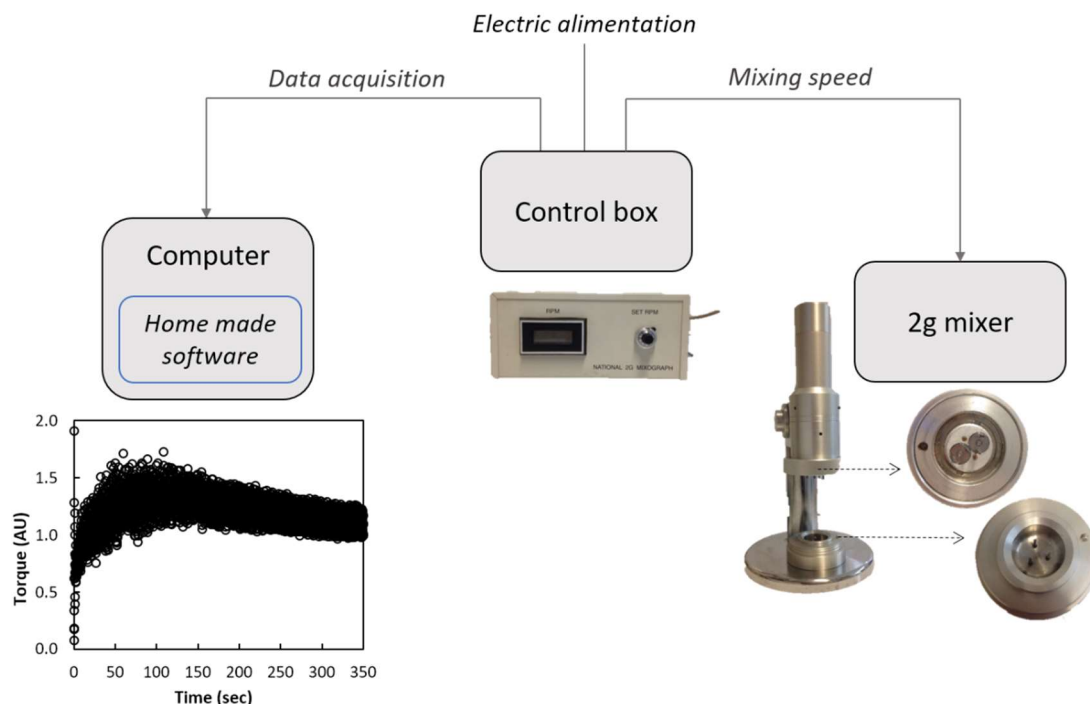


Figure 3. The 2g mixograph assembly

ii. Dough fabrication protocol

Two major parameters are usually determined to obtain an optimally developed dough: the mixing time and the water absorption. The optimum mixing time corresponds to the time required for optimum dough development. The water absorption corresponds to the amount of water needed to obtain a dough which consistency is about 500 BU when optimally mixed using a Brabender farinograph. Unfortunately, the amount of available LPC was not enough to perform farinographic assays for each dough composition. Attempts were made to transpose the farinograph water absorption measurement method to the 2 g-mixograph scale as previously described (Reiko, Aya, Hideki, & Masaharu, 2016) but they were unsuccessful. We decided to work with constant water content of 67% (db) for all dough compositions. This value was determined after empirical tests using the 2 g-mixograph to obtain a homogeneous cohesive dough. The optimum mixing time was determined to be 220 sec on average measurements performed on several doughs.

The dough making protocol is shown in **Figure 4**. The powders, wheat semolina and protein concentrate, are first mixed. The water is then added and the mixture is homogenised in the mixing bowl for 6 s at 54 rpm at ambient temperature. The mixture is left to rest for 15 min at 40 °C and cooled to 20 °C over a period of 25 min to ensure the hydration of the semolina particles. The dough was then mixed at 20°C at 54 rpm for 220 s. The hydration step was necessary to obtain a non-sticky and homogeneous dough.

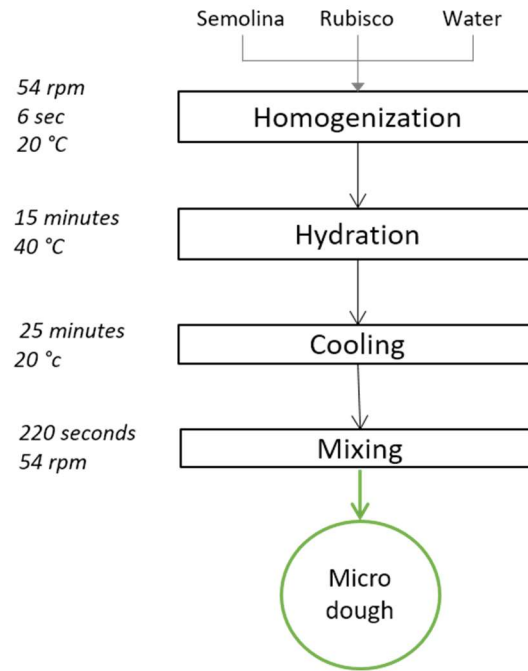


Figure 4. Steps for producing the micro wheat doughs using the 2g mixograph equipped with a double-walled jacket coupled with a temperature-controlled water bath.

To study the effect of heat on dough protein structure, a thermal treatment was applied to the mixed dough. A hermetically sealed aluminium container was built to contain 1.2 g of dough. Directly after mixing, the device was filled with 1.2 g of dough and immersed in a water bath at 80 °C for 3 min. The dough sample was then removed and immersed in liquid nitrogen before freeze-drying. The biochemical, spectroscopic and digestibility studies were performed on freeze-dried doughs.

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Chapter 3. Characterisation of the leaf protein concentrate

Preamble

Since its discovery, numerous characterisation studies have been carried out on highly purified RuBisCO. These studies aimed at understanding the structural basis of the ability of RuBisCO to fix CO₂. In the field of food science and technology, many studies report the improvement of extraction techniques in relation to functional properties of the leaf protein concentrate (LPC) (reviewed in Chiesa & Gnansounou, 2011). However, the characterisation of the LPC resulting from these processes is fragmented and the reported properties varies depending on LPC origin. The behaviour of proteins in food systems are closely related to their biochemical and physical-chemical characteristics. Therefore, the present section describes the biochemical and physical-chemical characterisation of the leaf protein concentrate extracted from *Cichorium endivia* leaves obtained from Florette (Lessay, France) in the framework of the GreenProtein project.

Main questions:

- What is the composition of our LPC ?
- What are the biochemical characteristics of the LPC protein ?
- What physical-chemical characteristics define the RuBisCO dispersion ?

A combination of techniques based on biochemical assay, chromatography, spectroscopy and optics were used to investigate the composition of the LPC and the main parameters related to RuBisCO.

Main results:

- ✓ The leaf protein concentrate contains 74.1% of protein and sugars are the major non-protein contaminant.
- ✓ The major protein of LPC is RuBisCO; the small and large chains of RuBisCO were identified as well as a large-chain dimer.
- ✓ Parameters such as UV-visible absorbance, refractive index and electrophoretic mobility display unconventional values, presumably due to the presence of non-protein contaminants such as phenolic compounds.
- ✓ Chromatography experiments under different physical-chemical conditions suggest that these compounds are bound to the proteins via both covalent interactions and weak bounds.

Biochemical and physical-chemical characterisation of leaf proteins extracted from *Cichorium endivia* leaves

The results presented in this section are under submission.

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1 Introduction

Leaf proteins were first described by Rouelle in 1773. Researches on leaf proteins were stimulated in the early 1940s when Norman Pirie discussed the potential of leaf protein concentrates (LPC) as a source of proteins for human consumption to overcome wartime food shortages (Pirie, 1942). Proteins constitute between 15 to 20% of the leaf dry mass and most of them (75%) are located in the chloroplast (Ellis, 1979). Leaf proteins are often classified according to their affinity with water. Water-insoluble proteins, also called "green proteins", are mainly embedded in the membrane of plant organelles and often associated with lipophilic pigments such as chlorophyll (Thornber, 1975). Water-soluble proteins, also called "white proteins", are mainly composed of chloroplastic and cytoplasmic enzymes. The first commercial LPC production was developed in 1967 on alfalfa leaves (U.S. Congress, 1983) resulting in a sole green concentrate rich in proteins and xanthophylls for monogastric and poultry feed (Knuckles, Spencer, Lazar, Bickoff, & Kohler, 1970). Once improved, the pilot-scale process allowed the fractionation of water-soluble and water-insoluble proteins, resulting in a green LPC with lower protein content and in a white protein concentrate, suitable for human consumption (de Fremery et al., 1973; Edwards et al., 1975). This white LPC is mainly composed of a protein named Ribulose-1,5-BISphosphate Carboxylase/Oxygenase (RuBisCO).

RuBisCO is a key enzyme in photosynthetic carbon assimilation, catalysing the first step of CO₂ fixation in the Calvin cycle (Trown, 1965). RuBisCO is found in most autotrophic organisms from prokaryotes to eukaryotes. Despite its deficient specificity and its low carboxylase activity, RuBisCO fulfills its carbon fixation functions in plants thanks to its high level of expression in the growing leaf. The enzyme represents up to 30-50% of the soluble proteins in leaves. RuBisCO is claimed to be the most abundant protein on Earth (Kung, 1976; Phillips & Milo, 2009). Estimations report that there would be constantly 5 kg of RuBisCO per human (Phillips & Milo, 2009). RuBisCO is a hexadecameric protein, consisting of 8 large chains (LC) and 8 small chains (SC) arranged around a four-fold axis. The quaternary structure of RuBisCO mainly relies on electrostatic interactions although it was shown that LC tends to cross-link by disulphide bridges in oxidative stress conditions (Mehta, Fawcett, Porath, & Mattoo, 1992). The active site of RuBisCO is formed by the LC folding (Andersson et al., 1989). However, the SC is expected to have a structural role by maintaining the assembly of catalytic units (Schneider et al., 1990). In

addition to its abundance, it was mentioned from the early 1940s that RuBisCO was interesting for human nutrition because of its balanced amino acid profile (Pirie, 1942). Some authors even compare the nutritive value of RuBisCO to that of casein (Ershoff, Wildman, & Kwanyuen, 1978; Hood, Cheng, Koch, & Brunner, 1981).

Numerous characterisation studies have been carried out on highly purified RuBisCO to understand its structure and its enzymatic activity to improve its carbon fixation rate. In the field of Food Science and Technology, many studies address the extraction techniques in relation to functional properties of the LPC (reviewed in Chiesa & Gnansounou, 2011). However, the characterisation of the LPC resulting from these processes is fragmented and, to our knowledge, no exhaustive study characterises in details the biochemical and physical-chemical properties of one concentrate. The present paper describes in detail the biochemical and physical-chemical characterisation of a leaf protein concentrate extracted from *Cichorium endivia* leaves using a pilot scale process. A combination of techniques based on biochemical assay, chromatography, and spectroscopy were used to investigate the composition of the LPC and the main physical chemical properties of RuBisCO. The experimentally measured parameters are compared with values from the literature as well as with theoretical values calculated from known RuBisCO sequences.

2 Material and methods

2.1 Leaf protein concentrate extraction and storage

Leaf protein concentrate (LPC) was provided by Florette (Lessay, France). LPC was extracted from *Cichorium endivia* leaves using the extraction conditions of the WO 2014/104880 patent but excluding the hydrophobic column adsorption step. Briefly, the leaves were first pressed to disrupt the plant cells and obtain a juice. This step was carried out in the presence of a reducing agent, sodium metabisulfite, to avoid oxidation reactions. Then, the juice was heated to precipitate chlorophyll-containing membranes and the soluble fraction was recovered. Ultrafiltration and diafiltration were then performed to remove most of the salts and phenolic compounds. The juice was then freeze-dried. To ensure constant moisture content, at least 14 days before the experiment, the protein powder was placed in a humidity-controlled chamber with a K_2CO_3 saturated salt solution at 20 °C to maintain 43.2% relative humidity.

2.2 Amino acid composition and nitrogen to protein conversion factor determination

Amino acid analysis was carried out with an amino acid analyser (L-8900, Hitachi, Paris, France) as described in (Margier et al., 2018). Briefly, four types of protein hydrolysis were performed before the analysis. Acid hydrolysis with 6 N HCl was performed for 24 hours at 110 °C to determine the amount of most amino acids. Oxidation with performic acid was performed before the hydrolysis to assay sulphur-amino acids. Leucine, Isoleucine and valine were quantified after an acid hydrolysis with 6 N HCl for 48 hours at 110 °C. The amount of tryptophan was determined after basic hydrolysis with 4 N $Ba(OH)_2$ for 16 hours at 110 °C. For each hydrolysis, norleucine was used as internal standard. The amino acid composition of RuBisCO from *lactuca sativa* was calculated using the sequences of its subunits available on UniProt (*lactuca sativa*; accession numbers of LC and SC in UniProt database: P48706 and Q40250). The whole RuBisCO sequence was obtained by adding the sequences of 8 large chains and 8 small chains, excluding the signal peptides.

The Nitrogen to Protein conversion factor (N:P factor) was computed from the ratio of total anhydrous mass of amino acids to the total mass of nitrogen, as described in (Sosulski & Imafidon, 1990). The anhydrous molecular weight of an amino acid corresponds to its molecular weight from which the molecular weight of a molecule of water (18 g.mol^{-1}) is subtracted. The Asparagine (Asn) and Glutamine (Gln) were assayed in acidic form. Therefore, they could not be distinguished from Asparagic acid (Asp) and Glutamic acid (Glu) content in the amino acid profile analysis. We estimated the content in Asn et Gln by taking the proportion of Asn/Asp and Gln/Glu from lettuce RuBisCO amino acid sequence.

From the amino acid composition, the chemical score of essential amino acids (EAA) was calculated as the ratio between the EAA content in the concentrate over its content in the reference amino acid profile established by the French Food Safety Agency (AFSSA, 2007).

2.3 Composition of the leaf protein concentrate

Water and ash content were determined by thermogravimetric analyses (TGA 2050, TA instruments, New Castle, England) under nitrogen atmosphere. About 10 mg of powder was heated at $3 \text{ }^{\circ}\text{C}/\text{min}$ until $130 \text{ }^{\circ}\text{C}$ for 30 min. The measured mass loss was attributed to water content. The water content was at 8.6%. The powder was further heated at $600 \text{ }^{\circ}\text{C}$ for 180 min to determine ash content. Measurements were done in triplicates. The protein content was analysed using the Kjeldahl procedure (NF V 03–050, 1970). Uronic acid was determined by an automated m-hydroxybiphenyl method (Thibault, 1979). Neutral sugars were analysed as to their alditol acetate derivatives by gas-liquid chromatography after hydrolysis (Englyst & Cummings, 1988). The total free phenolic compounds were determined using the Folin-Ciocalteu reagent. Polyphenols were extracted in acetone (80%) with a liquid:solid ratio of 40. The extraction was performed at 4°C applying in a ultrasounds bath for 45 minutes. The mixture was centrifuged ($9000 \times g$, 15 minutes) and the extraction was repeated once. Both supernatants were combined and evaporated under nitrogen stream. Dry extracts were then diluted in ultrapure water so as to obtain an absorbance within the linearity range of the test. In a microplate, $25 \mu\text{L}$ of sample was mixed with $125 \mu\text{L}$ of Folin Ciocalteu (10%) and $100 \mu\text{L}$ Na_2CO_3 (75 g.L^{-1}). The microplate was then incubated at $40 \text{ }^{\circ}\text{C}$ for 5 minutes before absorbance reading at 735 nm. Gallic acid was used as standard and results were expressed as gallic acid equivalent. Measurements were done in triplicates.

2.4 SDS-PAGE and Western Blot

SDS-PAGE was performed in non-reducing and reducing conditions. LPC was solubilised at 2 g.L^{-1} in sodium phosphate buffer (0.1 M, pH 8, 1 mM EDTA) and diluted twice in Laemmli buffer. β -Mercaptoethanol at $50 \mu\text{g.mL}^{-1}$ was added to the Laemmli buffer for electrophoresis under reducing conditions. Both unreduced and reduced samples were then heated at $100 \text{ }^{\circ}\text{C}$ for five minutes. Gels (4-12 % Bis-tris-Plus, Bolt) were run in MES 1% running buffer at a constant voltage of 200 V for 40 minutes. For the non-specific labeling of proteins, electrophoresis gel was incubated one hour in Coomassie blue stain (InstantBlue, Expedeon, San Diego, CA, USA), rinsed several times in distilled water and scanned.

A Western-Blot was performed using rabbit anti-RuBisCO large chain (anti-Rbcl) as described in (O'Donnelly et al., 2014) with some modifications. Briefly, the gel was equilibrated in Transfer buffer (25mM Tris, 192mM Glycine, SDS 0.1% (w/v), Ethanol 20% (v/v)) for 15 minutes after SDS-PAGE

running. Polypeptides fractionated on SDS-PAGE gels were transferred to nitrocellulose membranes at 250 mA for 90 minutes. The membrane was then washed with saturating solution (5% (w/v) milk powder in PBS 1X-Tween20 0.1%) for 30 minutes at room temperature, and rinsed three times 10 min in PBS-tween buffer. The membrane incubation with primary antibody solution (rabbit anti-Rbcl dissolved in PBS 1X, 2% milk powder at a ratio 1/10000) was performed for 1 hour. The membrane was then rinsed 3 times 10 minutes in PBS-Tween and incubated with secondary Antibody Solution (AntiLapin HRP, dissolved in PBS 1X, 2% milk powder at a ratio 1/25000) during 1 hour before being rinsed twice in PBS-tween and once in PBS. Western Bright™ Quantum (Advansta) reagent was mixed with the membrane for 2 minutes ; the membrane was then imaged.

2.5 UV-visible spectra

Determination of the specific extinction coefficient

The specific extinction coefficients were determined on LPC dispersion at pH 5 (25 mM acetate buffer), pH 7 (10 mM phosphate buffer) and pH 8 (10 mM phosphate buffer). If needed, minimal amount of NaCl was added to reach a ionic strength of 25 mM. LPC was dispersed overnight at room temperature at 3 mg.mL⁻¹ ; dispersions were centrifuged the day after for 30 minutes at 39191 x g. The total nitrogen content of supernatant was determined using the Kjeldahl procedure (NF V 03-050, 1970). Protein content was obtained using a N:P factor determined from LPC amino acid profile.

UV-visible spectra were measured on the supernatant and after six dilutions in appropriated buffers (30, 12, 6, 4, 3 and 1.5 times). UV-visible spectra were acquired using a UV-compatible microplate and a plate reader (Spark®, Tecan Trading, Ltd., Switzerland). The specific extinction coefficient at 280 nm was determined by establishing the Beer-Lambert law $A = \epsilon \cdot l \cdot c$, where A is the absorbance, ϵ the specific extinction coefficient (L.mg⁻¹.cm⁻¹), l the pathlength (cm), and c the protein concentration (mg.L⁻¹). The liquid pathlength in a standard cuvette is 1 cm, whereas the liquid pathlength in a microplate is not fixed. The liquid pathlength in the microplate wells depends on the volume of liquid, the dimensions of the microplate wells and on the shape of the meniscus at the liquid/air interface. Therefore, the liquid pathlength in each well was determined as described in Lampinen, Raitio, Perälä, Oranen, & Harinen (2012). Pathlengths were calculated as followed: $l = \frac{A_{977} - A_{900}}{A_{\text{water}; \text{cm}^{-1}}}$ with A₉₇₇ (resp. A₉₀₀) the absorption of the sample at 977 nm (resp. 900 nm) and A_{water; cm-1} the difference in water absorption at 977 and 900 nm measured on a standard cuvette with a pathlength of 1 cm.

Calculation of the theoretical UV-visible spectrum

The theoretical UV-visible spectrum of RuBisCO was calculated from LPC amino acid profile. First, the concentration of tyrosine, tryptophan and phenylalanine was calculated for 1 g.L⁻¹ LPC dispersion. Then, the theoretical UV-visible spectrum was calculated as a linear combination of individual spectrum of each AA extracted from the photochemcad database (<http://photochemcad.com/>).

2.6 Refractive index increment

The refractive index increment (dn/dc) was determined at 589.3 nm using a multi-wavelength Abbe refractometer (Anton Paar GmbH, Graz/AUSTRIA) at 20 °C for LPC dispersions, similarly to the specific extinction coefficients, at pH 5 (25 mM acetate buffer), pH 7 (10 mM phosphate buffer) and pH 8 (10

mM phosphate buffer). Theoretical dn/dc was calculated from the amino acid composition of LPC according to Zhao, Brown, & Schuck (2011).

2.7 Protein solubility

LPC was solubilised at 5 g.L^{-1} in water for 30 minutes at room temperature. The pH was adjusted to the desired value with 0.1 or 0.5 M NaOH or HCl. Dispersions were mixed on a rotary shaker for 2 hours; the pH was checked and adjusted if needed at the end. Dispersions were then centrifuged ($10000 \times g$, 20°C , 15 min) to recover soluble proteins. The nitrogen content of total dispersion and of the supernatant was analysed according to the Dumas method using a rapid MAX N exceed (Elementar, Langensfeld, Germany). About 1 mL of protein dispersion was placed in a steel crucible and injected into an oven under a stream of oxygen and helium. The combustion was operated at 900°C with an oxygen flow rate of 100 mL/min for 120 s. The gas mixture was then reduced in a reduction tube and separated on selective trap columns. Glycine was used as a standard for nitrogen content. Measurements were done in triplicates.

2.8 Electrophoretic mobility

To determine LPC isoelectric point, electrophoretic mobility was measured on LPC dispersions at pH ranging from 3 to 11. LPC was solubilised at 5 g.L^{-1} in water for two hours before being centrifuged at $12000 \times g$ for 20 minutes. One milliliter of supernatant was adjusted to the desired pH using 0.1 M HCl or 0.1 M NaOH. Electrophoretic mobility was measured in triplicate at each pH using a zetasizer Nano Series (Nano-ZS, Malvern instrument, Germany). The conductivity was about $0.7 \pm 0.3 \text{ mS.cm}^{-1}$. The whole experiment was performed twice. The theoretical net charge of lettuce RuBisCO as a function of pH was computed using the webserver pdb2pqr (<http://server.poissonboltzmann.org/pdb2pqr>), for unfolded and folded spinach RuBisCO (ProteinDataBank entry for spinach RuBisCO: 1aus). The computation was performed on spinach RuBisCO because the 3D-structure of lettuce RuBisCO was not available. Note that the theoretical net charges of unfolded spinach RuBisCO and unfolded lettuce RuBisCO were similar.

2.9 Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed using a zetasizer Nano Series (Nano-ZS, Malvern instrument, Germany). To study the effect of ionic strength, LPC was dispersed in 0.01 M phosphate buffer pH 7 to which was added NaCl to achieve given ionic strengths: 25 mM, 50 mM, 100 mM or 150 mM. The final protein concentration of all samples was $2.2 \pm 0.1 \text{ mg.mL}^{-1}$. To determine the effect of pH, LPC was dispersed in MES buffer 0.03 M pH 6, phosphate buffer 0.01 M pH 7 or phosphate buffer 0.01 M pH 8. The DLS signal was weak and noisy on protein dispersion at lower pH. The protein content of the dispersions were $1.4 \pm 0.1 \text{ mg.mL}^{-1}$ at pH 6, $1.6 \pm 0.0 \text{ mg.mL}^{-1}$ at pH 7 and $2.2 \pm 0.0 \text{ mg.mL}^{-1}$ at pH 8. The effect of protein content was measured on dispersions at 1.5 mg.mL^{-1} , 3 mg.mL^{-1} and 9 mg.mL^{-1} in phosphate buffer 0.01 M pH 7. All LPC dispersions were performed overnight, at room temperature before being centrifuged at $12000 \times g$ for 20 minutes. The measurements were performed at 20°C in manual mode: 10 runs of 30 seconds were collected and repeated twice for each measurement. From the correlation function, the size distribution was calculated using the “general purpose” analysis, provided by the commercial software.

2.10 Size-exclusion chromatography analyses

SE-HPLC of LPC dispersion in denaturing conditions

LPC proteins were extracted as described in Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'H (2000) with some modifications. Proteins were first extracted in 0.1 M sodium phosphate buffer pH 6.8 with 1% SDS at a solid to liquid ratio of 8. Extraction was performed on a rotary shaker set at 60 rpm at 60 °C for 80 minutes. The supernatant containing SDS-soluble proteins was recovered after centrifugation (39191 x g, 30 min, 20 °C). The SE-HPLC apparatus (Waters model LC Module1 plus) was equipped with an analytical column, TSK G4000-SW (7.5 x 300 mm) and a guard column, TSK G3000-SW (7.5 x 75 mm) (both from Merck, Darmstadt, Germany). The columns were eluted at ambient temperature with 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% SDS. The flow rate was 0.7 mL.min⁻¹ and absorbance was recorded from 210 nm to 700 nm. Apparent molecular weights were assessed with column calibration as described in Redl, Morel, Bonicel, Vergnes, & Guilbert (1999).

SEC of low-molecular weight species of the LPC dispersions

To remove low molecular weights contaminants, LPC was desalted by size-exclusion chromatography. LPC proteins were first solubilised in phosphate buffer (0.1 M, pH 6.8) for 3 hours at 10 °C on a rotation wheel. The LPC dispersion was then centrifuged at 12000 x g for 20 minutes. The protein concentration of supernatant was determined by absorbance using specific extinction coefficient, previously determined. The supernatant was then diluted to reach a protein concentration of 1.5 mg.mL⁻¹. Sample aliquots of 0.5 mL were injected at a flow rate of 5 mL.min⁻¹ on a Hitrap Sephadex G25 Superfine column (GE Healthcare, 1.6 x 2.5 cm, 5 mL) equilibrated in phosphate buffer (0.1 M, pH 6.4) using an Akta Avant 25 (GE Healthcare, USA). Absorbance was recorded at 260 nm, 280 nm and 325 nm during elution. The excluded volume (EV) contains the protein fraction with molecular weights higher than 5 kg.mol⁻¹. Six fractions of 1.5 mL were recovered at different elution time and their UV-visible spectra were recorded as described previously. The whole experiment was performed in triplicate.

3 Results and discussion

3.1 Composition of the leaf protein concentrate

The composition of the LPC was determined by analysing the amino acid composition, the content in nitrogen, ashes, and sugars. Results are expressed in dry matter. Amino acid composition of LPC is detailed in **Table 1** and is compared to the theoretical amino acid composition of lettuce RuBisCO. Experimental data obtained on LPC are very similar to the calculated amino acid composition of lettuce RuBisCO, except for the cysteine content. A 2.5 fold higher amount of cysteine is found in LPC than the amount calculated for lettuce RuBisCO. According to the literature, this difference in amino acid composition is not related to species (Gerloff, Lima, & Stahmann, 1965). The composition in amino acids allows the calculation of the N:P factor, which is essential for calculating the protein content from a nitrogen assay (Mariotti, Tomé, Mirand, Mariotti, & E, 2008). From the amino acid composition describes in **Table 1**, we obtained a nitrogen-to-protein conversion factor of 5.79. This value is within the range of nitrogen-to-protein conversion factor estimated for 90 plant species, which varies from 5.15 to 5.93 (Yeoh & Wee, 1994). The nitrogen content of LPC is of 12.8% according to Kjeldahl method, which leads to 74.1% of protein. LPC contains about 9.6% sugars. The significant content of sugars, detected in the LPC, probably results from leaf polysaccharides. Ashes represent 7.8% of the LPC mass

and 8.5% of LPC dry mass is of unknown origin. The LPC contains less than 5 mg of free phenolics (in gallic acid equivalents) in 100 g of powder.

Table 1. Amino acid composition determined on LPC in mg.g⁻¹ of protein as compared to calculated amino acid composition of lettuce RuBisCO (based on lettuce RuBisCO sequence found in UniProt database: *lactuca sativa*; accession numbers of LC and SC: P48706 and Q40250).

Amino acids	Amino acid residues content (mg.g ⁻¹ total amino acids residues)	
	<i>Leaf protein concentrate</i>	<i>Lettuce RuBisCO</i>
Ala	55.6	49.7
Arg	56.7	76.7
Asp	82.3	83.6
Cys	45.0	18.4
Glu	116.6	116.9
Gly	49.9	46.7
His	33.7	36.7
Ile	45.4	53.9
Leu	91.1	84.2
Lys	62.9	64.8
Met	29.3	25.4
Phe	56.7	61.3
Pro	63.9	46.2
Ser	24.4	29.8
Thr	55.5	54.2
Trp	21.4	33.2
Tyr	48.4	60.7
Val	61.4	57.5

3.2 Chemical score of LPC essential amino acids

The amino acid composition was used to compute the chemical score of LPC, reported in **Table 2**. Chemical scores of all essential amino acids are above 100%, meaning that LPC includes all essential amino acids needed for human diet. LPC has remarkable high chemical scores in sulphur amino acids (cysteine and methionine) and aromatic amino acids (tyrosine, phenylalanine, and tryptophan). The balanced amino acid profile of LPC has already been described, but sulphur amino acids, specifically methionine were found to be limiting (Gerloff et al., 1965; Hood et al., 1981). Although the experimental cysteine content is higher than expected for unclear reasons, the theoretical cysteine content of lettuce RuBisCO also gives rise to a chemical score above 100%.

Table 2. Chemical scores of LPC essential amino acids: composition in essential amino acids of LPC as compared to an ideal amino acid composition proposed by (AFSSA, 2007).

Amino acids		Chemical score
Methionine + Cysteine		322%
His	Histidine	193%
Ile	Isoleucine	167%
Leu	Leucine	154%
Lys	Lysine	137%
Phenylalanine + tyrosine		246%
Thr	Threonine	224%
Trp	Tryptophane	336%
Val	Valine	249%

3.3 Evaluation of protein purity and estimation of the molecular weights of RuBisCO sub-units

LPC dispersions were analysed by SDS-PAGE in non-reducing and reducing conditions to evaluate the protein purity and determine the molecular weight of the major proteins in denaturing conditions. Results of these analyses are shown in **Figure 1A**. Three major bands are observed on SDS-PAGE gels in both non-reducing and reducing conditions at 50, 40 and 14 kg.mol⁻¹. Bands at 50 and 14 kg.mol⁻¹ may respectively correspond to the large-chain (LC) and the small-chain (SC) of RuBisCO (Baker, Eisenberg, Eiserling, & Weissman, 1975). A Western Blot analysis was performed using anti-Rbcl (RuBisCO LC) antibody. Both bands at 50 and 40 kg.mol⁻¹ are revealed by anti-Rbcl antibody (**Figure 1B**), confirming the presence of the RuBisCO LC at 50 kg.mol⁻¹. The band at 40 kg.mol⁻¹ also contains RuBisCO LC. It may correspond to a hydrolysed form of RuBisCO LC, as previously suggested by Hood et al. (1981). Several small bands of medium intensity are observed in non-reducing conditions around 100 kg.mol⁻¹. Their intensity decreases upon reduction and they are revealed by the antibody against RuBisCO LC. These bands may correspond to disulphide-bonded dimers of LC. Dimers of RuBisCO LC have already been identified *in vivo* and *in vitro* (Mehta et al., 1992; Rintamaki, 1989). Authors related these dimers to an oxidative stress *in planta* and/or during the extraction process. A low intensity band is observed at ~35 kg.mol⁻¹. The intensity of this band increases after reduction and anti-Rbcl antibodies reveal this band. This band may also correspond to a hydrolysed form of LC, which would be part of the disulphide-bonded aggregates in non-reducing conditions. Apart from identified RuBisCO bands, less intense bands were observed between 10 and 55 kg.mol⁻¹. It is not possible to precisely quantify the RuBisCO purity of LPC based on SDS-PAGE patterns due to the protein-type dependency of Coomassie staining (Fountoulakis, Juranville, & Manneberg, 1992). However, the SDS-PAGE analysis highlights that RuBisCO is the major protein of LPC.

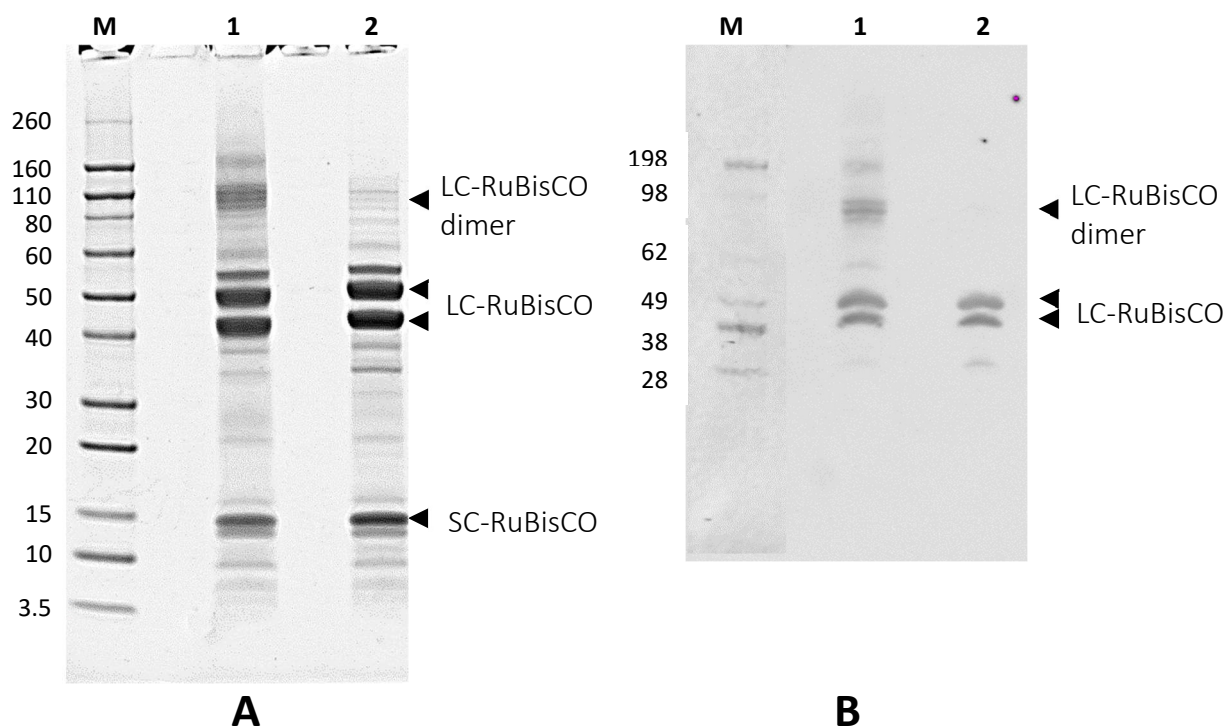


Figure 1. SDS-PAGE (A) and Western blot (B) patterns of LPC in non-reducing (lane 1) and reducing conditions (lane 2). Arrows indicate RuBisCO sub-units. LC-RuBisCO stands for RuBisCO large chain and SC-RuBisCO stands for RuBisCO small chain. Lane M corresponds to molecular weights standards in $\text{kg}\cdot\text{mol}^{-1}$.

3.4 UV-visible spectra and specific extinction coefficient determination

In the wavelength range of 235-300 nm, the UV-visible spectrum of a protein is mostly driven by its content in UV-absorbing amino acids namely tyrosine, tryptophan, and phenylalanine. The UV-visible spectrum of a protein also varies with the environment of the aromatic amino acids and their degree of exposure to the solvent but this variation is small (<5%) (Pace, Vajdos, Fee, Grimsley, & Gray, 1995). Therefore, from the proportions of the three UV-absorbing amino acids in the protein and their respective individual spectra, a theoretical UV-visible spectrum of the protein was calculated. The calculated spectra are represented in **Figure 2A** for a $1 \text{ g}\cdot\text{L}^{-1}$ LPC dispersion. LPC theoretical spectrum shows that a maximum of absorbance, between 250 and 290 nm, is expected at 275 nm due to the major contribution of tryptophan and tyrosine. Experimental UV-visible spectra of LPC dispersion in water at several protein concentrations are represented in **Figure 2B**. Above 245 nm, the absorbance reaches a maximum, at 258 nm and displays residual absorption between 300 and 420 nm. Other spectra obtained at pH 5, pH 7 and pH 8 present the same profile with a maximum absorbance around 258 nm (**Supplementary 1**). The maximum at 258 nm and the residual absorption between 300 and 420 nm, experimentally measured, suggest a contamination by non-protein compounds that absorb around 260 nm and 300-450 nm. Phenolic compounds are non-protein contaminants common in LPC (Pedone, Selvaggini, & Fantozzi, 1995). Some of them, such as rutin, have high absorption around 260 nm and 400 nm and could correspond to the non-protein UV-absorbing species (Mirgorod, Borodina, & Borsch, 2013).

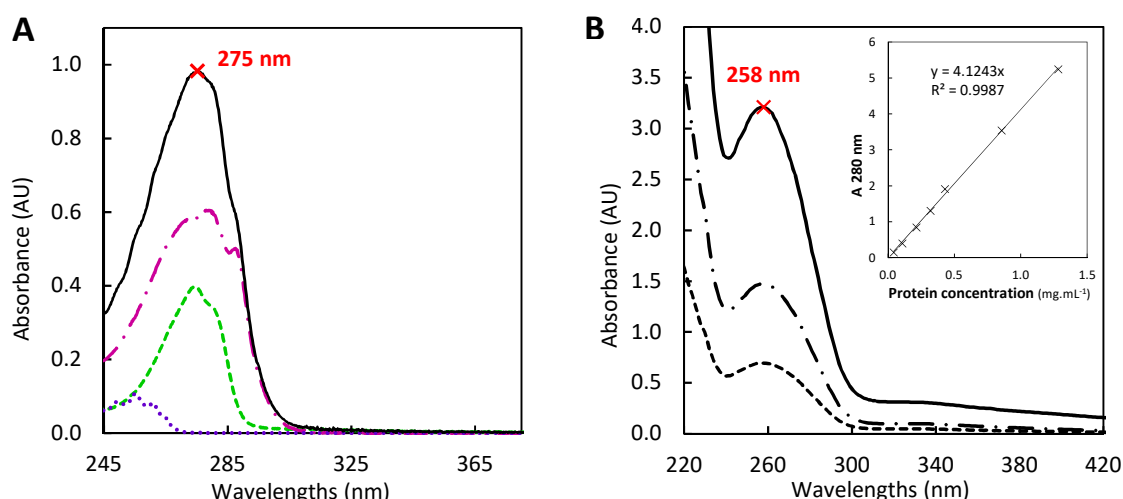


Figure 2. **A.** UV-visible spectra calculated for phenylalanine (dotted line), tyrosine (dashed line) and tryptophan (dashdotted line) for a 1 g.L⁻¹ LPC dispersion; and total calculated UV-visible spectra (solid line) for a 1 g.L⁻¹ LPC dispersion. Calculations are based on the amino acid composition of LPC. **B.** UV-visible spectra of LPC dispersion in water at several protein concentrations: 0.43 (full line), 0.21 (dash-dotted line) and 0.11 (dashed line) mg.mL⁻¹. The insert represents the Beer-Lambert law established for RuBisCO in water.

These results suggest that absorption at 280 nm of the LPC dispersion is not only the result of protein absorption. Therefore, the use of extinction coefficients values found in literature or calculated from the amino acid sequence is excluded. The specific extinction coefficient at 280 nm was determined for LPC in buffers at pH 5, pH 6 and pH 7. The linearity of Beer Lambert's law was checked in triplicate in each physical-chemical condition (insert in **Figure 2**). The specific extinction coefficients correspond to the slopes of absorption at 280 nm versus the protein mass concentration. It is given in **Table 3** for LPC dispersed in buffers at pH 5, pH 7 and pH 8. They range from 4.58 to 5.82 cm⁻¹ g⁻¹ L depending on the pH. Several studies carried out on purified RuBisCO report specific extinction coefficients in the range of 1.41 to 1.82 cm⁻¹ g⁻¹ L depending on the species and the method of extraction of the proteins (reviewed in Douillard & de Mathan, 1994). These values are much lower than those measured in our study, supporting the presence of non-protein UV-absorbing species.

Some methods have been proposed to correct the interference of nucleic acid and/or amino acid composition on UV-visible absorbance of proteins based on differential absorbance. In particular, it was shown that the difference in absorbance at 235 nm and 280 nm ($A_{235\text{nm}} - A_{280\text{nm}}$) would provide an accurate determination of protein concentration (Whitaker & Granum, 1980). Similarly, a linear correlation ($R^2 > 0.988$) was found between the protein concentration measured in Kjeldahl and the differential absorbance ($A_{235\text{nm}} - A_{280\text{nm}}$) measured on LPC dispersion as shown in **Supplementary 1B**. A slope of 2.67 was found independently of the pH. This coefficient is higher than 2.51, found by (Whitaker & Granum, 1980); this may be related to the nature of the species interfering in the UV-visible spectra of LPC. The distortion of the maximum of absorbance of the UV-visible spectra is not specific to LPC. It was observed on other plant protein isolates (**Supplementary 2**). Therefore, this approach of differential absorbance may be valuable to quantify protein concentration in the broader framework of plant proteins.

Table 3. Specific extinction coefficients and refractive index increments (dn/dc) of LPC at several pH

Buffer	$\epsilon_{280\text{ nm}}$			$\text{dn/dc}_{589.3\text{ nm}}$		
	$\text{cm}^{-1} \cdot \text{g}^{-1} \cdot \text{L}$			$\text{L} \cdot \text{g}^{-1}$		
pH 5	4.96	$\pm$	0.08	0.39	$\pm$	0.00
pH 7	5.82	$\pm$	0.19	0.31	$\pm$	0.01
pH 8	4.58	$\pm$	0.22	0.27	$\pm$	

3.5 Determination of the refractive index increment

We computed the dn/dc from the amino acid composition of LPC and obtained $0.189 \text{ g} \cdot \text{mL}^{-1}$ at 589.3 nm and 25°C. This value is close to the consensus value of $0.185 \text{ g} \cdot \text{mL}^{-1}$ generally accepted for proteins (Zhao et al., 2011). The dn/dc values measured on LPC dispersions at several pH values are reported in **Table 3**. They vary from 0.27 at pH 8 to 0.39 at pH 5. These values are high as compared to calculated values. As the refractive index is related to the polarizability of the molecules, this high value may indicate that non-protein molecules increase the refractive index of the medium. Phenolic compounds and polysaccharides can contribute to the refractive index of the medium (Greenwood & Hourston, 1975; Stalikas, 2007).

3.6 Effect of pH on protein solubility and surface charge

The solubility of LPC proteins was determined as a function pH. For $\text{pH} > 7$, the solubility was higher than 90% as shown in **Figure 3A**. A minimum of solubility was obtained for pH comprised between 4 and 5. The overall features of the solubility curve are consistent with literature data (Kiskini, 2017; Van de Velde, Alting, & Pouvreau, 2011). The minimum of solubility of the protein is consistent with the electrophoretic mobility measurements as presented in **Figure 3B** (empty symbols). LPC dispersion has a negative charge from $\text{pH} > 4.0$; its isoelectric point (IEP) is around pH 3.8. For comparison, the pH-dependence of the calculated total net charge of spinach RuBisCO is reported (full symbols). The pH value at which spinach RuBisCO, with no adsorbed ionic species, has an equal number of positive and negative charges, is called the isoionic point (IIP). For unfolded spinach RuBisCO, it is expected to be at pH 6.6. As the net charge of proteins depends on their conformation, the theoretical charge for a folded spinach RuBisCO was also calculated, using PROPKA software (version 3.0). The protein conformation is expected to shift the IIP from 6.6 to 6.0. The experimentally measured IEP of LPC is much lower than the theoretical IIP of RuBisCO. Discrepancies between IEP and IIP have been observed experimentally and may be related to adsorbed ions and/or charge regulation phenomenon (Salis et al., 2011). The IEP of the LPC is also lower than other experimental values obtained using isoelectric focusing: between 5 and 5.5 for spinach RuBisCO (Iwanij, Chua, & Siekevitz, 1974; Matsumoto, Sugiyama, & Akazawa, 1969; Miziorko, Nowak, & Mildvan, 1974), around 7 for lettuce RuBisCO (Rabinowitz, Reisfeld, Sagher, & Edelman, 1975), and 6 for alfalfa RuBisCO (reviewed in Douillard & de Mathan, 1994). The isoelectric point of maize RuBisCO was reported around 4.6 as obtained by isoelectric focusing (Reger, Ku, Pottert, & Evans, 1983). The IEP of the LPC is however in the range of sugar beet leaves protein concentrate as determined by zeta potential measurement (Kiskini, 2017). In the latter study, the shift between the experimental surface charge and the theoretical charge was ascribed to the presence of uronic acid in the protein powder, suggesting the presence of pectin.

Mixtures of pectin with positively charged proteins are known to reduce the charge density (Amine, Boire, Kermarrec, & Renard, 2019). The discrepancy between experimental and theoretical charges observed in our study could also be related to adsorbed ionic molecules or to chemical modification of the protein. For example, several studies report an acidification of proteins after their derivatisation by phenolic compounds. Higher degrees of derivatisation induced lower IEP of soybean glycinins (Harshadrai M Rawel, Czajka, Rohn, & Kroll, 2002) and of lysozyme (Harshadrai M. Rawel, Kroll, & Rohn, 2001). Also, sodium metabisulfite that is used during the extraction process may have react with cystine residue to produce S-sulfocysteine adding a negative charge to the protein (Cole, 1963; Nakazawa, 2006).

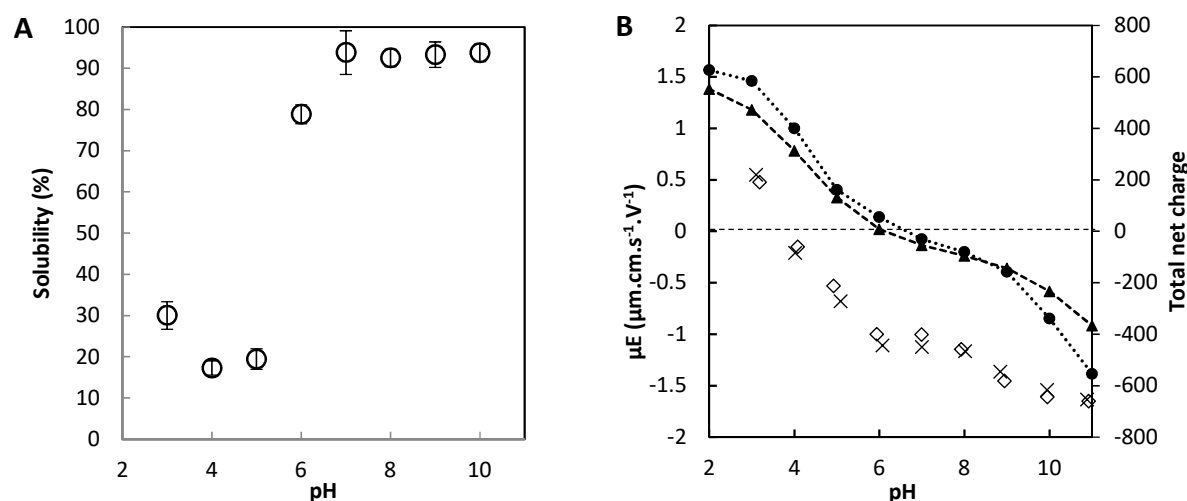


Figure 3. **A.** Evolution of the LPC protein solubility in water from pH 3 to pH 10. **B.** Experimental electrophoretic mobility (empty symbols) of LPC dispersion (at 2.9 g.L⁻¹ for diamonds and 2.8 g.L⁻¹ for crosses) according to pH and estimated total net charge (full symbols) of unfolded (circles, pointed line) and foled (triangles, dashed line) spinach RuBisCO. Spinach RuBisCO total net charge was estimated using PROPKA (3.0) with spinach RuBisCO structure from PDB (entry: 1AUS).

3.7 Impact of pH on the size distribution of proteins

DLS measurements were performed to highlight the effect of pH on LPC dispersion hydrodynamic properties. Representative correlation functions are shown in **Figure 4**. Whatever the pH, a double exponential fit well the data suggesting two size populations in the dispersion (data not shown). In DLS, the scattered intensity is dominated by the largest particles. Therefore, the determination of hydrodynamic radius on polydisperse mixtures is complex. The size distribution of the dispersed particles can be estimated from the correlation functions. Two major peaks are observed with a mean diameter of 15 and 90 nm, respectively. An increase in pH from 6 to 8 increases the proportion of smaller particules. Electrostatic repulsion at basic pH, as suggested by the electrophoretic mobility results, may prevent protein aggregation. At pH 8, the dominant population could correspond to the folded RuBisCO. At other pH, part of the RuBisCO may no longer be in the form of hexadecamer. The protein concentration, up to 9.5 g.L⁻¹, and the ionic strength, up to 150 mM, do not affect the size distribution of the LPC dispersion (**Supplementary 3**).

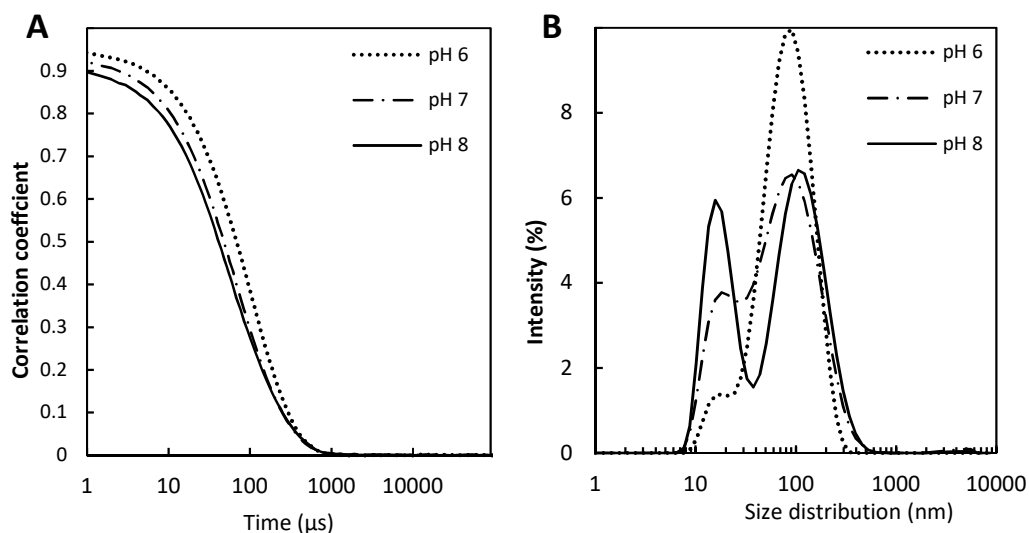


Figure 4. Correlation functions (A) and size distribution (B) obtained by dynamic light scattering according to pH. Correlation functions are representative of at least six observations. Size distribution curves are the mean of six observations. For readability reasons, the standard deviations are not shown; they suggest a significant difference between the mean curves.

3.8 Insight into the interactions between UV-absorbing species and RuBisCO

The above results suggest that LPC contains non-protein UV-absorbing species. To evaluate the affinity of RuBisCO for these compounds, several chromatography techniques combined with spectral analyses were performed on LPC dispersions.

Covalent interaction between UV-absorbing species and RuBisCO

To identify possible covalent bound between RuBisCO and UV-absorbing species, LPC proteins were extracted in denaturing buffer and analysed using size-exclusion HPLC. Signal was recorded from 200 to 700 nm. The elution profile obtained at 214 nm is represented on **Figure 5A**. Three major peaks are visible. The main fraction was eluted at 16.4 min and was attributed to RuBisCO SC, with a molecular weight of about 15 kg.mol⁻¹. The peak eluted at 14.3 min (~45 kg.mol⁻¹) was attributed to the RuBisCO LC (Barbeau & Kinsella, 1988) and the peak eluted at 13.15 min (~100 kg.mol⁻¹) corresponds to the LC dimer. A shoulder is visible between the LC and the SC peaks, corresponding to a specie of about 28 kg.mol⁻¹. Smaller peaks are detected below 10 kg.mol⁻¹, they correspond to small peptides or non-protein species.

UV-visible spectra recorded for these four main peaks are represented on **Figure 5B**. Spectra associated with RuBisCO LC and LC dimer have the same profile with a maximum absorbance at 276 nm, close to the expected wavelength of whole LPC dispersion (275 nm). This result suggests that RuBisCO LC and LC-dimer are not covalently linked with any UV-absorbing species. In contrast, the absorption spectra associated with the RuBisCO SC displays a maximum at 265 nm. In case of covalent protein-polyphenol interactions, an increase in the molecular weight is expected (H. M. Rawel, Kroll, & Riese, 2000; Harshadrai M. Rawel, Czajka, Rohn, & Kroll, 2002; Harshadrai M. Rawel et al., 2001) and there is no molecular weight modifications of RuBisCO SC. The SC peak is surrounded by two peaks with a high absorbance at 260 nm resulting in high $A_{260\text{ nm}}/A_{214\text{ nm}}$ ratio (0.37 and 0.79 as compared to

0.16 for SC peak). It is therefore likely that the distortion of the RuBisCO SC spectra is due to the absorbance of the two adjacent peaks rather than to the formation of a SC-phenolics complex.

The absorption spectra associated with the peak detected around 28 kg.mol⁻¹ displays a maximum at 259 nm. A similar 28 kg.mol⁻¹ molecule is also detected on a commercial RuBisCO (Sigma-Aldrich, St. Louis, MO) but its maximum absorbance is measured at 277 nm, supporting a proteic nature (**Supplementary 4**). The blue shift of this molecular specie suggests the addition of non-proteic UV absorbing compound. Finally, spectra associated with the two small peaks detected below 10 kg.mol⁻¹ display maximum at 258 nm for one and 248 and 330 nm for the other (**Supplementary 5**). The absorbance at 214 nm is related to peptides bonds of proteins and absorbances at 280 or 260 nm are related to aromatic residues absorption. Thus, a change in the absorbance ratios $A_{260\text{ nm}}/A_{214\text{ nm}}$ or $A_{280\text{ nm}}/A_{214\text{ nm}}$ in function of elution time may suggest a change in amino acids compositions of proteins, and thus a change in protein species. Also, a high $A_{260\text{ nm}}/A_{214\text{ nm}}$ ratio may suggest that the specie is not a protein. LC and SC peaks have $A_{260\text{ nm}}/A_{214\text{ nm}}$ ratios of 0.06 and 0.16 respectively. Molecules below 10 kg.mol⁻¹ display a $A_{260\text{ nm}}/A_{214\text{ nm}}$ ratio above 0.45, suggesting they are not proteins.

These data suggest that RuBisCO sub-units are not covalently bonded with a UV-absorbing species. In contrast, a protein specie of about 28 kg.mol⁻¹ may be bounded to a UV-absorbing species. Small non-protein UV-absorbing species are detected with molecular weights below 10 kg.mol⁻¹.

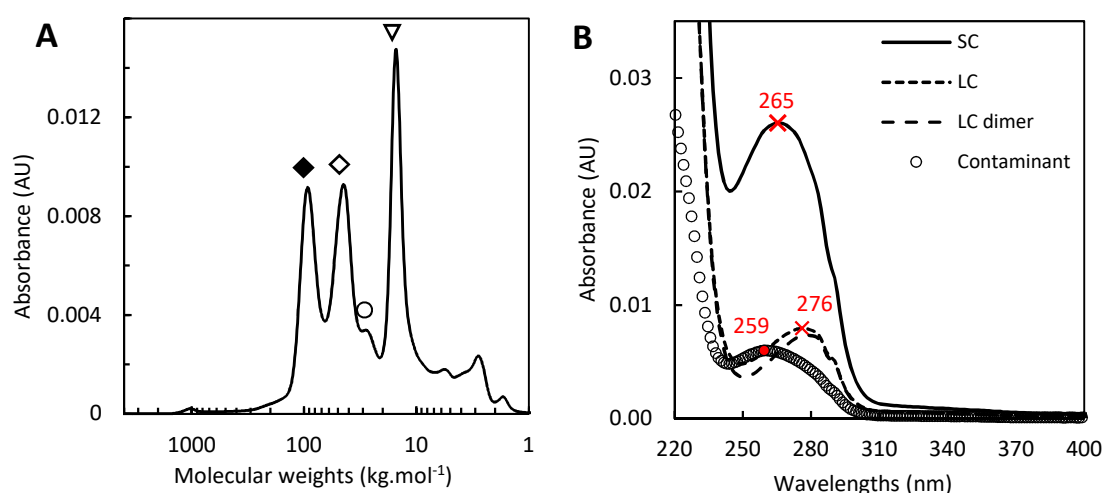


Figure 5. **A.** Elution profile of SDS soluble proteins extracted from LPC. Symbols represent peaks of interest: RuBisCO small chain (SC; ▽), possible UV-absorbing species (○), RuBisCO large chain (LC; ◇) and RuBisCO large chain dimer (LC dimer; ◆). **B.** UV-visible spectra acquired at the elution times corresponding to the peaks at 214 nm of SC, contaminant, LC and LC dimer.

Removal of small UV-absorbing species using desalting column

The above results highlight the presence of small species (< 10 kg.mol⁻¹), probably non-protein, with high $A_{260\text{ nm}}/A_{214\text{ nm}}$ and $A_{280\text{ nm}}/A_{214\text{ nm}}$ absorbance ratios. The content of free phenolic compounds was shown to be low, suggesting that these compounds are bound to proteins by weak bonds. To remove these free small contaminants, LPC dispersion was injected on a desalting column, and the signal was recorded at 260 nm, 280 nm, and 325 nm. The excluded volume (EV) contains the protein fraction with molecular weights higher than 5 kg.mol⁻¹. Elution profiles and ratios of absorbance $A_{260\text{ nm}}/A_{280\text{ nm}}$ and $A_{325\text{ nm}}/A_{280\text{ nm}}$ are represented in **Figure 6**. Different absorbance ratios indicate different molecular

species. The ratio $A_{325\text{ nm}}/A_{280\text{ nm}}$ of fraction 1 (F1) is up to six times higher than for the excluded volume. Also, the ratio $A_{260\text{ nm}}/A_{280\text{ nm}}$, is three times higher for fraction 2 (F2) and fraction 3 (F3) than for the excluded volume. These results show that the excluded volume, F1 and F2 might contain different species. Besides, the UV-visible spectra of F1 and F2 confirm that eluted fractions contain the expected species displaying two maxima at 260 and 325 nm (**Supplementary 6B**). However, the maximum absorption of the UV-visible spectrum of desalted protein fraction (EV) remains around 260 nm (**Supplementary 6A**). Therefore, the desalting step did not remove UV-absorbing species responsible for the distortion of the UV-visible spectrum. Adsorption of LPC dispersion on ion exchange resin shifted the maximum absorbance from 260nm to 267nm (**Supplementary 7**). This suggests that part of the UV-absorbing species is bounded by ionic interactions to proteins. The desalting experiment was performed with a ionic strength of 180 mM ; this ionic strength may not be sufficient to screen the charge-charge interactions involved in these complexes. In addition, no change of spectrum was observed with uncharged hydrophobic resin (**Supplementary 7**), although it has already been proven to be effective (D'alvise et al., 2007).

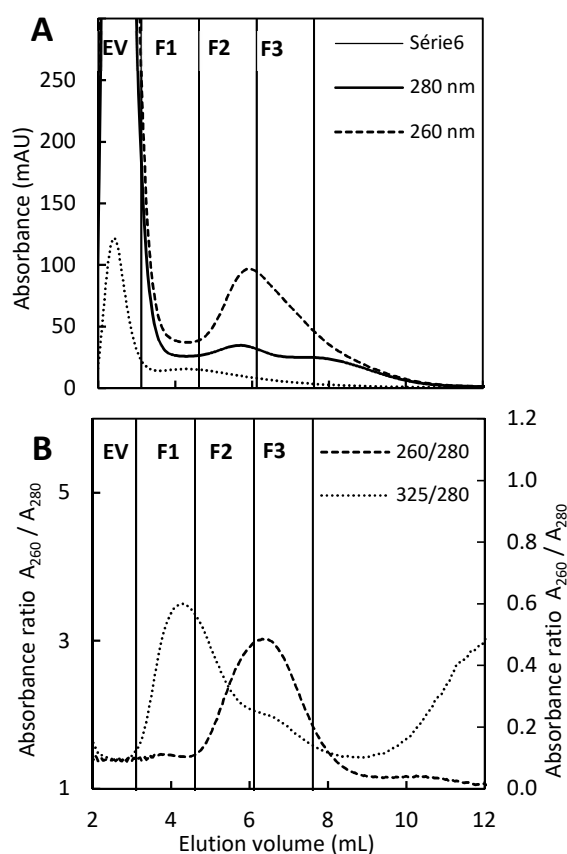


Figure 6. **A.** SEC profile of LPC recorded at 3 wavelengths: 280 nm (solid line), 260 nm (dashed line) and 325 nm (dotted line). EV: excluded volume, Fi: fraction i. **B.** Absorbance ratio during elution: $A_{260\text{ nm}} / A_{280\text{ nm}}$ (dashed line) and $A_{325\text{ nm}} / A_{280\text{ nm}}$ (dotted line).

4 Conclusion

We showed that LPC powder has a content of proteins higher than 74%. Polysaccharides and ashes constitute major contaminants, with 9.6 and 7.8 % in weight proportion, respectively. In terms of protein composition, RuBisCO is the major protein of the LPC powder. Disulphide bonded stabilised dimers of LC RuBisCO were highlighted, similarly to previous reports. This suggests that disulphide bond exchanges occurred *in planta* or during the process. The UV-visible spectrum of LPC dispersions suggests the presence of polyphenols or other UV-visible absorbing species. RuBisCO sub-units are not covalently bonded with a UV-absorbing species as shown by size exclusion chromatography in denaturing conditions. The overall charge of LPC dispersion is significantly lower than that conventionally measured or calculated on RuBisCO. The solubility of LPC dispersion is minimal close to the isoelectric point (pH ~ 4) ; a solubility higher than 80% was observed for pH > 6.0.

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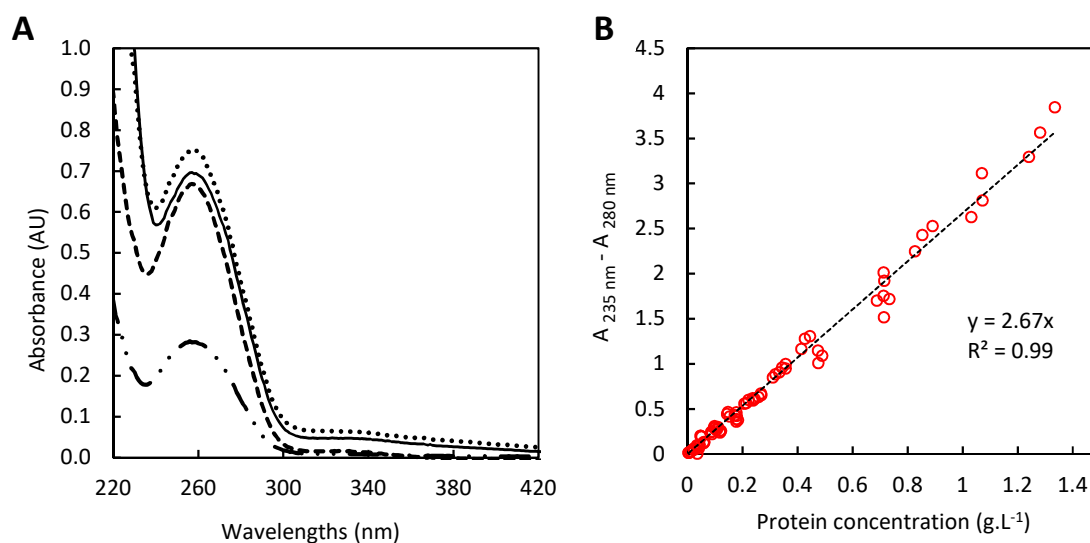
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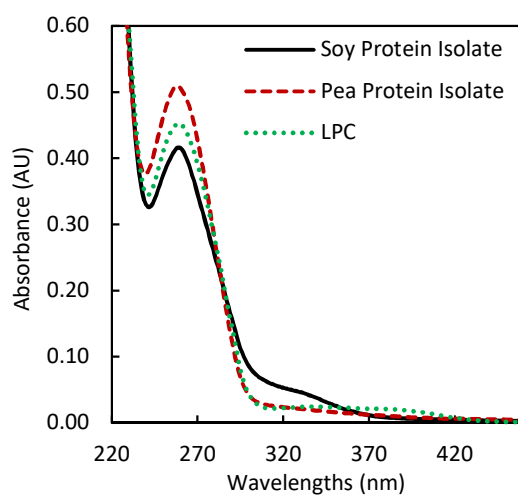
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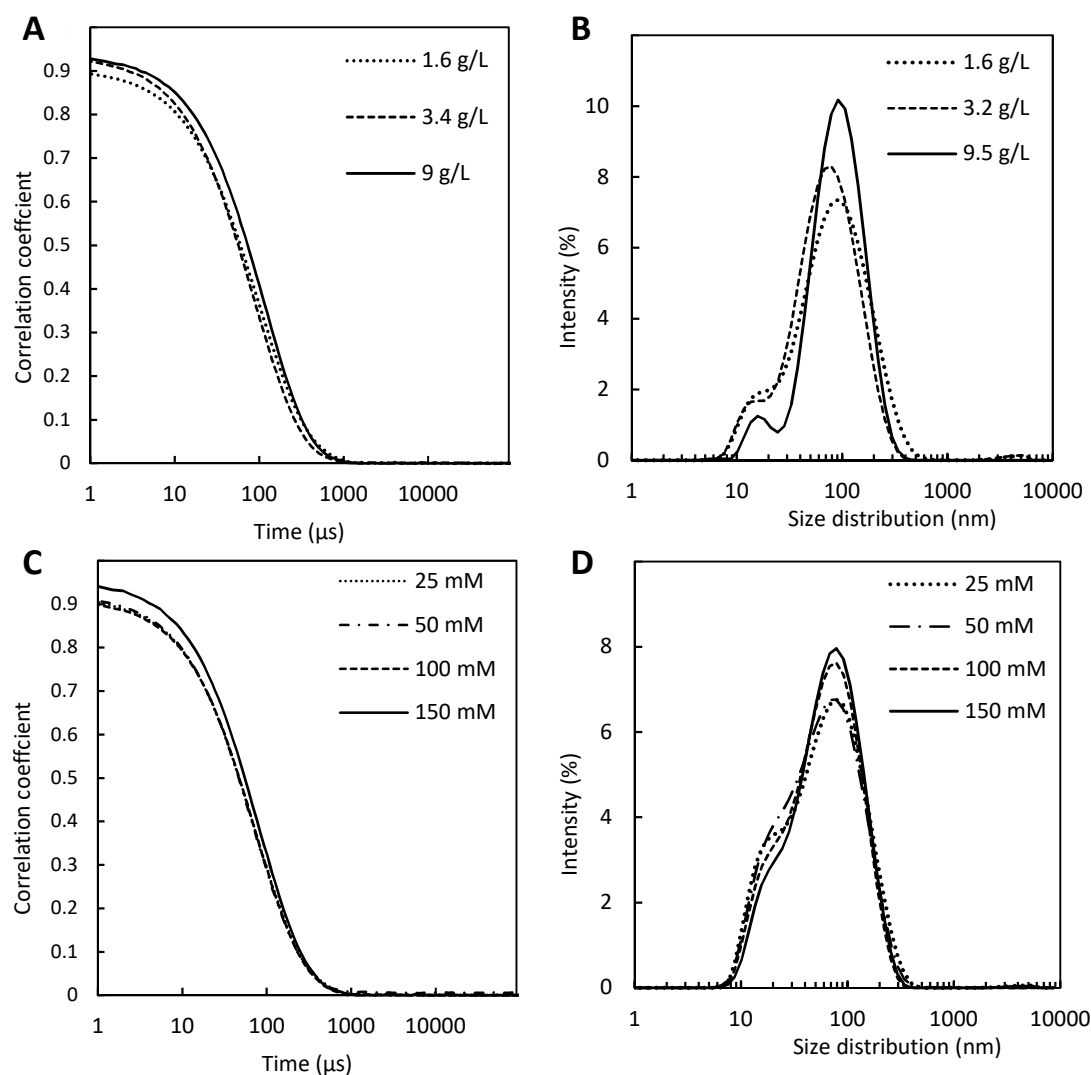
Supplementary material



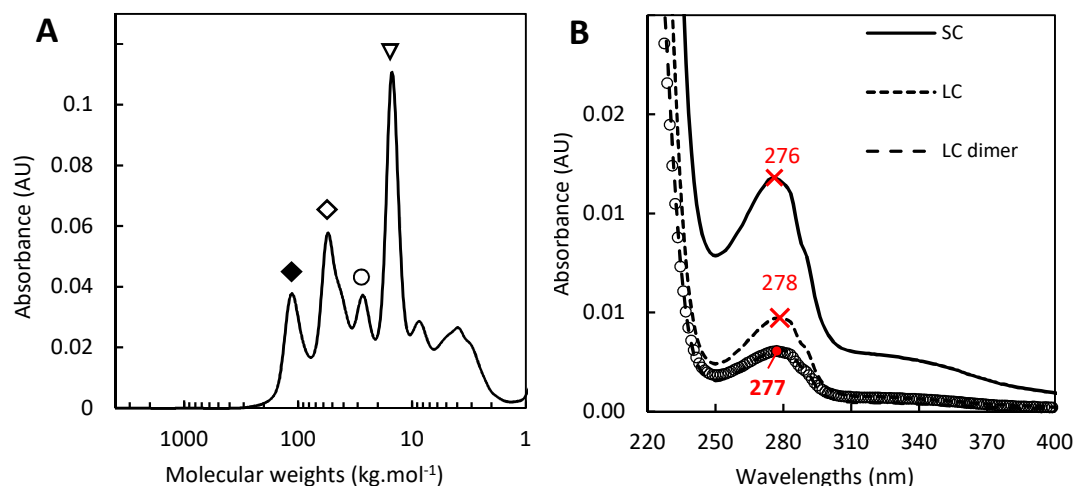
Supplementary 1. A. UV-Visible spectra of LPC dispersion in buffer at pH 5 (dash and dotted line) at 0.03 mg.mL⁻¹; pH 7 (dashed line) at 0.06 mg.mL⁻¹; pH 8 (dotted line) at 0.09 mg.mL⁻¹ and in water (solid line) at 0.11 mg.mL⁻¹. **B.** Linear correlation between the LPC dispersion protein concentration measured in Kjeldahl and the differences in absorbance at 235 nm and 280 nm ($A_{235\text{ nm}} - A_{280\text{ nm}}$).



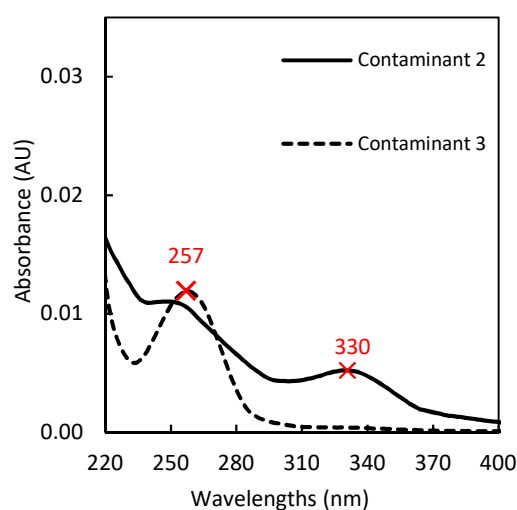
Supplementary 2. UV-visible spectra obtained on Soy protein (black full line) and pea protein (red dashed line) concentrates as compared to the UV-visible spectrum of LPC (green dotted line).



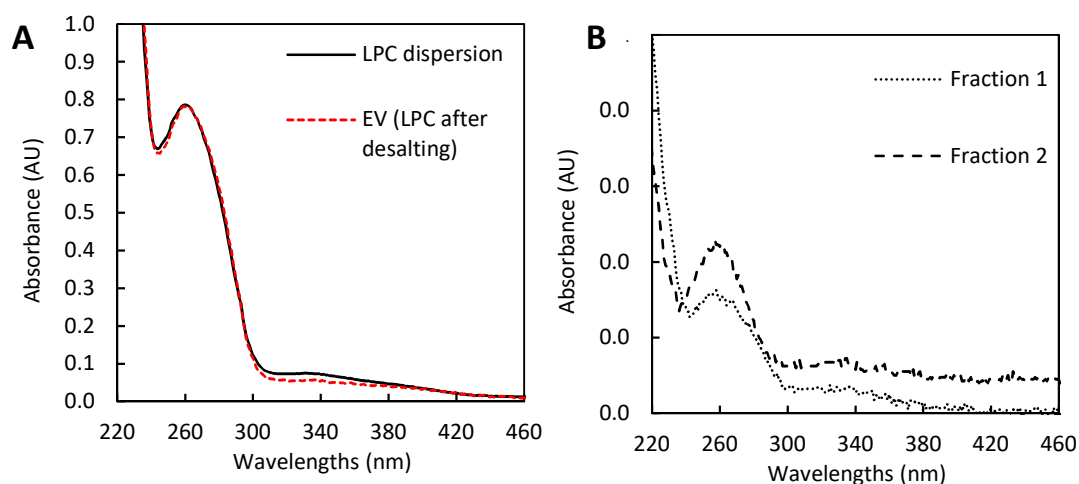
Supplementary 3. Correlation functions (on the left) and size distribution (on the right) obtained by dynamic light scattering according to protein concentration (A; B) and to ionic strength (C; D). Correlation functions are representative of at least six observations. Size distribution curves are the mean of six observations. For readability reasons, the standard deviations are not shown; they suggest no difference between the mean curves.



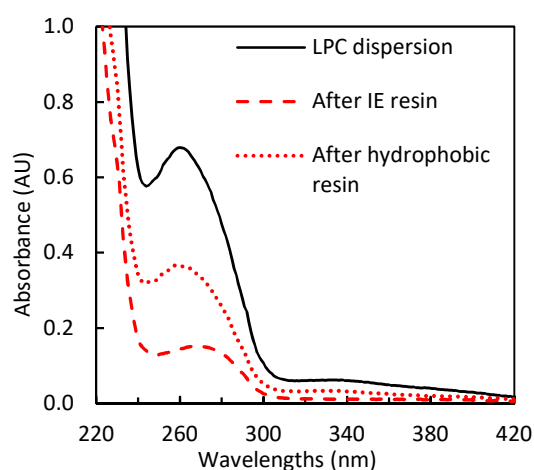
Supplementary 4. A. Elution profile of SDS soluble proteins extracted from a commercial RuBisCO concentrate (R8000, Sigma-Aldrich, St. Louis, MO). Symbols represent peaks of interest: RuBisCO small chain (SC; ∇), unidentified protein ($\circ$), RuBisCO large chain (LC; $\diamond$) and RuBisCO large chain dimer (LC dimer; $\blacklozenge$). **B.** UV-visible spectra acquired at the elution times corresponding to the peaks at 214 nm of SC, unidentified protein, LC and LC dimer.



Supplementary 5. UV-visible spectra acquired at 18.3 (contaminant 2) and 19.5 minutes (contaminant 3) of the LPC dispersion elution profile, corresponding to species with molecular weight below 10 kDa. Their low absorbance at 214 nm in comparison to their absorbance at 260 nm suggest they are non protein species.



Supplementary 6. A. UV-visible spectrum of LPC dispersion in phosphate buffer (0.1 M, pH 6.8) (black solid line) and UV-visible spectrum of excluded volume of LPC dispersion after desalting (red dashed line). **B.** UV-visible spectra of fraction 1 and fraction 2 recovered after LPC dispersion desalting.



Supplementary 7. UV visible spectra of LPC dispersion in phosphate buffer (0.1 M pH 6.8; black solid line), of LPC after incubation with ionic-exchange resin (incubation overnight on Amberlite IRA 900 CL after three changes of resin; red dashed line) and hydrophobic resin (incubation overnight on Amberlite XAD16 after three changes of resin; red dotted line).

Chapter 4. Characterisation of mechanical properties and protein structures of RuBisCO-enriched wheat doughs

Preamble

The previous chapter shows that RuBisCO has a balanced amino acid profile. It could therefore be used to improve the amino-acid profile of wheat-based food. In many cases, protein enrichment of wheat-based food dilutes the gluten network, leading to a decrease of the food quality (Laleg et al., 2017; Rayas-Duarte, Mock, & D., 1996). The aim of this chapter was to evaluate the changes in mechanical properties and protein structures caused by a RuBisCO enrichment of wheat dough.

Main questions:

- Are the dough mechanical properties modified due to a RuBisCO enrichment?
- Are protein-protein interactions affected by RuBisCO in dough?
- Is the microstructure of the protein network modified by RuBisCO?
- Are the secondary protein structures changed following RuBisCO enrichment?

This chapter will be divided into two parts. The first part constitutes a published scientific article. The protein enrichment impact on dough structuring during heating was studied using dynamic thermo-mechanical analysis (DMTA). Protein polymerisation in doughs was studied by protein sequential extraction in denaturing and thiol-reducing buffers followed by size-exclusion chromatography both before and after dough thermal treatment. The overall structure of the protein network was visualised by confocal scanning light microscopy (CSLM). The second part of this chapter focuses on the study of protein secondary structure by Fourier-transform infrared spectroscopy (FTIR). Experimental data of this part were acquired at the Bohdan Dobrzanski Institute of Agrophysics (Lublin, Poland) during a scientific stay in the framework of Agreenium international research facility (École internationale de recherche d'Agreenium, EIR-A).

Main results:

- ✓ RuBisCO is part of the gluten network formed during dough mixing through the establishment of weak and disulphide bonds
- ✓ RuBisCO subunits form covalent bonds during the heat treatment and enhance protein polymerisation, leading to an increase in large size disulphide-linked polymers
- ✓ RuBisCO addition does not affect the increase in dough elasticity during heating
- ✓ RuBisCO addition enhances the protein secondary structure changes under the effect of heat treatment

Part 1. RuBisCO: a promising plant protein to enrich wheat-based food without impairing dough viscoelasticity and protein polymerisation

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1 Introduction

Foods of animal origin are a major source of proteins in today's Western diet. Nevertheless, their overconsumption raises public health (Friel et al., 2009) and environmental issues (Aiking, 2014; John Reynolds, David Buckley, Weinstein, & Boland, 2014). A panel of experts recently underlined the need to shift to a diet richer in plant proteins (Willett et al., 2019). Among other things, this food transition requires the development of alternatives to meat proteins in Western countries. Development of meat analogues and dairy substitutes is part of the solution but there are still technological challenges to address to meet consumer preferences (Wild et al., 2014). Another strategy to meet nutritional needs is to enrich traditional food with plant proteins, leading to foods with higher protein content and a balanced amino acid profile. These enriched foods are currently mainly based on a combination of proteins from legumes and wheat (ING Economics department, 2017). The association of legumes with wheat counterbalances the lack of lysine or threonine in wheat proteins (Bahnassay, Khan, & Harrold, 1986). However, adding legumes in wheat-based foods may impair the organoleptic quality of the product. As recently reviewed, fortifying durum wheat pasta with legumes increases cooking loss and reduces the cooked weight of pasta (Monnet, Laleg, Michon, & Micard, 2019). This reflects higher leaching of solids from the pasta into the cooking water due to the weakening of the gluten network (Bahnassay & Khan, 1986; Laleg et al., 2017). Weakening of the protein network is also responsible for the degradation of the textural properties of pasta enriched with legumes (Laleg et al., 2017; Petitot, Boyer, Minier, & Micard, 2010). Similarly, the addition of legume proteins in bread dough affects the volume of the loaf by reducing its gas retention capacity as recently reviewed (Boukid, Zannini, Carini, & Vittadini, 2019). The ability of wheat semolina or flour proteins to form a gluten matrix is the main factor influencing the strength and elastic properties of wheat dough that ensure the quality of wheat-based foods. However, the mechanisms through which extrinsic proteins affect the gluten network remain unclear. The addition of non-wheat components may dilute gluten proteins, thereby impairing and weakening the overall protein network (Laleg et al., 2017). It is therefore a challenge to develop protein-enriched wheat-based foods with minimized or even no gluten dilution effects.

Considerable attention has been paid to legume proteins for their nutritional quality and their availability. In the same way, leaf proteins have worthwhile nutritional qualities for human food (Gerloff, Lima, & Stahmann, 1965; Lexander et al., 1970). Leaf protein can be extracted from numerous crops, green by-products and aquatic plants (Ellis, 1979). The fractionation of wet green crops aims at

recovering a juice with both high dry matter and protein content (Fiorentini & Galoppini, 1983). The soluble part is mainly composed of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), an enzyme involved in carbon fixation, which is richer in sulphur-containing amino acids than wheat proteins, and also rich in lysine, threonine and tryptophan (Gerloff et al., 1965). Although several extraction processes have been described (R. H. Edwards et al., 1975; Knuckles, Bickoff, & Kohler, 1972; Knuckles, De Fremery, Bickoff, & Kohler, 1975), RuBisCO is still not used as an ingredient in human food because of the difficulty of extracting a functional white concentrate with no off-flavours. However, technological advances in extraction processes and recent studies on functionality are promoting the use of leaf protein concentrates as an ingredient in human food (Firdaous et al., 2017; Hadidi, Ibarz, Conde, & Pagan, 2019; Kiskini, Vissers, Vincken, Gruppen, & Wierenga, 2016; Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019; Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016; Udenigwe et al., 2017). Besides its nutritional interest, RuBisCO has been found to have interesting functional properties (Barbeau & Kinsella, 1988; Douillard, 1985; Knuckles & Kohler, 1982). RuBisCO has a foaming capacity similar to egg white proteins (Sheen & Sheen, 1985), good solubility at food pH and is able to form gels at low concentrations and low temperatures in aqueous buffers at several pH (Martin, Nieuwland, & De Jong, 2014). The thermal denaturation and gelling properties of proteins are crucial to the structure and texture of wheat-based foods. To our knowledge, the functionality of RuBisCO when incorporated in a dense food matrix has not yet been studied.

The aim of this study was thus to investigate the impact of introducing RuBisCO on the structure of wheat proteins. Micro wheat semolina doughs were used as a model food system to investigate the interactions between proteins and other components in protein-enriched wheat matrices. In addition to RuBisCO concentrate, the effects of adding gluten and pea proteins were also studied as a comparison. Their impact on dough structuring during heating was studied using dynamic thermo-mechanical analysis (DMTA) at several enrichment rates ranging from 0 to 33% of total proteins. Protein polymerisation in doughs was studied by protein sequential extraction followed by size-exclusion chromatography both before and after dough thermal treatment. The overall structure of the protein network was visualised by confocal scanning light microscopy (CSLM).

2 Material and methods

2.1 Material

Wheat semolina was provided by *La Semoulerie de Bellevue* (Panzani, Marseille, France) and passed through a 200 µm sieve before use. Pea protein concentrate (Nutralys, F85F) was purchased from Roquette (Lestrem, France) and RuBisCO concentrate was provided by Florette (Lessay, France). RuBisCO was extracted from *Cichorium endivia* leaves using the extraction conditions of the WO 2014/104880 patent but excluding the hydrophobic column adsorption step. The gluten was extracted by hand from a durum wheat semolina dough based on Auger, Morel, Dewilde, & Redl (2009) with some modifications. Briefly, doughs were prepared at 55% water content (wet basis). After a premixing step at 28 rpm during 2.5 min, the dough was mixed at 90 rpm until optimum development time. The dough was then diluted and washed under demineralized water. Gluten was recovered on 800µm sieve and freeze-dried. To ensure constant moisture content, at least 14 days before the experiment, wheat semolina and protein concentrates were placed in a humidity-controlled chamber with a K₂CO₃ saturated salt solution at 20°C to maintain 43.16% relative humidity. The water content of powders was determined in triplicate by weighing after drying at 105°C for three hours. The protein content of

all the raw materials was analysed using the Kjeldahl procedure (NF V 03-050, 1970) with a conversion factor of 5.7 for wheat semolina, pea protein and gluten concentrates. A conversion factor of 5.8 was used for the RuBisCO concentrate based on the amino acid profile previously determined (data not shown). Analytical grade sodium dodecyl sulfate (SDS), sodium phosphate dibasic (Na_2HPO_4) and monosodium phosphate (NaH_2PO_4) were purchased from VWR international (Leuven, Belgium). Other chemicals were purchased from Sigma-Aldrich (Bornem, Belgium) and Milli-Q water was used (Millipore Systems, Guyancourt, Molsheim, France).

2.2 Accessible free thiol content assay

Free thiol contents of protein concentrates were measured using Ellman's reagent, 5,5-dithiobis-2-nitrobenzoic (DTNB) (Ellman, 1959). Sodium phosphate buffer (0.1M, pH 8) containing 1mM EDTA was degassed under vacuum before use. Protein concentrates were dispersed with a solid:liquid ratio of 2.5 in sodium phosphate buffer containing DTNB (0.3 g.L^{-1}). Tube headspaces were filled in with argon or nitrogen and the tubes were incubated on a rotary shaker at 8°C for 15 min in the dark. The tubes were then centrifuged ($12000 \times g$, 20 min) and supernatant absorbance was measured at 412 nm. The absorbance of the buffer and of the proteins in a non-reactive buffer (free of DTNB) was subtracted from the absorbance of the samples. The concentration of the released thionitrobenzoate ion (TNB^{2-}) was calculated using a molar absorption coefficient of $13600 \text{ M}^{-1}\text{cm}^{-1}$. Results are expressed in micromoles of accessible free thiols per gram of protein.

2.3 Sample preparation

i. Dough formulation

Dough samples (3 g) were prepared using wheat semolina, water and variable amounts of protein concentrates. A standard water content of 67% was used (dry mass basis) to obtain a homogeneous cohesive dough. Protein enrichments were carried out by replacing 0 to 10% of the semolina mass with a protein concentrate. Protein enrichment is hereafter expressed as the amount of added protein as a percentage of the total protein content of the protein-enriched dough. It ranged from 0% (control dough) to 33%. When analysing protein interactions, the protein used for enrichment is called "extrinsic protein".

ii. Dough production

Doughs were prepared using a "2-g Mixograph" (TMCO, Lincoln, NE, USA) equipped with a homemade aluminium double-walled jacket coupled with a water-regulated bath to control the temperature of the bowl. Wheat semolina and the protein concentrate were first mixed. The water was then added. The mixture was homogenised in the mixing bowl for six seconds at 54 rpm at ambient temperature. The mixture was left to rest for 15 min at 40°C and cooled to 20°C over a period of 25 min. The dough was then mixed at 54 rpm for 220 s.

To study the effect of heat on protein polymerisation, a thermal treatment was applied to the mixed doughs. A hermetically sealed aluminium container (internal dimensions: 9 mm radius, 3 mm height) was made to contain 1.2 g of dough. Directly after mixing, the device was filled with 1.2 g of dough and immersed in a water bath at 80°C for three minutes. It was then cooled down on ice and the dough sample was removed and immersed in liquid nitrogen before freeze drying.

2.4 Dough mechanical properties

The mechanical properties of the raw doughs were analysed immediately after mixing. Dynamic mechanical thermal analysis (DMTA) with oscillatory measurements were performed as described in (Shehzad, Chiron, Valle, Lamrini, & Lourdin, 2012). A controlled strain dynamic mechanical analyser (DMTA MK 4, Rheometric Scientific, USA) was used in compression mode at a frequency of 1Hz with a 17mm diameter plate-plate geometry. A sinusoidal strain was applied with strain amplitude of 0.10%. The behaviour of wheat dough in dynamic measurements at this strain amplitude can be considered as linear (Lefebvre, 2006). A piece of dough weighing 0.90g ($\pm$ 0.02g) was placed between the two plates and the gap was set to 3.7 mm before the experiment. The dough faces in contact with the air were covered with grease to prevent water loss during measurement. Stress was recorded during a temperature ramp of 3°C/min from ambient temperature to 140°C.

To relate the oven temperature to the internal temperature of the dough, the temperature of two samples was measured using a thermocouple placed in the core of the dough. Using a polynomial model, an equation was determined to obtain the dough temperature from the oven temperature for all samples. The dynamic storage modulus, or elastic modulus (E'), was calculated using RSI Orchestrator software (Rheometric Scientific, USA).

2.5 Analysis of protein polymerisation and protein/protein interactions

i. Extraction of the gluten-like fraction after dough mixing

Samples of control and protein-enriched doughs were washed by hand under deionized water for 15 min to remove most of the starch and soluble material. The rubbery mass that remains is called the gluten-like fraction (GLF) because it is probably composed of other proteins than gluten ones when extracted from protein-enriched doughs. The GLF was then recovered on a 200 μ m sieve, immersed in liquid nitrogen and freeze-dried.

ii. Protein analysis in denaturing conditions

SE-HPLC analysis of proteins was performed on protein concentrates, ground freeze-dried doughs and ground freeze-dried GLF. Protein was extracted as described in Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'H (2000) with some modifications. Proteins were first extracted in 0.1M sodium phosphate buffer pH 6.8 with 1% SDS. Solid to liquid ratios of 8, 0.8 and 1.5 were used for the freeze-dried dough, protein concentrates and extracted gluten, respectively. Extraction was performed on a rotary shaker set at 60 rpm at 60°C for 80 min. SDS-soluble proteins were recovered in the supernatant after centrifugation (39191 x g, 30 min, 20°C). SDS disrupts non-covalent bonds and allows small aggregates to dissolve (Khan, Huckle, & Freeman, 1994). A second extraction was performed on pellets at 60°C for 60 min, in the same buffer including 20 mM dithioerythritol (DTE), to break the disulphide bonds. The pellets were then sonicated (Vibracell 72434, Bioblock Scientific, Illkirch, France) for three minutes at 50% power setting 20 kHz. Supernatants were recovered after centrifugation as described previously and diluted twice with 0.1 M phosphate, 1% SDS buffer including 40mM of iodoacetamide (IAM), to prevent reformation of the disulphide bonds. For readability, proteins insoluble in SDS but soluble in SDS and DTE are hereafter called "SDS+DTE soluble proteins".

The SE-HPLC apparatus (Waters model LC Module1 plus) was equipped with an analytical column, TSK G4000-SW (7.5 x 300 mm) and a guard column, TSK G3000-SW (7.5 x 75 mm) (both from Merck,

Darmstadt, Germany). The flow rate was 0.7 mL/min and absorbance was recorded at 214 nm. Apparent molecular weights were assessed with column calibration as described in Redl, Morel, Bonicel, Vergnes, & Guilbert (1999). For the analysis, six peaks were defined on chromatograms of SDS soluble proteins: peak P1 corresponds to proteins eluted in the void volume, peak P2 corresponds to proteins whose molecular weight ranged from 157 kDa to 763 kDa, peak P3 from 82 to 157 kDa, peak P4 from 50 to 82 kDa, peak P5 from 20 to 50 kDa and peak P6 from 6 to 20 kDa. The signal recorded after P6 does not correspond to proteins. The sum of the areas of the elution profiles of SDS-soluble and SDS+DTE soluble proteins, from peak P1 to peak P6, corresponds to the amount of total extractable proteins. The peak boundaries were defined according to the position of the peaks in the RuBisCO-enriched wheat dough, so they may not exactly correspond to the peaks of the other samples such as protein concentrates or control dough. The repeatability of dough production and protein extraction was assessed in triplicate on several samples (**Supplementary fig. 1**). Dough fabrication and protein extraction were then performed only once on each sample.

iii. Elution profile analysis

Elution profiles were normalised on the basis of their total protein content before any calculation. To evaluate if protein enrichment altered protein polymerisation in wheat dough, a differential SE-HPLC profile was calculated.

First, theoretical elution profiles of wheat proteins and extrinsic proteins of enriched dough were built. They correspond to the elution profile of the 100% wheat control dough and to the elution profile of the extrinsic protein concentrate, both normalised based on their respective proportion in the enriched dough studied. This is illustrated in Diagram 1 for wheat dough enriched at 20% RuBisCO. If the protein enrichment of the wheat dough does not alter the polymerisation of the proteins, then its elution profile corresponds to the sum of these two theoretical profiles.

Second, we subtracted the theoretical elution profile of wheat proteins from the experimental elution profile of protein-enriched dough. The resulting profile, called the differential profile, was compared to the elution profile of the extrinsic protein concentrate used for the enrichment. Any difference between the two latter profiles identifies changes in protein polymerisation induced by protein enrichment of the dough.

A similar approach was used to analyse the gluten-like fraction extracted from RuBisCO-enriched dough. As it cannot be excluded that some proteins were lixiviated during dough washing, the final proportion of RuBisCO and wheat proteins in the GLF is unknown. To calculate differential profiles, we considered that the extraction yield of wheat proteins in RuBisCO-enriched GLF was similar to that in the control wheat GLF. The resulting differential profile was compared to the elution profile of the RuBisCO concentrate. Considering the approximation used for the calculation, care must be taken in the interpretation of such differential profiles.

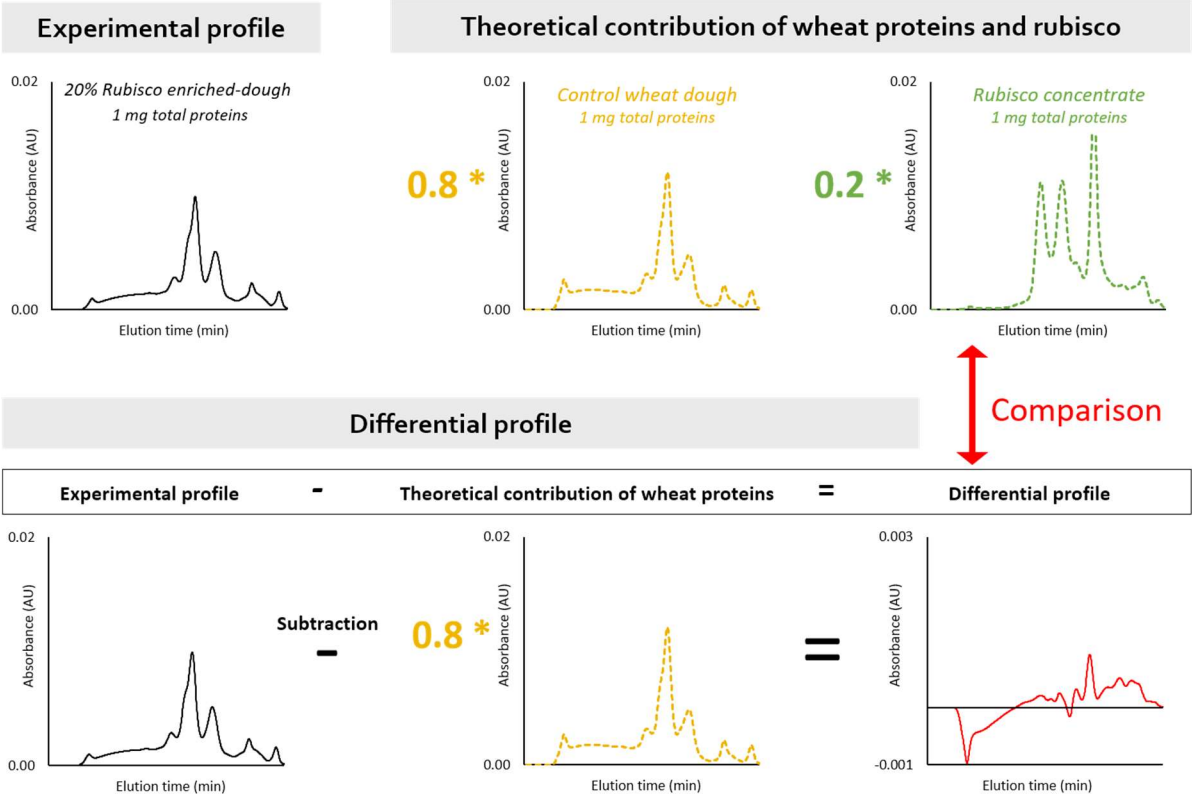


Diagram 1. Method for the analysis of elution profiles of protein-enriched samples: Example for a wheat dough enriched at 20% RuBisCO.

2.6 Microstructure of doughs observed by confocal laser scanning microscopy (CLSM)

CLSM was performed on control, 29.6% gluten-enriched dough, 33% pea protein-enriched doughs and 31.9% RuBisCO-enriched doughs.

Frozen doughs were used for non-specific labelling of proteins. Samples were cut into 80µm thick slices at -20°C using a cryotome (Microm HM 500 OM, Microm International GmbH, Germany). They were then placed on a cold glass coverslip and proteins were stained with 0.2µL of Alexa Fluor™ 546 NHS Ester (Thermo Fisher Scientific, USA). The slices were then mounted on microscope slides.

RuBisCO was specifically labelled to visualise its location compared to wheat proteins. RuBisCO concentrate was first dispersed at 25 g.L⁻¹ in 0.1M sodium carbonate buffer at pH 9 for five hours at ambient temperature. The dispersion was then centrifuged (12000 x g, 20 min). The supernatant was mixed with a 1% Fluorescein isothiocyanate (FITC) solution in dimethyl sulfoxide (DMSO) at a ratio of 0.015:1. After one hour of incubation, the sample was dialysed using a 6-8 kDa molecular weight cut off tubing (Spectra/Por®, VWR, Leuven, Belgium) against water to remove free dyes for 26 hours with several changes of bath water. Specifically labelled RuBisCO was concentrated by osmotic compression against a 10% (wt/v) polyethylene glycol 20000 solution, until a protein concentration of 135 mg.mL⁻¹ was obtained. The protein concentration was estimated from the dry mass recovered after drying at 105°C for five hours.

Doughs were prepared as described in 2.3.2, using a mix of unlabelled RuBisCO and fluorescein isothiocyanate (FITC) specifically labelled RuBisCO.

Images were acquired at least five minutes after staining using a Nikon A1 microscope equipped with a NIS-Element AR software (Nikon, Germany). Samples were observed in confocal mode with a 561 nm and a 488 laser for Alexa Fluor™ 546 NHS Ester and FITC label respectively. Emitted light was recovered with a filter at 515/30 and 595/50 nm, respectively. Images (2048 pixels) were taken at a magnification of 40 and resolution of 0.16 $\mu\text{m}/\text{px}$ in duplicate.

3 Results

3.1 Characterisation of protein concentrate

Protein concentrates acquired from commercial or laboratory sources can vary considerably. Their protein content, the amount of accessible free thiols or their state of aggregation affects their functionality. For this reason, the concentrates used in this study were first characterised. Gluten, pea protein and RuBisCO concentrates contained respectively 67.2, 77.5 and 74.1% of protein (dry basis) as reported in **Table 1**.

Gluten proteins do not contain free thiols, or an amount below the limit of detection. When propan-2-ol was used as a solvent, a low free thiol content of $0.94 \pm 0.1 \mu\text{mol.g}^{-1}$ of vital gluten was reported (Morel, Bonicel, Micard, & Guilbert, 2000). Pea proteins contain an average of 11.2 μmol of free thiols per gram of protein as reported previously (O’Kane, Vereijken, Gruppen, & van Boekel, 2005). RuBisCO has a remarkably high free thiol content of $67.2 \pm 0.9 \mu\text{mol.g}^{-1}$. Lower free thiol contents ($13.66 \mu\text{mol.g}^{-1}$) have been reported in the literature for RuBisCO from alfalfa (Hood, Cheng, Koch, & Brunner, 1981). Gluten protein concentrate contains $88.9 \pm 0.7\%$ of SDS soluble proteins as a function of total extractable proteins. In this study, pea protein concentrate contains $76.6 \pm 0.7\%$ of SDS soluble proteins, whereas higher SDS solubility values (91.9%) have been reported for protein from pea flour (Kristiawan et al., 2018). This suggests that industrial pea protein extraction causes higher aggregation of proteins. RuBisCO has the highest solubility in SDS with $96.4 \pm 0.7\%$ of extractable proteins.

SE-HPLC profiles of SDS soluble and SDS+DTE soluble proteins of the three protein concentrates are shown in **Figure 1**. SE-HPLC profile of wheat proteins is characterised by a large polydispersity comprising proteins from six to more than 1000 kg mol^{-1} . This is related to the intrinsic diversity of wheat proteins, in particular of wheat prolamins. Peaks P1, P2 and P3 of SDS soluble proteins include proteins with molecular weights higher than 80 kg.mol^{-1} (**Figure 1A**). These peaks were attributed to glutenin polymers (Morel, Dehlon, et al., 2000). Peaks P4 and P5 correspond to proteins with molecular weights ranging from 20 to 80 kg.mol^{-1} and were attributed to gliadins. Peak P6, including proteins from six to 20 kg.mol^{-1} , was attributed to wheat albumins and globulins. Three major peaks can be seen in the SDS+DTE soluble protein elution profile. The first eluting material extends from the upper column molecular exclusion size (about 2,000 kg.mol^{-1}) to a peak centred at about 86 kg.mol^{-1} . This peak is hypothesised to correspond to the high molecular weight (HMW) glutenin subunits released from glutenin polymers after disulphide bond reduction. The main following peak could correspond to low molecular weight (LMW) glutenin subunits of about 45 kg.mol^{-1} . Finally, albumin and globulin that were initially disulphide-bonded to glutenin polymers were possibly eluted at about 15 kg.mol^{-1} (Veraverbeke & Delcour, 2002). The elution profiles of pea proteins are presented in **Figure 1B**. The SDS soluble fraction includes two major peaks at 58-60 kg.mol^{-1} (P4) and 36-38 kg.mol^{-1} (P5) which may be attributed to alpha/beta dimers of legumin and to vicilin subunits, respectively (John A. Gatehouse, Croy, Morton, Tyler, & Boulter, 1981). A shoulder is detected around 70-80 kg mol^{-1} which may be attributed to convicilin subunits (Croy, Gatehouse, Tyler, & Boulter, 1980). Smaller polypeptides at 22,

17 and 14 kg.mol⁻¹ are also visible. These polypeptides cannot be attributed firmly based on molecular weights as they could correspond to alpha, beta chains of legumins (Matta, Gatehouse, & Boulter, 1981), to vicilin peptides (John A. Gatehouse et al., 1981) or pea albumins (Croy, Hoque, Gatehouse, & Boulter, 1984; J. A. Gatehouse, Gilroy, Hoque, & Croy, 1985). A fraction of proteins displayed a molecular weight higher than 100 kg.mol⁻¹, probably corresponding to aggregated proteins. The fraction of SDS-insoluble pea protein released after reduction of DTE contains several peaks, the same peaks as those in the SDS soluble protein profile, along with large species (> 200 kg.mol⁻¹), suggesting that some of the SDS-insoluble aggregates are stabilised by, at least, disulphide bridges. Industrial pea proteins are complex mixtures of proteins. In reduced conditions, it is difficult to attribute the nature of polypeptides based on their molecular weight. Other technics based on immunospecificity would be required. The elution profile of RuBisCO SDS-soluble protein fraction includes three well defined peaks (**Figure 1C1**). The main fraction was eluted in P6 (16.4 min) and was attributed to the small chain of RuBisCO (SC), with a molecular weight of about 15 kg.mol⁻¹. The peak eluted between P4 and P5 (14.3 min, ~ 45 kg.mol⁻¹) was attributed to the RuBisCO large chain (LC) (Barbeau & Kinsella, 1988). Peak P3 (13.15 min, ~100 kg.mol⁻¹) could corresponds to large-chain dimers that may be formed during the extraction process (Rintamaki, 1989). This dimer was not detected when proteins were directly extracted in the presence of SDS and DTE (data not shown). Moreover, only two peaks attributed to the small and large subunits are present in SDS+DTE soluble fraction (**Figure 1C2, inset**), suggesting that interchain disulphide bonds would stabilise LC dimer. A shoulder visible between P5 and P6 in the SDS soluble protein profile corresponds to a molecule of about 28 kg.mol⁻¹ of unknown origin. At the elution time of this peak, the ratio of intensity at 260 nm with 214 nm is much higher than for the other peaks (data not shown). This suggests that this molecule is richer in groups that absorb at 260 than other proteins of the extract. The molecular weight is too high to correspond to phenolic compound but this molecule could be a protein linked with phenolic compounds.

Table 1. Characterisation of protein concentrates: protein content, accessible free thiol content and protein sequential solubility in denaturing buffer (SDS) and reducing buffer (SDS+DTE). "N.d" stands for "not determined". Standard deviations are expected to be similar to the standard deviations obtained on RuBisCO concentrate.

	Protein content			Free thiol content			SDS soluble protein			SDS+DTE soluble proteins		
	<i>mg/100 g of concentrate, dry basis</i>			<i>μmol / g of protein</i>			<i>in % of extractable proteins</i>					
Gluten	67.2	±	2.4	0	±	0.1	88.9	±	n.d	11.1	±	n.d
Pea proteins	77.5	±	0.3	11.2	±	0.1	76.6	±	n.d	23.4	±	n.d
RuBisCO	74.1	±	0.7	67.2	±	0.9	96.4	±	0.7	3.6	±	0.7

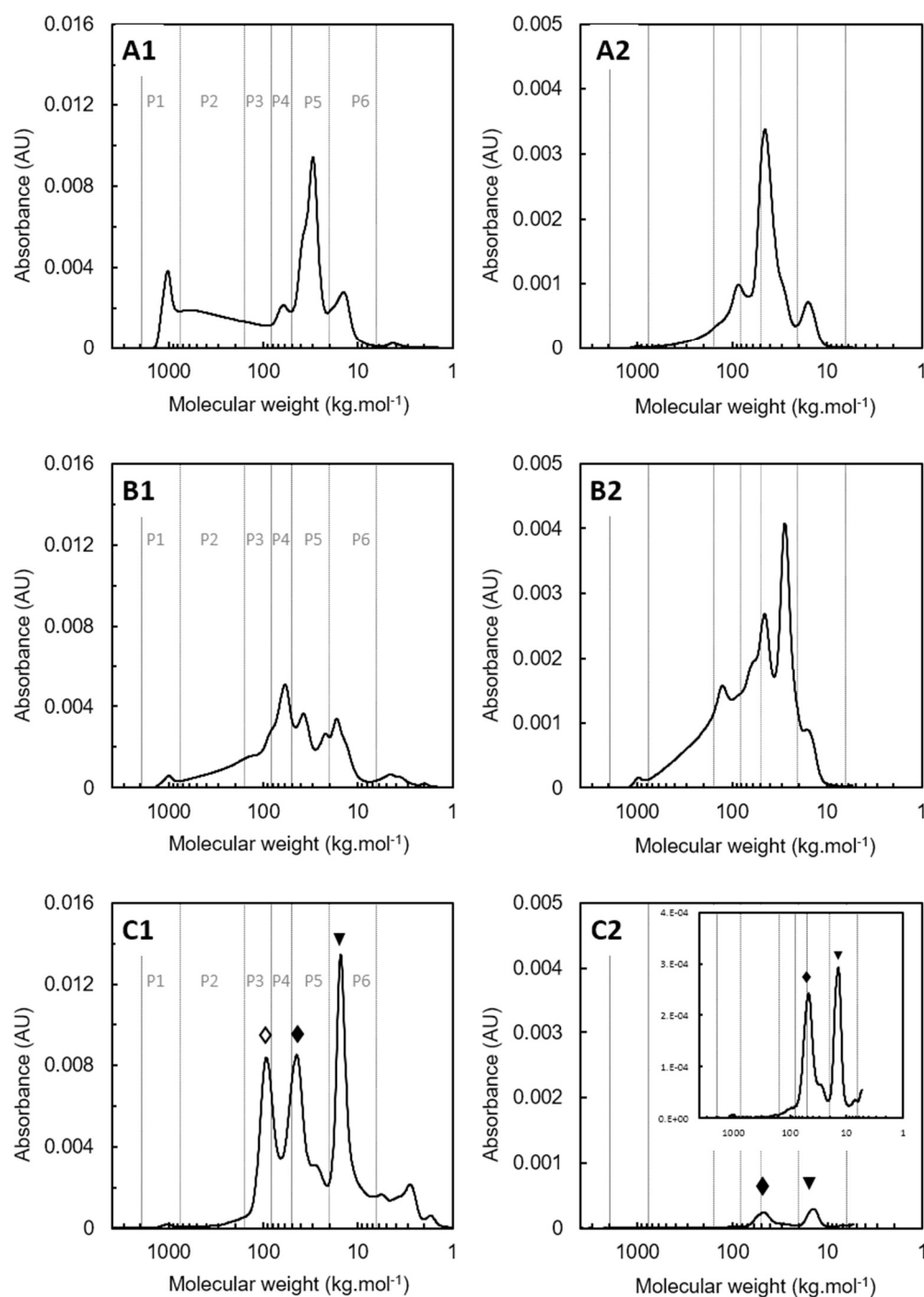


Figure 1. Elution profiles of SDS soluble protein fractions (1; left) and SDS+DTE soluble protein fractions (2; right) extracted from gluten (A), pea protein (B) and RuBisCO (C) concentrates. The inset in C2 is a close-up of the elution profile. The symbols in figure C1 and C2 represent RuBisCO subunits: small chain (SC: ▼), large chain (LC: ◆) and large chain dimer (LC dimer: ◇). For the sake of readability, the two types of elution profiles are not represented at the same scale and the elution profile of SDS+DTE soluble proteins is represented only from 6 kg.mol⁻¹. Every experimental elution profile is represented after normalisation based on 1mg of total protein in the sample.

3.2 Influence of protein enrichment on dough stiffening during heating

Temperatures above 50°C are known to trigger protein polymerisation and starch gelatinisation, and thus to affect wheat dough mechanical properties (Bloksma, 1972). Therefore, to understand the effect of protein enrichment on the structuring of dough during thermal treatment, changes in mechanical properties of doughs prepared at several enrichment rates were assessed during a temperature ramp. Representative curves of elastic modulus (E') as a function of the core sample temperature for control and ~32% extrinsic protein-enriched doughs are shown in **Figure 2A**. All samples exhibited the same trend. When the temperature was increased up to 50°C, E' decreased (Zone I) due to an increase in water and polymer chain mobility (Bloksma, 1990), followed by a marked increase in E' between 50°C and 75°C reflecting dough stiffening (Zone II). This increase corresponds to the cumulative effect of starch gelatinisation and protein aggregation. However, there is no clear scientific consensus on which of the two phenomena dominates the elastic modulus response in these temperature ranges. The maximum value of the elastic modulus has been shown to be positively correlated with the starch content in starch-gluten blends while gluten is assumed to have little influence on E' values (Dreese, Faubion, & Hosene, 1988; Mario Jekle, Mühlberger, & Becker, 2016; Zanoletti et al., 2017). Conversely, some studies on wheat doughs reported that changes in E' with temperature are mainly due to the polymerisation of gluten proteins (Rouillé, Chiron, Colonna, Della Valle, & Lourdin, 2010). Beyond 75°C, the elastic modulus decreased almost linearly (zone III). The mechanism related to this change is not well established in the literature as reviewed in Vanin, Michon, & Lucas, (2013). Several studies on gluten, pea or RuBisCO proteins system report an increase or a stabilisation of E' values at these temperatures (Felix, Perez-Puyana, Romero, & Guerrero, 2017; Martin et al., 2014; Wang et al., 2017). Therefore, elasticity drop above 75°C may be associated with the softening of swollen starch granules. The peak of elastic modulus is representative of the maximal structural hardening of the dough. The change in maximum value of E' ($E'_{\max}$), is shown on **Figure 2B** as a function of the protein enrichment and total protein contents for the three proteins studied. $E'_{\max}$ values of doughs enriched at 8% were lower than the control dough regardless of the type of protein used. Gluten, pea proteins and RuBisCO alter dough thermal structuring similarly at 8% of protein enrichment. Beyond 8%, two types of behaviour were observed depending on the protein used for enrichment. Enrichment in gluten and pea proteins caused a further decrease in $E'_{\max}$ of about 36% compared to the control dough, suggesting that either starch gelatinisation or gluten polymerisation rates decrease with the incorporation of more pea and gluten proteins. In contrast, enrichment with RuBisCO beyond 8% resulted in $E'_{\max}$ values similar to those of the control dough, demonstrating its capacity to preserve the elastic behaviour of dough.

The loss factor, $\tan(\delta)$, represents the viscous to elastic ratio of dough and thus gives an estimate of the extent of dough structuring (M. Jekle & Becker, 2011). **Figure 3** shows how it changed with temperature in control dough, 29.6% gluten-enriched dough, 33.0% pea protein-enriched dough and 31.9% RuBisCO-enriched dough. Its value was less than 1 and continuously decreased from 25 to 95°C in all samples. A $\tan(\delta)$ value below 1 is typical of elastic material and a decrease indicates a gain in dough stiffness. The gluten-enriched dough displayed the same changes in $\tan(\delta)$ as the control dough with slightly higher values, meaning that an increase in gluten content increases the liquid-like behaviour of dough. The $\tan(\delta)$ of pea protein-enriched dough was close to that of the control dough below 47°C. It then decreased but less markedly than in the control dough, ultimately leading to a substantially higher value of $\tan(\delta)$. Pea proteins reduce the gain in elasticity of the semolina dough

during heating. The loss factor of RuBisCO-enriched doughs was significantly higher than that of the control dough and remained stable from 25 to 40°C. It then dropped abruptly to reach a value similar to that of the gluten-enriched dough at 65°C. This suggests that, between 40 and 65°C, RuBisCO contributes to dough elasticity while below 40°C, it acts as a diluent increasing the liquid behaviour of the sample. It should be noted that beyond 70°C, $\tan(\delta)$ values of doughs enriched in either RuBisCO (31.9%) or gluten (29.6%) were exactly the same, supporting similar involvement of both types of protein in dough structure.

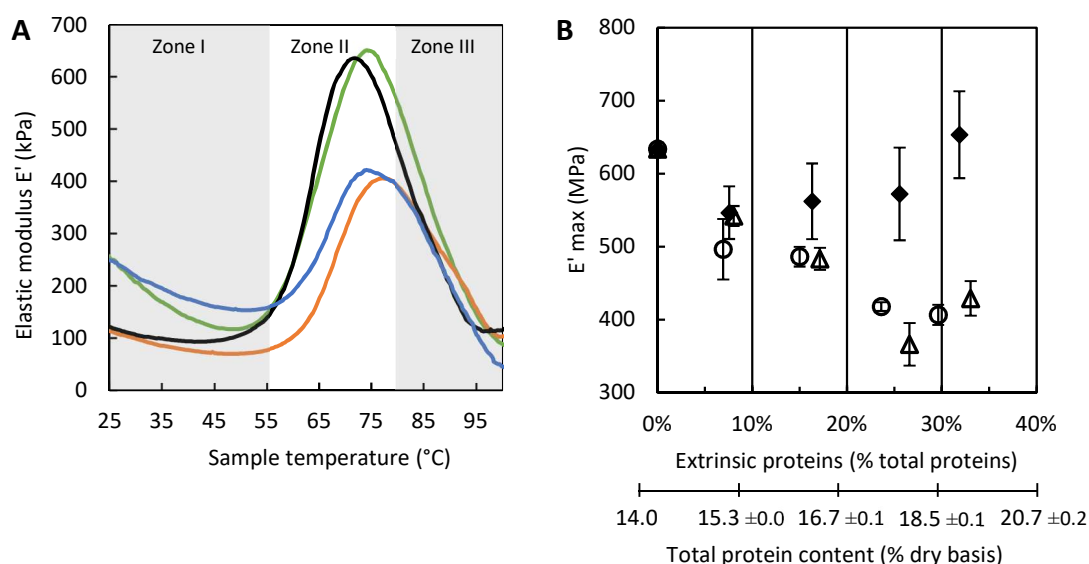


Figure 2. A: Thermal variation of elastic modulus determined by DMTA in control (black) sample and 29.6% gluten- (orange line), 33.0% pea protein- (blue line) and 31.9% RuBisCO- (green line) enriched doughs. The curves are representative of triplicate observations. **B:** Comparison of E' max values measured by DMTA on gluten-enriched doughs (○), pea protein-enriched doughs (Δ) and RuBisCO-enriched doughs (◆) at several enrichment rates.

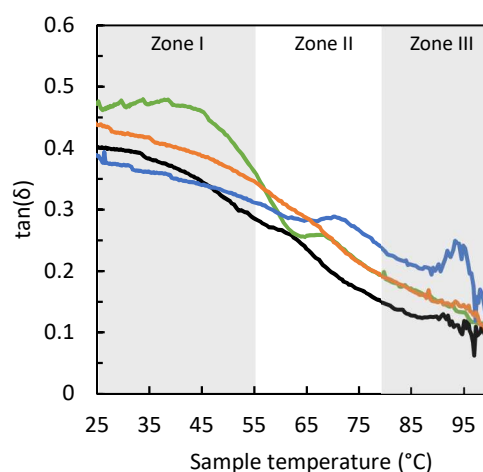


Figure 3. Thermal variation of loss factor $\tan(\delta)$ determined by DMTA in control (black) sample and 29.6% gluten (orange line), 33.0% pea protein (blue line) and 31.9% RuBisCO (green line) enriched doughs. The curves are averages of triplicate observations.

3.3 Protein polymerisation in thermally treated doughs

To identify heat-induced changes in protein interactions, proteins from dough samples treated at 80°C for three minutes were successively extracted in SDS and then in SDS+DTE buffers and analysed by size-exclusion chromatography. Experiments were performed on control and 14.9% gluten, 17.1% pea protein and 16.3% RuBisCO-enriched doughs.

The differential SE-HPLC profiles of each protein-enriched dough were calculated and compared to the SE-HPLC experimental profile of the extrinsic protein concentrate (as described in 2.5.3). Results concerning the SDS-soluble protein fractions are shown in **Figure 4A, B and C**. **Figure 4D** shows the same kind of profiles for SDS+DTE soluble proteins in RuBisCO-enriched doughs. Differences between differential and protein concentrate profiles, when present, can be linked to a change in the size distribution of the extrinsic protein or to the effect of enrichment on the overall heat-induced protein polymerisation whatever the protein considered, either extrinsic or wheat protein.

The impact of protein enrichment on protein polymerisation depends on the type of protein. The differential profile corresponding to gluten-enriched dough is below the gluten concentrate profile, especially at the location of glutenin polymers (**Figure 4A**). Hence, the areas of peaks P1 and P2 are respectively 46% and 24% lower than in the gluten concentrate profile. A decrease of the same magnitude was observed between raw control doughs and thermally treated control dough (data not shown). This suggests that the differences observed between the differential profile of gluten-enriched dough and the gluten concentrate profile could reflect the effect of heating on extrinsic glutenin polymers. The differential profile of pea protein-enriched dough does not differ much from the pea protein concentrate elution profile (**Figure 4B**). Peaks P4, P5 and P6 are only about 10% lower than those of the raw pea concentrate profile

The differential profile of the SDS-soluble fraction obtained from the heated RuBisCO-enriched dough contrasted sharply with that of raw RuBisCO as shown in **Figure 4C**. The differential profile shows negative P1 and P2 peaks, corresponding, in the mirror image, to peaks of wheat glutenin polymers. In addition, the typical RuBisCO peaks, P3, P4 and P6, almost disappeared, implying that enrichment in RuBisCO makes these wheat and RuBisCO protein fractions insoluble in SDS. Moreover, the differential profile of SDS+DTE soluble proteins is well above that of the SDS+DTE soluble protein in the RuBisCO concentrate (**Figure 4D**). In **Figure 4D**, peak P3 may correspond to high-molecular-weight glutenin subunits as described previously, while peak P5 and P6 may partly correspond to RuBisCO LC and RuBisCO SC. The fact that addition of DTE allowed the release of these subunits from the SDS-insoluble protein pellet indicates that protein insolubility was driven by disulphide crosslinking. The same analyses performed with increasing levels of RuBisCO enrichment showed that the concentration of RuBisCO affected the formation of disulphide-linked SDS-insoluble aggregates (not shown). Whether RuBisCO and wheat protein interact to form mixed aggregates was not established with the present analysis. However, it is likely that RuBisCO increases the heat-induced aggregation of wheat glutenin polymers.

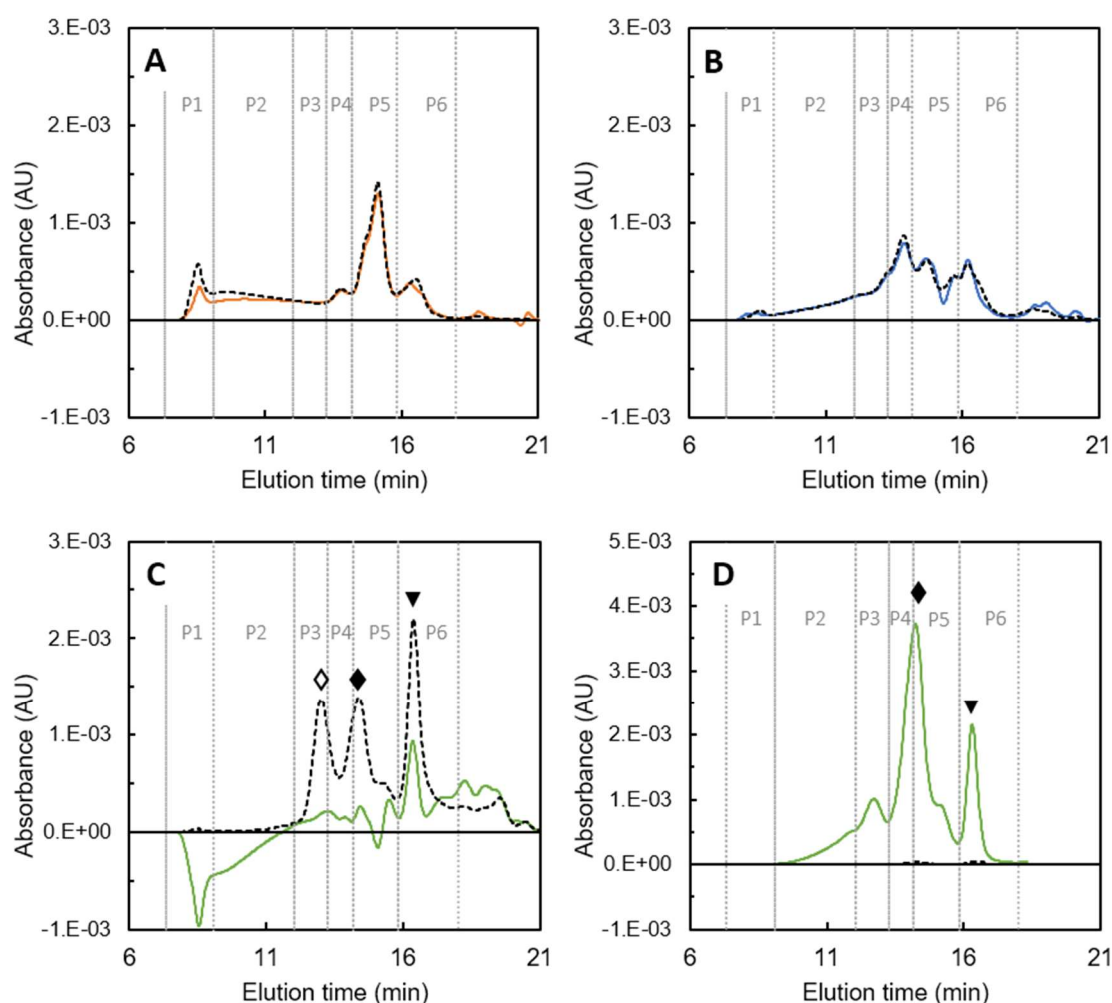


Figure 4. SDS soluble (A, B, C) and SDS+DTE soluble (D) differential profiles (solid lines) obtained from heat-treated doughs enriched with gluten (A), pea proteins (B) and RuBisCO (C, D) compared with elution profile of extrinsic protein extracts (dashed line) adjusted to the same extrinsic protein weight. Differential profiles were obtained by subtracting the SE-HPLC elution profile of heat-treated wheat control dough from the elution profile of the heat-treated enriched doughs (both adjusted to the same semolina weight). Doughs enrichment were 14.9% gluten (A), 17.1% pea protein (B) and 16.3% RuBisCO (C and D). Symbols represent RuBisCO subunits: small chain (SC: ▼), large chain (LC: ◆) and large chain dimer (LC dimer: ◇). For the sake of readability, the elution profile of figure D is represented only up to 18 min.

3.4 Effect of incorporating RuBisCO on protein interaction during hydration and dough mixing

Incorporating RuBisCO enhanced wheat protein polymerisation in thermally treated doughs. Experiments were conducted to check whether the modifications are related to heat treatment or take place during the hydration and dough mixing steps. To this end, the differential SE-HPLC profile of SDS soluble proteins from 37.4% RuBisCO-enriched raw dough was compared to the SE-HPLC profile of the RuBisCO concentrate (as described in iii). Noteworthy differences were observed between the differential profile and the elution profile of the concentrate, as shown in **Figure 5**. Like in thermally treated dough, peak P1 is negative on the differential profile, suggesting that the presence of RuBisCO may have reduced wheat glutenin polymer solubility in SDS. However, part of peak P2 is positive. Taken together, these features show that the addition of RuBisCO shifted the size distribution of wheat

glutenin polymers towards smaller polymers. Peak P3, corresponding to RuBisCO LC dimer, is slightly shifted on the left in the early elution stage, which could be due to incorrect separation between it and peak P2. Peaks P5 and P6, corresponding to RuBisCO LC and SC, respectively, are lower than in the RuBisCO concentrate profile. This suggests that RuBisCO LC and SC aggregate during mixing. These peaks are higher than in theory in SDS+DTE soluble protein elution profile (**Supplementary fig. 2**). RuBisCO LC and SC may therefore form SDS-insoluble disulphide-linked complexes, between each other or with wheat proteins, during hydration and mixing. Moreover, a negative peak can be seen after the RuBisCO LC peak (P5). It means that RuBisCO enrichment affected the molecular weight of proteins that are supposed to be eluted in this zone. It may correspond to a deficit in wheat gliadins and may also be due to the disappearance of RuBisCO LC.

In contrast to thermally treated doughs, the content of SDS-insoluble polymers did not increase with increasing doses of RuBisCO. However, a dose effect was observed for specific peaks (**Figure 6**). P2 and P3 areas are higher on the differential profiles than on the RuBisCO concentrate profile and the difference between the two profiles increased with increasing RuBisCO enrichment of the dough. The opposite was observed for P4 and P5 whose recovery decreased with increasing RuBisCO enrichment. With an increase in the RuBisCO/wheat protein ratio, the size of glutenin polymers decreased and the solubility of the LC and SC subunits in SDS decreased. Incorporating RuBisCO modified the molecular interactions during dough mixing with an effect of dose. The size of SDS soluble glutenin polymers decreased and RuBisCO LC and SC formed disulphide linked aggregates.

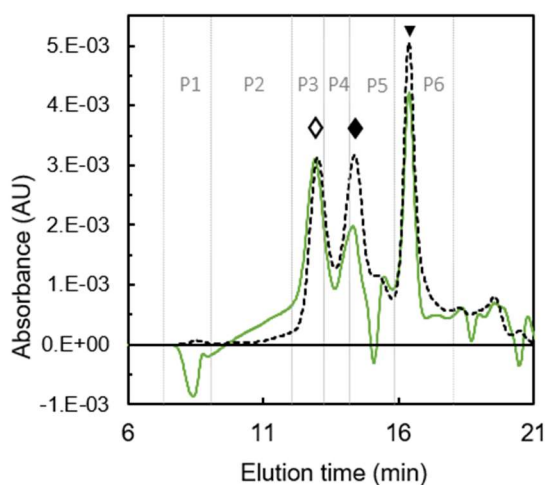


Figure 5. Differential SE-HPLC profile (green line) of raw dough enriched at 37.4% RuBisCO compared to SE-HPLC profile of RuBisCO protein concentrate (dashed black line) based on the same RuBisCO concentrate weight. The differential profile was obtained by subtracting the SE-HPLC profile of SDS-soluble proteins of wheat control raw dough from the profile of RuBisCO-enriched raw dough (both adjusted to same semolina weight). Symbols represent RuBisCO subunits: large chain dimer (LC dimer; ◇), large chain (LC; ◆), small chain (SC; ▼).

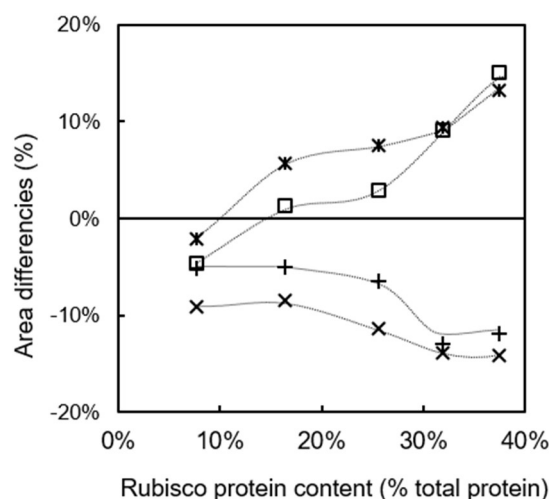


Figure 6. Differences of area under the curve for peaks P2 (□), P3 (×), P4 (+) and P5 (×) between experimental and theoretical elution profiles obtained for RuBisCO-enriched doughs at several RuBisCO enrichment rates. Theoretical profiles correspond to the sum of the elution profiles of the 100% wheat control dough and of the RuBisCO concentrate, both normalised based on their respective proportion in the enriched dough. Differences in peak area are expressed as a percentage of the theoretical profile. Dotted lines are included to guide the eye.

3.5 Weak bond formation during hydration and mixing

Previous results suggest that the introduction of RuBisCO affects molecular interactions in wheat dough. To highlight a possible effect of RuBisCO on gluten network specifically, gluten-like fraction (GLF) were recovered from control and RuBisCO-enriched raw doughs (16.3%). Extraction yields in dry mass and protein content are listed in **Table 2**. A higher amount of GLF was recovered from RuBisCO-enriched dough than from control dough. Given their similar protein content, it can be concluded that enriching the dough with RuBisCO increased the quantity of water-insoluble proteins. This could be related to an increased contribution of wheat proteins, and/or of RuBisCO in GLF.

Table 2. Extraction yield and protein content of gluten-like fractions extracted from control and RuBisCO-enriched doughs.

	GLF weight <i>g (db)</i>	Protein content <i>g/100g of GLF (db)</i>	Protein extraction yield <i>% of dough total proteins</i>
Control wheat dough	0.225 ± 0.009	75.0 ± 0.1	67.1 ± 2.9
16.3% RuBisCO-enriched dough	0.257 ± 0.002	75.1 ± 1.6	66.4 ± 0.9

To assess the presence of RuBisCO in the GLF of RuBisCO-enriched dough, proteins of GLF from control and enriched dough were sequentially extracted in SDS and SDS+DTE and analysed by SE-HPLC. Raw elution profiles of GLF from control and RuBisCO-enriched doughs are given in supplementary data (**Supplementary fig. 3**). We hypothesised that the extraction yield of wheat proteins in RuBisCO-enriched GLF was similar to that in the control wheat GLF (detailed in part 2.5.3). This equates to estimating that 84.5% of the proteins of the RuBisCO-enriched GLF are wheat proteins and 15.5% are RuBisCO. The differential profile of RuBisCO-enriched GLF is compared to the elution profiles of RuBisCO concentrate in **Figure 7**. The SDS-soluble fraction of RuBisCO-enriched GLF shows a higher peak P2, meaning that RuBisCO increases the concentration of medium size glutenin polymers in the GLF.

The presence of peaks of the RuBisCO subunits (P3, P5 and P6) on the differential SE-HPLC profile means they are co-extracted with wheat proteins during dough washing. The heights of peaks P3 and P6, corresponding to RuBisCO LC dimer and SC, are similar on the elution profiles of both differential and RuBisCO concentrate. Most LC dimers and SC are therefore water-insoluble but SDS-Soluble. This suggests that their water-insolubility is related to the establishment of weak interactions. In contrast, the height of the RuBisCO LC peak is lower on the differential profile than on the RuBisCO concentrate profile but higher on the SDS+DTE profile. This suggests that RuBisCO LC form water-insoluble aggregates stabilised by disulphide bounds during mixing.

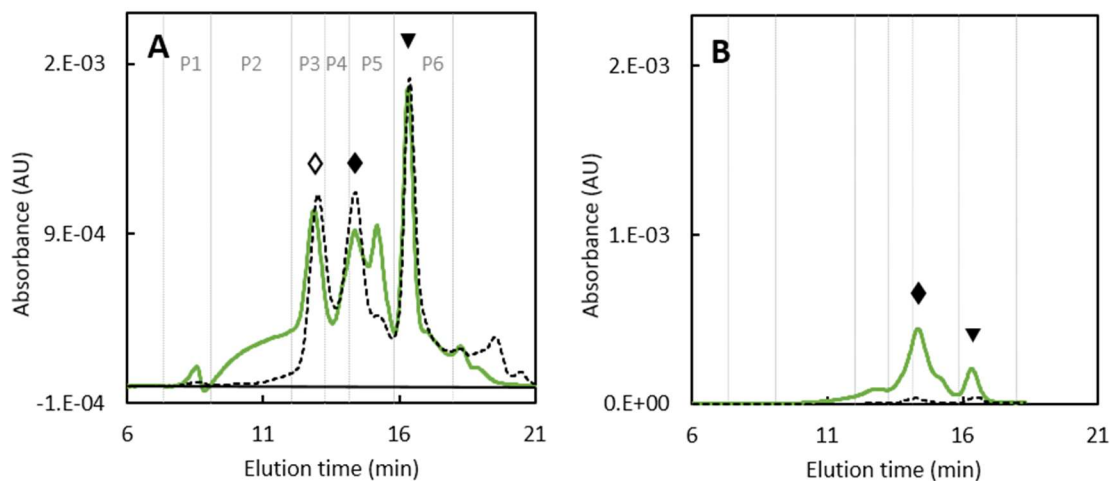


Figure 7. SDS soluble (A) and SDS+DTE soluble (B) differential profiles obtained for the gluten-like fraction (GLF) extracted from dough enriched with 16.3% RuBisCO (solid lines) compared with elution profile of RuBisCO concentrate (dashed lines) adjusted to the same RuBisCO weight. Differential profiles were obtained by subtracting the SE-HPLC elution profile of control GLF from the elution profile of the RuBisCO-enriched GLF. The calculation was made considering that the semolina protein extraction yields were the same for both gluten-like fractions. Symbols represent RuBisCO subunits: small chain (SC: ▼), large chain (LC: ◆) and large chain dimer (LC dimer: ◇).

3.6 Microstructure of the dough protein network

The microstructure of the protein network was visualized by confocal laser scanning microscopy (CLSM). The overall arrangement of the protein network was observed by non-specific covalent labelling of proteins from control dough, 29.6% gluten-enriched dough, 33% pea protein-enriched dough and 31.9% RuBisCO-enriched dough. The resulting images are shown in **Figure 8**. The protein network of the control wheat dough (**Figure 8a**) has a typical honeycomb arrangement embedding starch granules (McCann & Day, 2013). Gluten-enriched dough has a very similar structure (**Figure 8b**). Large protein particles are visible in the pea protein-enriched wheat dough micrograph (**Figure 8c**). Since the emission of these structures is intense, the gluten protein network must be examined at a lower intensity to avoid image saturation. Finally, the RuBisCO-enriched wheat dough has a slightly different protein network than the control wheat dough. Indeed, the extent of the protein network seems to be less uniform with higher lacunar zones. Further image analyses are needed to confirm this observation.

In order to reveal the segregation or co-location of RuBisCO and gluten, doughs were prepared using covalently-labelled RuBisCO. CLSM images (**Figure 8d1 and d2**) show that RuBisCO does not form

aggregated structures visible at the micro-scale in contrast with pea proteins. Instead, it is co-localized with the gluten protein network.

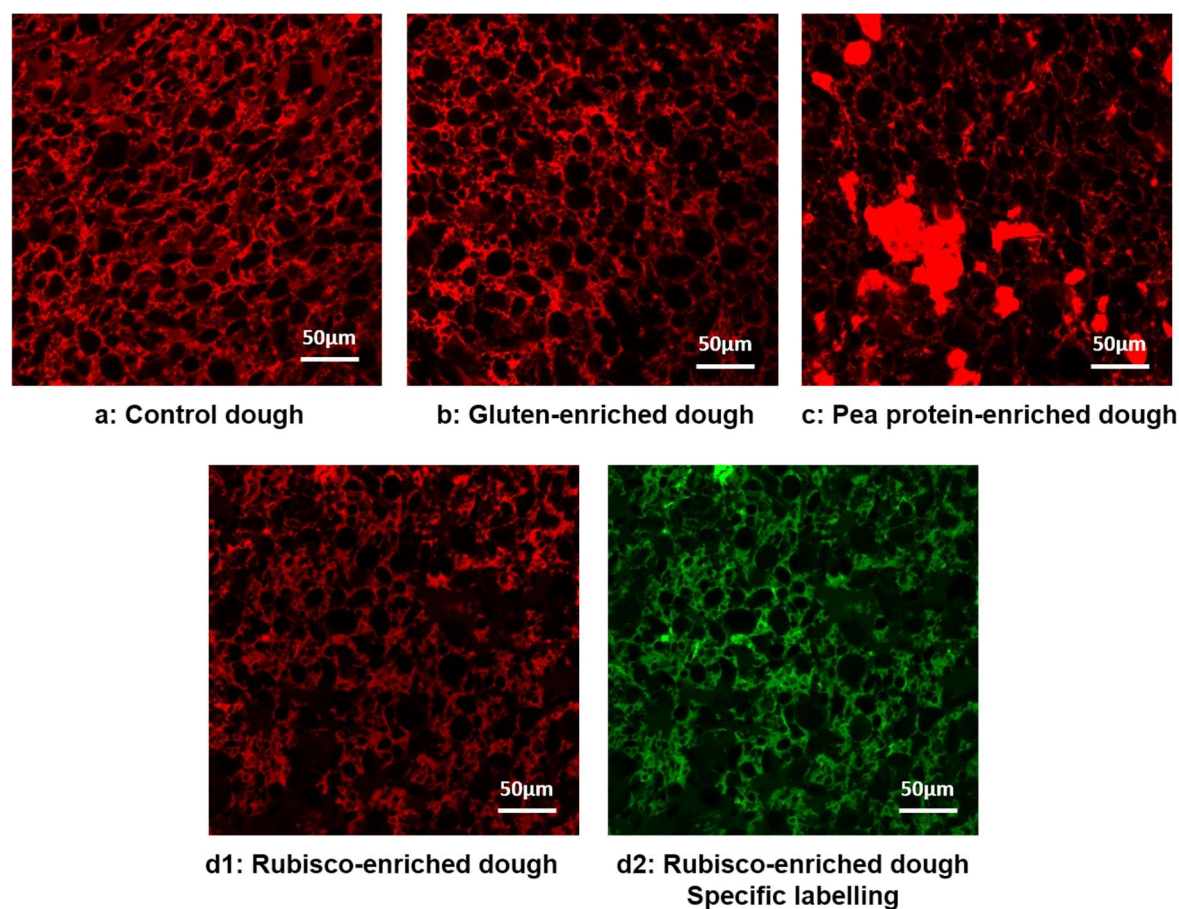


Figure 8. CLSM images of control wheat dough (a), 29.6% gluten-enriched dough (b), 33% pea protein-enriched dough and 31.9% RuBisCO-enriched dough (d1, d2). Proteins were stained using Alexa Fluor™ 546 NHS Ester (in red in the images) in all samples. Only the RuBisCO-enriched dough has double labelling: RuBisCO was specifically labelled with FITC (in green in the images) before it was incorporated in the dough.

4 Discussion

RuBisCO concentrate was used to improve the nutritional profile of wheat semolina dough. In comparison to gluten and pea proteins, RuBisCO exhibited specific behaviour. Indeed, adding RuBisCO preserved the elastic potential of wheat dough in contrast to adding gluten and pea protein. RuBisCO sub-units formed both weak and covalent bonds, with a probable interaction with wheat proteins.

Unlike pea proteins or gluten, adding RuBisCO enhanced wheat dough stiffening during a temperature ramp

Dilution of wheat semolina with increasing concentrations of extrinsic gluten reduced the rise in elastic modulus between 55° and 80°C and shifted $\tan(\delta)$ to higher values. However, the addition of gluten did not modify the drop of $\tan(\delta)$ during heating, which remained parallel to that of the standard wheat dough. Similar mechanical changes have already been reported in wheat doughs (N. M. Edwards, Dexter, Scanlon, & Cenkowski, 1999) and in protein-starch-water systems (Hibberd, 1970). Thus, a decrease in the starch-gluten ratio in wheat dough increases the liquid-like behaviour of dough (rise of $\tan(\delta)$) and limits the rise of the elastic modulus during heating. In addition, SE-HPLC analysis of SDS-

soluble proteins from thermally treated gluten-enriched wheat dough demonstrated that adding gluten does not alter protein crosslinking during heating. Furthermore, extrinsic gluten and native semolina proteins were shown to behave similarly, forming SDS insoluble aggregates under thermal treatment. Thus, the decrease in E' values in gluten-enriched wheat doughs is not related to a change in gluten protein polymerisation but rather to a change in starch gelatinisation. Several authors reported that adding gluten in starch-gluten blends can affect the starch gelatinisation rate and extent due to competitive hydration or by hindering water diffusion (Mario Jekle et al., 2016). Further analysis by differential scanning calorimetry (DSC) is now needed to determine if extrinsic gluten can act as a water trap.

Similarly, the enrichment of wheat dough in pea proteins reduced $E'_{\max}$ values with no major modification of wheat protein crosslinking upon heating. It can be concluded that like gluten, pea proteins compromise starch gelatinisation. Moreover, pea protein-enriched doughs presented the highest $\tan(\delta)$ values above a temperature of 65°C, meaning that the relative viscous part of the dough increased as a result of the addition of pea proteins. This could be related to the higher water-binding capacity of pea proteins compared to gluten proteins (Bravo-Núñez, Garzón, Rosell, & Gómez, 2019; Peters, Vergeldt, Boom, & van der Goot, 2017). In addition, CLSM images of enriched doughs revealed the presence of large protein particles, likely from pea proteins. They can affect the structure of the wheat gluten network due to steric hindrance. Several studies have shown that pea or faba bean enrichment reduces pasta quality by diluting the gluten network (Laleg et al., 2017; Petitot et al., 2010).

Finally, RuBisCO enrichment of less than 8% affected the mechanical properties of dough in the same way as gluten or pea proteins, suggesting that starch gelatinisation is similarly compromised. However, above 8%, the addition of RuBisCO maintained a notable rise in E' during heating in a similar way to control dough. RuBisCO contributes to the elastic and viscous properties of the system. RuBisCO may not act as a water trap like extrinsic gluten or pea proteins and may allow starch gelatinisation proceed with no hindrance. In addition, RuBisCO enhances the formation of disulphide-linked protein aggregates upon dough heating that may strengthen dough mechanical properties. Incorporating RuBisCO in dough also increases the $\tan(\delta)$ values up to a temperature of 65°C. This increase in the liquid-like behaviour of the dough may be related to the decrease in the concentration of HMW glutenin polymers. The further decrease in $\tan(\delta)$ values above 70°C may be related to the formation of large disulphide-linked polymers in thermally treated RuBisCO-enriched wheat doughs.

In this study, RuBisCO differed from pea proteins, which do not react with semolina protein. However, other legume proteins, such as soy proteins, are able to form disulphide-bonded polymers when incorporated in wheat dough (Ribotta, León, Pérez, & Añón, 2005). But contrary to RuBisCO, this enrichment impairs the gluten network by reducing the overall size of the polymers (Pérez, Ribotta, Steffolani, & Le, 2008), resulting in reduced E' values during the temperature gradient (Zhou, Liu, & Tang, 2018).

RuBisCO sub-units participate in the network by means of both non-covalent and covalent bonds in wheat dough

Analysis of SE-HPLC profiles in control and RuBisCO-enriched dough led us to conclude that RuBisCO triggers the formation of large polymers stabilised by disulphide bonds. RuBisCO subunits are involved in these large polymers by covalent bonding, sometimes stabilised by disulphide bridges.

The high concentration of free thiols in RuBisCO may partially explain RuBisCO's ability to form disulphide bonds when used to enrich wheat dough. Indeed, it has been suggested that free thiols enhance the formation of disulphide bond dynamics in the gluten network (Auvergne et al., 2008). It is known that an increase in temperature above 50°C increases the size of glutenin polymers (Bloksma, 1972). Up to 70°C, this increase in size is explained by sulfhydryl-disulphide interchange (Schofield et al., 1983). These interchanges are explained by radical and nucleophilic mechanisms (Auvergne et al., 2008). It has been assumed that nucleophilic mechanisms increase in the presence of thiolate groups in equilibrium with thiol functions. In that sense, incorporating RuBisCO could enhance the interchange of disulphide bonds by providing free thiol groups in the system. To validate this mechanism, controlled alkylation experiments should be performed on RuBisCO before it is incorporated in the dough. RuBisCO's participation in sulfhydryl-disulphide interchanges with gluten proteins would imply it is a co-protein network.

Besides covalent interactions, non-covalent bonds play a significant role in the mechanical properties of the gluten network (Belton, 1999; Belton et al., 1995; Shewry, Popineau, Lafiandra, & Belton, 2001; Tkachuk & Hlynka, 1968). The balance between the amino acid residues involved in hydrogen bonds with water molecules and inter-chain hydrogen bonds would lead to the formation of a "loops and trains" structure. The deformation and relaxation capacity of these structures would provide the elasticity to gluten molecules (Belton, 1999). Moreover, recent structural models of gluten suggest that weak interactions act as junction points that initiate the formation of the gluten network during hydration and mixing. Such weak bond nodes allow gluten polymers to percolate and to form a network (Dahesh, Banc, Duri, Morel, & Ramos, 2016; Létang, Piau, & Verdier, 1999; Ng, McKinley, & Ewoldt, 2011). In our study, RuBisCO subunits were not leached out by water. Most were recovered in the gluten-like fraction and eluted at their expected molecular weight. This suggests that RuBisCO subunits establish weak bonds with wheat protein during dough mixing before any thermal treatment. Electrostatic interactions are unlikely because gluten proteins are weakly charged. Hydrophobic and hydrogen interactions control RuBisCO heat gelation in dispersed systems (Libouga, Aguié-Béghin, & Douillard, 1996; Martin et al., 2014). The contribution of both hydrophobic and hydrogen interactions in RuBisCO reactivity in wheat dough during mixing can be assumed.

The formation of a co-protein network between gluten proteins and RuBisCO is the most likely hypothesis to explain our results concerning the interactions established by RuBisCO during mixing and heating and the properties of the resulting dough. This hypothesis is supported by protein network microstructure visualised by CLSM since RuBisCO and gluten are co-located in the network before thermal treatment. However, further investigations are needed to prove that RuBisCO and gluten interact specifically with each other.

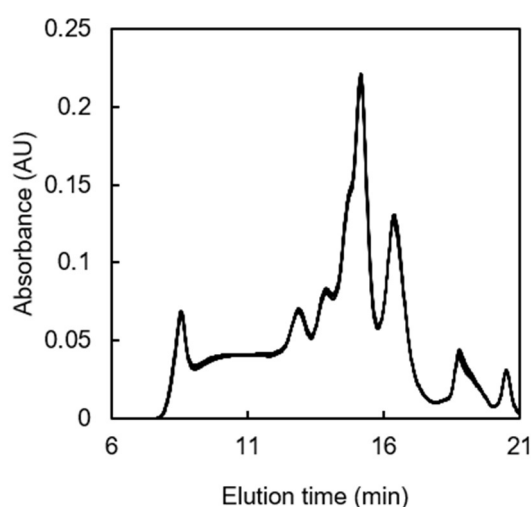
5 Conclusions

The study of the mechanical properties and protein interactions of RuBisCO-enriched wheat dough clearly highlights its potential ability to increase the plant protein content of cereal-based foods. RuBisCO behaviour is quite different from that of legume or even gluten proteins. Enrichment in pea proteins or gluten does not modify protein polymerisation even after thermal treatment. The thermo-mechanical properties of these pea or gluten-enriched doughs appear to be affected probably due to a modification of the distribution of water in the system, thereby limiting starch gelatinisation. The behaviour of RuBisCO is very different. Interestingly, RuBisCO is able to preserve the increase in elasticity of the dough during heating thanks to its own reactivity and to possible low competition with starch for water. Remarkably, hydrated and mixed with wheat semolina, RuBisCO formed both weak and disulphide bridges. It then joined the water-insoluble protein network. The concentration of large covalently linked polymers increased considerably during dough thermal treatment, because of the ability of RuBisCO to form new aggregates in these conditions. The colocation of gluten and RuBisCO proteins on micrographs supports the hypothesis that they even formed a co-protein network. To confirm the benefits of using RuBisCO to enrich cereal based food, it would be useful to test the mechanical properties of RuBisCO-wheat matrices at high deformations to better investigate the effect of protein enrichment on the rheological properties of the new food system in comparison to the better-known pea protein-enriched wheat matrices.

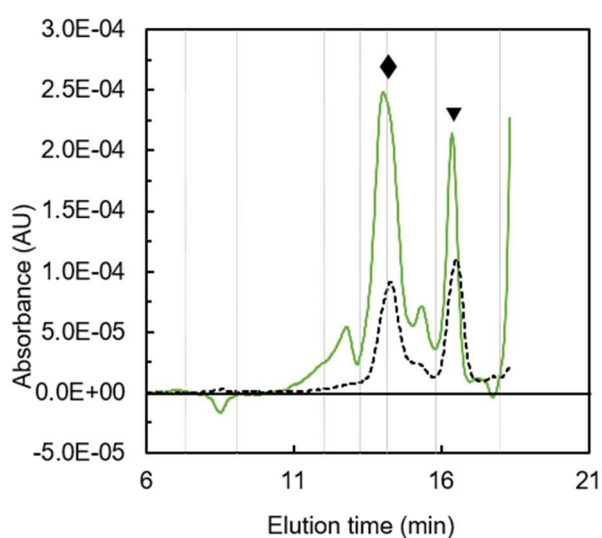
Acknowledgments

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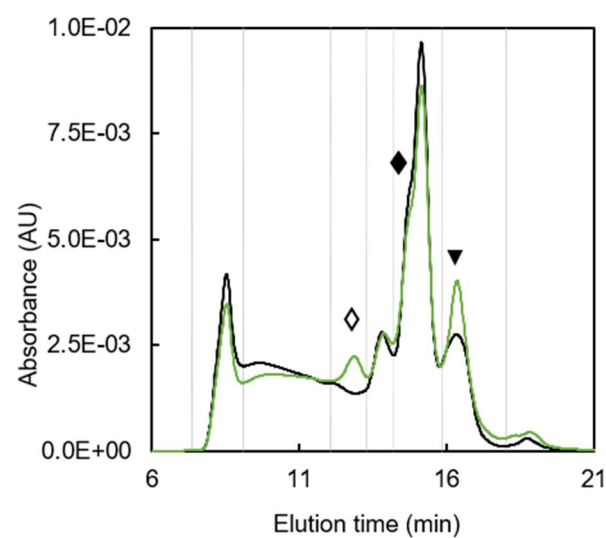
Supplementary material



Supplementary fig. 1 Comparison of three SE-HPLC elution profiles of SDS soluble proteins extracted from the same sample and two chromatograms extracted from another sample in exactly same conditions (16.3% RuBisCO-enriched dough, 65% water db).



Supplementary fig. 2 Differential SE-HPLC profile (solid line) of SDS+DTE soluble proteins from a 37.4% RuBisCO-enriched raw dough compared to the profile of RuBisCO protein concentrate (dotted line) adjusted to the same RuBisCO weight. Differential profiles were obtained by subtracting the SE-HPLC profiles of SDS+DTE soluble proteins of wheat control raw dough from the profile of RuBisCO-enriched raw dough (both adjusted to same semolina weight). For the sake of readability, the elution profile is represented only up to 18 minutes. Symbols represent RuBisCO subunits: small chain (SC: ▼) and large chain (LC: ◆).



Supplementary fig. 3 Experimental SE-HPLC profiles (solid lines) of SDS soluble proteins of gluten-like fraction extracted from raw control doughs (black line) and 37.4% RuBisCO-enriched dough (green line). Symbols represent RuBisCO subunits: small chain (SC; ▼), large chain (LC; ◆) and large chain dimer (LC dimer; ◇). Elution profiles after normalisation on the basis of 1mg of total protein in the sample.

Part 2. Changes in the secondary structures of proteins after the RuBisCO enrichment of wheat dough

Part of the experimental data presented in this section was acquired at the Bohdan Dobrzanski Institute of Agrophysics of the Polish Academy of Science (Lublin, Poland) during a scientific stay.

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1 Introduction

Protein covalent interactions, and notably disulphide bridges, play a major role in the formation of the gluten network and the establishment of the viscoelastic properties of the dough (Frater, Hird, Moss, & Yates, 1960; Wieser & Kieffer, 2001). Beside, gluten proteins polymerise during heat treatment counterpart leading to the formation of additional S-S bridges and others covalent linkages (Schofield et al., 1983; Tilley et al., 2001). Apart from intermolecular disulphide bridges, noncovalent interactions play an important role in gluten network structure (Belton et al., 1995; Wellner, Belton, & Tatham, 1996). Using Fourier-Transform Infrared Spectroscopy (FT-IR), it has been shown that hydrated gluten contains a high proportion of intermolecular β -sheets, β -turns and α -helices (Pézolet, Bonenfant, Dousseau, & Popineau, 1992). Moreover, gluten in dough displays less α -helices and β -turns and more β -sheets, especially inter-molecular, than in solution (Pézolet et al., 1992; Popineau, Bonenfant, Cornec, & Pezolet, 1994). Dough hydration and mixing would induce conformation changes leading to the decrease of β -turns and the increase of β -sheets (Georget, Underwood-Toscano, Powers, Shewry, & Belton, 2008; Jazaeri et al., 2015). Belton (1999) proposed a model structure in which gluten viscoelasticity relies on the high number of hydrogen bonds between glutenin subunits. In this model, the intermolecular hydrogen interactions are partly converted into protein-water interactions through hydration. These changes are accompanied by secondary structure changes of the glutamine residues from β -spiral structures to interchain β -sheet structures. Infrared spectroscopy can also be used to characterize the structural changes of other wheat components, such as starch after hydration or thermal treatment (Capron, Robert, Colonna, Brogly, & Planchot, 2007; Sevenou, Hill, Farhat, & Mitchell, 2002).

In the previous section, we showed that RuBisCO differs from pea or gluten proteins when used to enrich wheat dough. Enrichment in pea proteins or gluten does not modify the protein polymerisation even when a thermal treatment is applied to the dough. In contrast, hydrated and mixed with wheat semolina, RuBisCO forms both weak and disulphide bridges. Moreover, the content in large size covalently linked polymers highly increases during dough thermal treatment. This may be related to the ability of RuBisCO to form new aggregates in these conditions. Since changes in non-covalent interactions and secondary structures play an important role on gluten structure, the aim of this study was to investigate the impact of RuBisCO, gluten or pea proteins on the secondary structure of proteins in enriched wheat doughs. RuBisCO, gluten and pea protein-enriched doughs were analysed by FT-IR

both before and after thermal treatment. The analysis of the spectra was carried out by PCA or by calculation of differential spectra on the amide I band. An analysis was also carried out on the bands associated with the starch structures.

2 Material and methods

2.1 Material

The raw materials are the same as those described in (Ducrocq, Boire, Anton, Micard, & Morel, 2020). Briefly, wheat semolina was provided by La Semoulerie de Bellevue (Panzani, Marseille, France), pea protein concentrate (Nutralys, F85F) was purchased from Roquette (Lestrem, France) and RuBisCO concentrate was provided by Florette (Lessay, France). RuBisCO was extracted from *Cichorium endivia* leaves based on the extraction conditions of the WO 2014/104880 patent. The gluten was extracted by hand from a durum wheat semolina dough based on Auger, Morel, Dewilde, & Redl (2009). Wheat semolina and protein concentrates were stored in a humidity-controlled chamber with a K_2CO_3 saturated salt solution at 20°C to maintain 43.16% relative humidity.

The water content of powders was determined in triplicate by weighing after drying at 105°C for three hours. The protein content of all the raw materials was analysed using the Kjeldahl procedure (NF V 03-050, 1970) with a conversion factor of 5.7 for wheat semolina, pea proteins and gluten concentrates. A conversion factor of 5.8 was used for the RuBisCO concentrate based on the amino acid profile previously determined. Gluten, pea protein and RuBisCO concentrates contained respectively 67.2, 77.5 and 74.1% of protein (dry basis).

2.2 Sample preparation

Dough formulation

Dough samples (3 g) were prepared using wheat semolina, water and variable amounts of protein concentrates. A standard water content of 67% was used (dry mass basis) to obtain a homogeneous cohesive dough. Protein enrichments were carried out by replacing 3.5% (w:w) of the semolina mass with a protein concentrate. A 10% RuBisCO enriched dough was also prepared and analysed to assess a potential dose effect. Protein enrichment is hereafter expressed as a percentage of the total protein content of the protein-enriched dough. A 3.5% semolina substitution with protein concentrate leads to a 16.3% RuBisCO enriched dough, a 14.9% gluten-enriched dough or a 17.1% pea proteins enriched dough. A 10% semolina substitution leads to a 37.4% RuBisCO-enriched dough. The control dough corresponds to a non-enriched wheat dough (0 % enrichment).

Raw dough fabrication

Doughs were prepared using a 2-g Mixograph (TMCO, Lincoln, NE, USA) as described previously (Ducrocq et al., 2020). Briefly, wheat semolina, the protein concentrate and water were homogenised in the mixing bowl for six seconds at 54 rpm at ambient temperature. The mixture was left to rest for 15 min at 40°C and cooled to 20°C over a period of 25 min. The dough was then mixed at 54 rpm for 220 s.

To study the effect of heat on protein structure, a thermal treatment was applied to the mixed doughs. A hermetically sealed aluminium container was made to contain 1.2 g of dough. Directly after mixing, the device was filled with 1.2 g of dough and immersed in a water bath at 80°C for three minutes. It was then cooled down on ice and the dough sample was removed and immersed in liquid nitrogen

before freeze drying. Three samples were produced for each enrichment condition. All spectroscopy analyses were performed on freeze-dried doughs.

2.3 *Fourier transform infrared (FTIR) spectroscopy*

Spectra acquisition

FT-IR spectra were recorded using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Madison, WI, USA) equipped with a diamond attenuated total reflectance (ATR) attachment. Interferograms (128) were collected between 4000 cm^{-1} and 400 cm^{-1} at 4 cm^{-1} resolution and co-added before Fourier transformation. Five spectra were collected on each sample. The empty ATR crystal served as a reference. Samples were analysed randomly to avoid environmental effect on signals.

Spectra analysis

Infrared spectra were analysed either with Principal Component Analysis (PCA) or through differential spectra. Before applying a PCA, spectra were first truncated to the desired wavenumbers. Then, either a baseline correction or a second derivative was performed using the PLS-Toolbox v8.81 (Eigenvector Research, Inc) for MATLAB (v2015b 8.6.0, Mathworks). Otherwise specified, it is considered that the baseline correction method was used in this study. The spectra were then normalized on their total area, the data were mean centred and principal component analysis (PCA) were performed. The loadings of the principal components (PC) and their contribution in samples helps to understand the changes in sample spectra in relation with the observed variability. For the analysis of the PC loading, it is worth noticing that the second derivative of a spectrum displays opposite variations as compared to the initial spectra. Thus, a local maximum of intensity (a peak) of a spectrum will be represented by a local minimum on its second derivative. This is why, when a second derivative is performed on the sample spectra before PCA, the PC loading displays spectral patterns that are opposed to its actual contribution in the sample spectrum.

Alternatively, an analysis of the difference spectra was performed on Amide I band. Spectra were baseline corrected using OMNIC (v.8.2, Thermo Fischer Scientific Inc., Madison, WI, USA). Then, the five registered spectra for each sample were averaged. The spectra were truncated and normalized upon Amide I band area. The spectra of the three samples corresponding to the same conditions were averaged before being compared, by subtracting another condition.

3 Results

3.1 *Modification of starch structure induced by dough heating*

To observe the global variability of our spectra collection, we performed a PCA on FT-IR spectra from 1900 to 800 cm^{-1} acquired on samples with the same protein content. The analysis was performed on raw and thermally treated doughs enriched with either 14.9% gluten, 17.1% pea protein or 16.3% RuBisCO.

Figure 9A shows the scatterplot of the second principal component (PC2) versus the first principal component (PC1) from the PCA, explaining 21.6% and 36.9% of the total variance, respectively. Two major groups of spectra can be observed, one group contains raw samples while the other is composed of thermally treated samples. Inside these groups, samples composed of different proteins are not differentiated. The major variability of spectra between 1900 and 800 cm^{-1} is therefore due to the thermal treatment of doughs.

Plot of PC1 and PC2 loadings according to the wavenumbers is represented in **Figure 9B**. Both loadings display positive and negative peaks in two major wavenumbers ranges: from 950 to 1200 cm^{-1} and from 1500 to 1750 cm^{-1} . The bands around 1000 cm^{-1} mainly originate from the vibrational state of the glucose monomer unit of starch (reviewed in Kizil, Irudayaraj, & Seetharaman, 2002). The loading of PC1 opposes bands at 1020 cm^{-1} and 1039 cm^{-1} , respectively negative and positive. These bands can be related to amorphous and ordered structure of starch respectively, then to its gelatinisation degree (Wilson, Goodfellow, & Belton, 1988). The opposite pattern is observed for PC2 with a supplementary negative peak at 995 cm^{-1} , associated with the ordered structure of starch. Thermally treated sample group lays on the highest values PC1. A second derivative was performed on spectra before PCA, thus the principal components display spectral patterns that are opposed to its actual contribution in the sample spectrum. PC1 displays positive and negative bands related to the ordered and amorphous structures of starch, respectively. PC1 has a high contribution in thermally treated samples therefore, it can be concluded that thermal treatment induces a decrease of ordered structure in starch. PC2 displays nearly an opposite spectral pattern as compared to PC1 and thermally treated samples lay on lowest values of PC2. Therefore, same interpretation is drawn from the variation in PC2. This result is not surprising since an increase in temperature under the hydration conditions of the samples is supposed to induce starch partial gelatinisation. However, the screening of mechanical properties of doughs according to temperature suggested that gluten and pea proteins enrichment might partially reduce starch gelatinisation due to the competition for water. This effect is not highlighted here: PCA highlights the spectral changes associated with starch gelatinisation but does not separate the samples according to their protein composition. This may be related to the different methods of thermal treatment used for the analysis of doughs in spectroscopy (80°C for 3 minutes followed by freeze drying) or for the screening of mechanical properties (temperature ramp on raw dough in an oven).

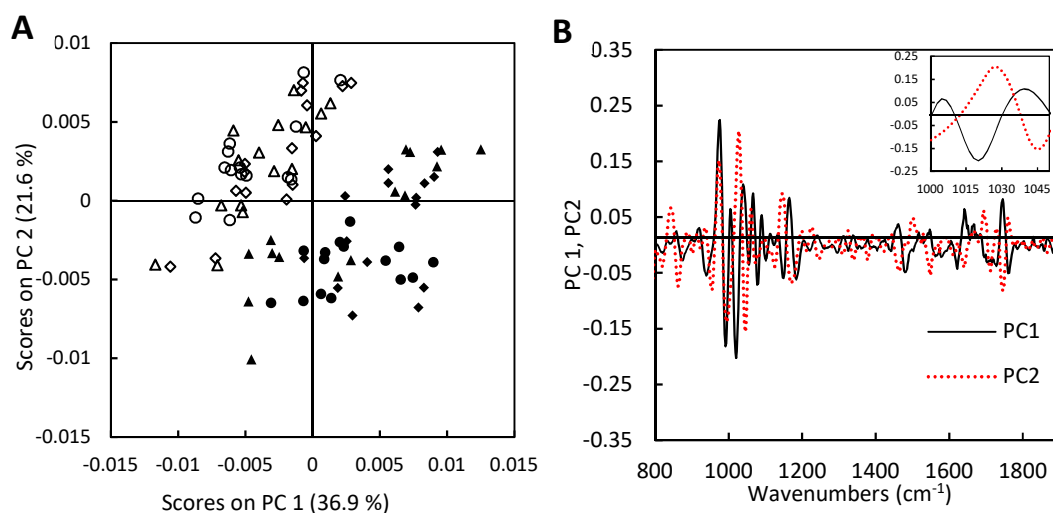


Figure 9. PCA (A) and the two first component loadings (B) performed on raw (empty symbols) and cooked (full symbols) doughs enriched with RuBisCO (diamonds), gluten (circles) and pea proteins (triangles) at 16.3 %, 14.9 % and 17.1 %, respectively. Analyses were performed between 800 and 1900 cm^{-1} . The two principal components explain 58 % of the total variance.

The variations of the components around 1500 and 1700 cm^{-1} correspond to changes in Amide I band related to the protein structures. However, the major intensity of the signal is due to the spectral

characteristics of the starch ($950 - 1200\text{ cm}^{-1}$). To analyse data related to protein structures, an analysis of the Amide I band excluding the starch signal was performed.

3.2 Changes in the secondary structure of proteins induced by heating

A PCA was performed on the Amide I band, from 1570 cm^{-1} to 1720 cm^{-1} , so that the signal of starch is discarded. The analysis was performed on raw and thermally treated doughs enriched with either 14.9% gluten, 17.1% pea protein or 16.3% RuBisCO. Spectra were baseline corrected before analysis. The scatterplot resulting from the PCA is shown in **Figure 10A**. The first and the second components explain 76.5 % and 15.6 % of the variability, respectively. As for the analysis performed on a wider wavenumbers range, the two first components do not differentiate samples according to their protein composition. The first component does not separate the samples on any of their known characteristics, indicating that PC1 could reflect the overall variability of all recorded spectra. However, the second component (PC2) differentiates the spectra according to whether they are thermally treated or not.

Plot of PC2 loading according to the wavenumbers is represented in **Figure 10B**. The loading opposes bands at 1651 cm^{-1} and 1695 cm^{-1} , respectively positive and negative. These wavenumbers are characteristics of α -helix and anti-parallel β -sheet in wheat protein structures (Barth & Zscherp, 2002; Popineau et al., 1994). PC2 has a lower contribution in thermally treated samples than in raw samples, meaning that thermal treatment lead to a decrease in bands intensity at 1651 cm^{-1} (α -helix) and an increase in bands intensity at 1695 cm^{-1} (anti parallel β -sheet). Anti-parallel β -sheet structures are formed due to heating at the expense of α -helices. It has been reported an increase in anti-parallel β -sheets in thermally treated gluten (Luo et al., 2016; Zhao, Wang, Zhu, Guo, & Hu, 2020). Similarly, the formation of anti-parallel β -sheets at the expense of α -helices has already been observed in gluten after the enrichment of dough with citrus pectin (Nawrocka, Mis, Szymanska-Chargot, Wilczewska, & Markiewicz, 2017).

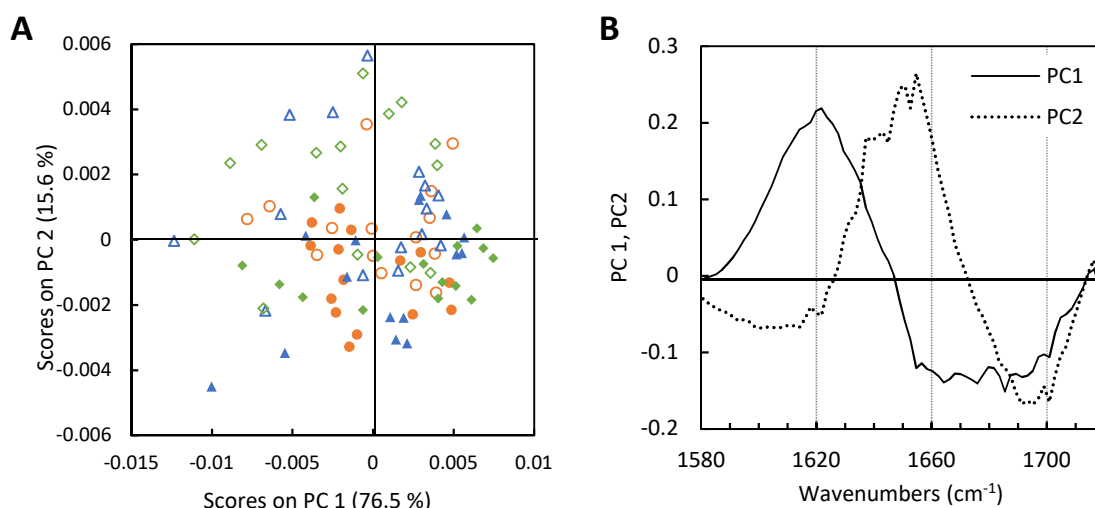


Figure 10. Projections of the two first components (A) and their loadings (B) of PCA performed on raw (empty symbols) and cooked (full symbols) doughs enriched with RuBisCO (green diamonds), gluten (orange circles) and pea proteins (blue triangles) at ~16 % enrichment. Analysis were performed in the Amide I region ($1570 - 1720\text{ cm}^{-1}$). The two principal components explain more than 92% of the total variance.

Several studies report the formation of β -sheets, related to bands around 1626 cm^{-1} , at the expense of α -helices following the heat treatment of gluten (Georget & Belton, 2006) or pasta (Laleg, Barron,

Santé-Lhoutellier, Walrand, & Micard, 2016), indicating the protein aggregation during heating (Mallamace, Fazio, Mallamace, & Corsaro, 2018). Although, the PC2 spectrum is negative around 1620 cm^{-1} , it does not show any peak. The analysis of differential spectra between raw and heat-treated doughs also highlights differences in spectral patterns. **Figure 11** shows the differential spectra between raw and thermally treated protein-enriched doughs. Difference spectra are noisy, probably due to the low difference existing between the two spectra. Therefore, variations observed on the difference spectra must be interpreted as trends. Three major peaks are identified on enriched dough differential spectra. All spectra display a positive peak around 1650 cm^{-1} , attributed to α -helix structures. A negative peak is observed around 1695 cm^{-1} , attributed to anti-parallel β -sheets. Interestingly, a negative peak is highlighted around 1620 cm^{-1} that could correspond to β -sheets. This suggests that thermal treatment enhances the β -sheets formation in protein-enriched doughs. The PCA did not reveal the formation of β -sheets following thermal treatment probably due to the variability of the overall spectra in this area, as evidenced by PC1 major peak around 1620 cm^{-1} . In addition, the difference spectra show lower intensities for pea and gluten enriched doughs than for RuBisCO enriched doughs. This would suggest that the effect of heat treatment is more intense in RuBisCO enriched doughs than in other doughs. This may be related to the thermal reactivity of RuBisCO at temperatures above 65°C (Béghin et al., 1993; Libouga et al., 1996; Martin et al., 2014). More surprisingly, the difference spectrum of the gluten-enriched dough shows less intense peaks than that of the control dough (not shown). Gluten enrichment may decrease the temperature sensitivity of the system. This effect is also visible in **Figure 10 A** where the cluster of raw and thermally treated gluten-enriched dough are more superimposed than the other enriched doughs. Moreover, spectra of gluten enriched dough shows less variability in PC2 values than other samples. This result is surprising because gluten-enriched dough is expected to show similar or more intense variations than the control dough. It has been shown that the introduction of gluten does not alter the covalent polymerisation of proteins during thermal treatment and that the added gluten behaves like the intrinsic gluten of semolina under heating (Ducrocq et al., 2020). Thus, it can be assumed that the addition of gluten decreases the temperature sensitivity of the system with respect to the conformational changes of the proteins without affecting the establishment of the covalent bonds.

The same analysis was performed after another data pre-processing (second derivative spectra) to better emphasize spectral changes, but similar results were obtained.

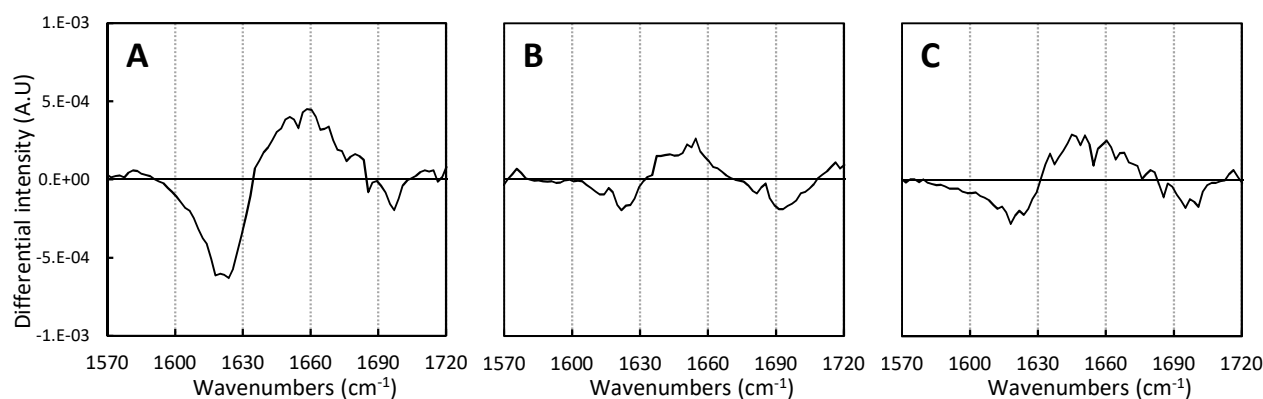


Figure 11. Differential spectra between raw and thermally treated doughs: 16.3% RuBisCO-enriched dough (A), 14.9% gluten-enriched dough (B) and 17.1% pea protein-enriched dough (C).

3.3 *RuBisCO enrichment may affect protein secondary structures in enriched dough*

To evaluate a dose response of RuBisCO on protein structures, a PCA was carried out on spectra of the Amide I region (1570 – 1720 cm^{-1}) from control dough and RuBisCO-enriched doughs at 16.3% and 37.4%. Gluten and pea protein-enriched doughs at 14.9 and 17.1% respectively, were added to the group to ensure that the differences observed are specific to RuBisCO. A second derivative was applied on spectra before the analysis. **Figure 12** shows projections of the two first components according to sample protein composition. PC1 and PC2 explain 44.9 % and 20.6 % of the total variability, respectively. Loadings of the two components are represented in **Figure 13**. Spectra from raw samples, whatever the protein composition, present similar values of PC1 and PC2. Raw samples were not differentiated according to the value of PC1 or PC2. As in previous analyses, the introduction of non-wheat proteins does not lead to structural differences in raw doughs that could be outlined in our analyses.

However, the PC1 separates raw and thermally treated samples of control and RuBisCO enriched doughs, with a lower PC1 values for thermally treated samples. It does not differentiate raw and thermally treated samples in the case of gluten and pea protein enriched doughs. PC1 loading (**Figure 13**) displays a positive peak at 1624 cm^{-1} and a negative peak at 1658 cm^{-1} . As a second derivative was performed on spectra before analysis, the effect of the heat treatment is associated with an increase of bands intensity at 1624 cm^{-1} and a decrease of bands at 1658 cm^{-1} on the spectra. These two peaks respectively correspond to β -sheet and α -helix structures. Thermal treatment induces the formation of β -sheets at the expense of α -helices. This result is in accordance with the result previously obtained and the literature. Moreover, results also underline that the structure modifications seem to be higher in control and RuBisCO enriched doughs than in gluten and pea protein enriched doughs. Scores on PC1 also separate the thermally treated samples according to their RuBisCO enrichment rates. An increased RuBisCO dose results in a lower PC1. Thus, protein structure in presence of RuBisCO may be more sensitive to heat treatment. The increase of β -sheets at the expense of α -helices in thermally treated RuBisCO –enriched doughs may also be related to the increase in SDS-insoluble protein content measured when increasing RuBisCO enrichment rates (Ducrocq et al., 2020). Indeed, it has already been shown that the α -helices/ β -sheets ratio was negatively correlated with the SDS-insoluble protein content of wheat flour (Liu et al., 2016).

The second component also slightly separates raw and thermally treated doughs. However, the overlapping between raw and thermally treated groups of samples is much lower for RuBisCO enriched doughs than for the other doughs. PC2 loading shows a positive peak at 1640 cm^{-1} and two negative peaks at 1624 cm^{-1} and 1695 cm^{-1} . Thermal treatment of RuBisCO-enriched dough leads to an increase of PC2 values, thus related to a decrease of band intensity at 1640 cm^{-1} and an increase of band intensity at 1624 cm^{-1} and 1695 cm^{-1} . In the literature, there are differing opinions on the assignment of the band at 1640 cm^{-1} . Indeed, bands at 1640 cm^{-1} were reported to be associated with weakly bonded β -strands in dough (Robertson, Gregorski, & Cao, 2006), with β -turns in purified gliadins (Wellner et al., 1996) and to random coil structure in gluten gel (Wang et al., 2017). As we are working on doughs, we can assume that the variations observed at 1640 cm^{-1} correspond to variations in β -strands structures. Thermal treatment may induce the formation of β -sheets and anti-parallel β -sheets at the expense of weakly bonded β -strands in RuBisCO-enriched doughs.

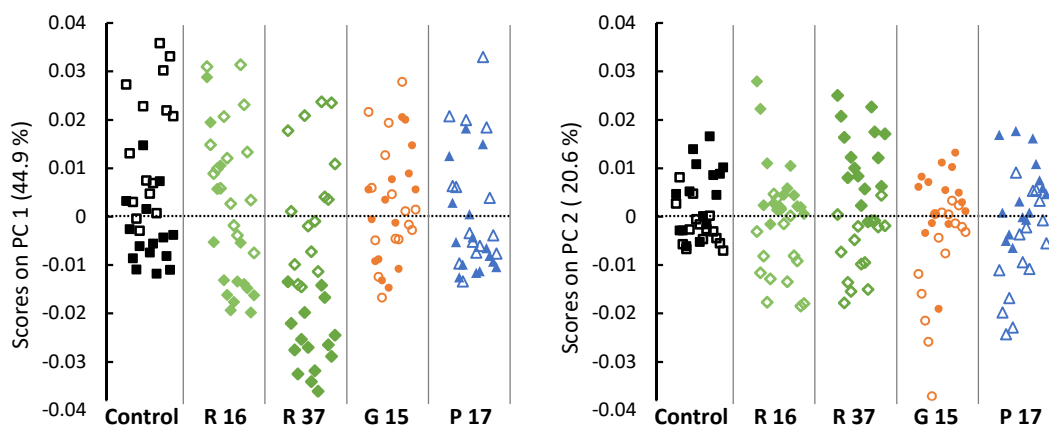


Figure 12. PC1 (on the left) and PC2 (on the right) of PCA performed on control and protein-enriched raw (empty symbols) and cooked (full symbols) doughs. “Control” stands for control dough; “R 16” and “R 37” stand for 16.3% and 37.4% RuBisCO enriched doughs respectively; “G 15” stands for 14.9% gluten-enriched dough and “P 17” stands for 17.1% pea protein enriched dough. Analysis were performed in the Amide I region ($1570 - 1720 \text{ cm}^{-1}$). The two principal components explain 65% of the total variance.

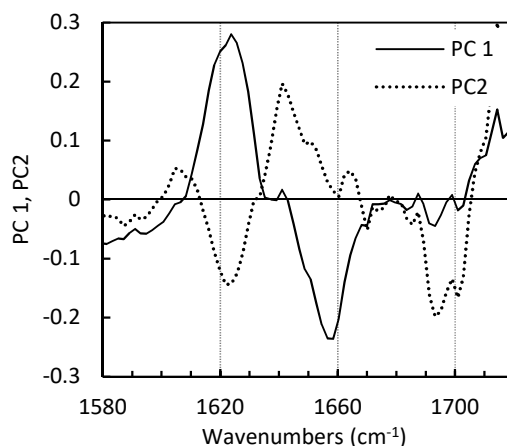


Figure 13. Loadings of the first and second components of the PCA performed on raw and thermal-treated control doughs and doughs enriched with gluten and pea proteins at 14.9 and 17.1%, respectively and dough enriched with RuBisCO at 16.3 and 37.4% enrichment, respectively. Analyses were performed in the Amide I region ($1570 - 1720 \text{ cm}^{-1}$). The two principal components explain more than 65% of the total variance.

The third component does not allow the spectra to be separated on any of their known characteristics. The projections of the fourth component of the PCA is represented on **Figure 14A** according to sample protein composition. This component only explains 6.2% of the total variance. Contrary to PC1 and PC2, it allows separating spectra according to the protein composition. Indeed, RuBisCO spectra, whether thermally treated or not, have higher PC4 contribution than spectra of control, gluten-enriched and pea protein-enriched doughs. PC4 loading is represented in **Figure 14B**. PC4 shows two positive peaks around 1610 and 1668 cm^{-1} . There are four negative peaks around 1589 , 1631 , 1655 and 1700 cm^{-1} . Enrichment of dough with RuBisCO, was thus related to a decrease of bands intensity at 1610 and 1668 cm^{-1} and to an increase of bands intensity at 1589 , 1631 , 1655 and 1700 cm^{-1} . The differential spectrum between semolina and pure RuBisCO is represented in **Figure 15**. The difference spectra displays bands at 1587 , 1608 , 1633 , 1670 , 1695 and 1708 cm^{-1} . Except for the band at 1695 cm^{-1} and 1708 cm^{-1} , these bands are at wavenumbers very close to those observed on PC4 and their sign

(positive or negative) correspond to the sign of PC4 contribution. Thus, it can be assumed that the PC4 component raises structural differences mainly related to the structure of RuBisCO itself rather than to modifications of secondary structures induced of the dough proteins by the addition of RuBisCO.

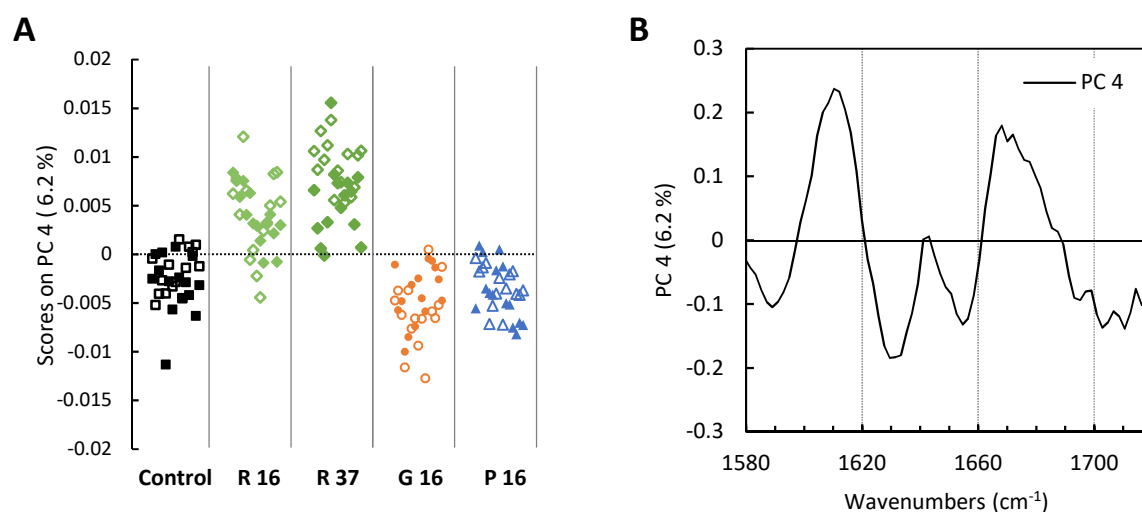


Figure 14. PC4 (A) and PC4 loading (B) of PCA performed on control and protein-enriched raw (empty symbols) and cooked (full symbols) doughs. “Control” stands for control dough; “R 16” and “R 37” stand for 16.3% and 37.4% RuBisCO enriched doughs, respectively; “G 15” stands for 14.9% gluten-enriched dough and “P 17” stands for 17.1% pea protein enriched dough. Analyses were performed in the Amide I region (1570 – 1720 cm^{-1})

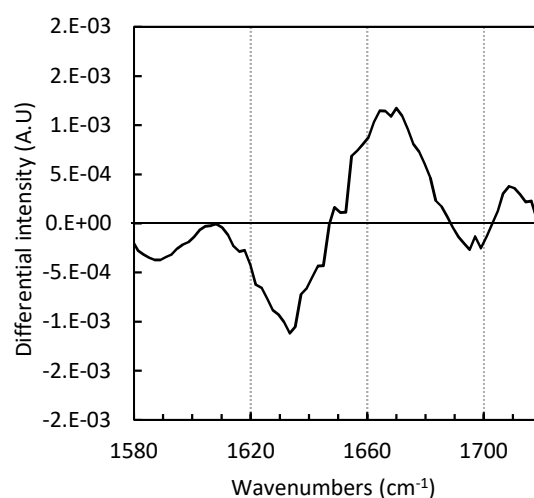


Figure 15. Difference spectra between semolina spectrum and RuBisCO spectrum.

4 Conclusion

The present study investigated the changes in the secondary structure of wheat dough proteins after addition of RuBisCO, gluten or pea proteins. The use of PCA highlighted spectral changes associated with starch gelatinisation due to heat treatment in all protein-enriched doughs whatever the protein used. The analysis of the amide I bands suggested that β -sheet structures are formed at the expense of α -helices in all protein-enriched doughs following heat treatment, as regularly observed in literature. However, the structure modifications seem to be more significant in RuBisCO enriched doughs than in control dough or gluten and pea protein enriched doughs. In addition, structural

changes are enhanced by increasing RuBisCO enrichment rate. These results could be related with those obtained in a previous study, showing that RuBisCO introduction enhanced the covalent protein polymerisation in dough under thermal treatment. This study illustrates that the singular behaviour of RuBisCO in wheat matrices is also visible at the level of protein secondary structure and highlights once again the increase in the thermal reactivity of the proteins induced by RuBisCO enrichment.

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Chapter 5. Protein *in vitro* digestibility of RuBisCO-enriched wheat doughs

Preamble

An enrichment in legume protein of wheat-based food often leads to an increase of the degree of hydrolysis of proteins. The authors relate the higher protein hydrolysis degree to the weakening of the gluten network in the food (Laleg et al., 2017; Patil, Brennan, Mason, & Brennan, 2016; Rayas-Duarte, Mock, & D., 1996). We have shown in the previous chapter that the addition of RuBisCO at 16.3% enrichment rate changes the protein aggregation state in thermally treated doughs. In particular, RuBisCO forms disulphide-linked aggregates, leading to a global decrease of SDS-soluble proteins. In contrast, gluten and pea protein additions do not modify the wheat protein polymerisation. The objective of the following chapter is to assess whether these protein structural changes affect the protein *in vitro* digestibility in dough.

Main questions:

- Does the protein enrichment of wheat doughs affect the degree of *in vitro* hydrolysis of peptide bonds?
- Is the nitrogen solubilisation during digestion modified by a RuBisCO enrichment?
- Does the RuBisCO enrichment of wheat dough increase the amount of digestible peptide?

Micro wheat doughs were enriched at 37.4% RuBisCO, 35.0% gluten or 38.7% pea proteins. The matrices were digested using the standardised static *in vitro* digestion protocol developed by the COST INFOGEST network (Brodkorb et al., 2019). Nitrogen and primary amino group contents were determined on the soluble fractions of digesta. The *in vitro* digestibility was assessed from the content of soluble nitrogen in the fraction of molecules smaller than 10 kDa.

Main results:

- ✓ Pea protein and RuBisCO enrichments improve the dough essential amino acid profile
- ✓ The protein enrichment of dough does not affect the protein *in vitro* digestibility, regardless of the protein used. The enrichments with RuBisCO or pea proteins thus increases the PDCAAS-like of dough.
- ✓ The degree of hydrolysis is not altered by the addition of RuBisCO. However, the rate of nitrogen solubilisation is increased, suggesting that RuBisCO introduction leads to an increase of the average peptide size at the end of digestion.
- ✓ There is no clear relation between the protein aggregation state in dough and the degree of proteolysis in our matrices.

Protein *in vitro* digestibility of RuBisCO-, pea protein- and gluten-enriched wheat doughs

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1 Introduction

For ecological and health reasons, several studies highlighted the need to increase the proportion of plant proteins in our diet, at the expense of animal proteins (Aiking, 2014; Friel et al., 2009; Willett et al., 2019). Thus, protein-rich plant ingredients are commonly used to enrich our foods (Alves & Tavares, 2019). Dairy products (Chihi, Sok, & Saurel, 2018; Jose, Pouvreau, & Martin, 2016; McClements, 2020) and wheat-based products (Berrazaga et al., 2020; Laleg et al., 2017; Monnet, Laleg, Michon, & Micard, 2019; Mubarak, 2001; Pérez, Ribotta, Steffolani, & Le, 2008; Petitot, Boyer, Minier, & Micard, 2010) are mainly concerned. Wheat-based foods are widely consumed in culinary traditions around the world. Wheat is a source of carbohydrates and proteins and some products, such as durum wheat pasta, have a low glycaemic index (Jenkins et al., 1981; P. R. Shewry, 2009). Enriching wheat-based products with plant proteins would contribute to the global effort of increasing the proportion of plant protein in our diet.

Wheat-based products are deficient in lysine and threonine. Their enrichment in plant proteins may thus improve their essential amino acid profile in addition to increase their protein content. Legumes are often used for this purpose because of their relatively high lysine and threonine content. However, an extensive literature reports the potential negative effects of adding legume flours or protein concentrates on the textural and culinary properties of wheat-based foods (Boukid, Zannini, Carini, & Vittadini, 2019; Chavan, 1993; Monnet et al., 2019). The addition of non-wheat proteins often alters the gluten network and more precisely leads to the decrease in the large size covalently linked polymers (Laleg, Barron, Santé-Lhoutellier, Walrand, & Micard, 2016). These structural changes would also be responsible for changes in digestion patterns such as the increase of both the protein hydrolysis degree and the rate of starch digestion (Laleg et al., 2017). Although the exact mechanisms through which added proteins affect the gluten network remain unclear, a simple effect of gluten dilution by non-wheat protein incorporation is often argued (Bahnassey, Khan, & Harrold, 1986; Laleg et al., 2017; Petitot et al., 2010; Rayas-Duarte et al., 1996).

Although special attention has been paid to legume proteins for their nutritional quality, other plant proteins, such as leaf proteins, have interesting potential for human nutrition (Gerloff, Lima, & Stahmann, 1965; Lexander et al., 1970). RuBisCO, Ribulose-3.5-bisphosphate Carboxylase-Oxygenase, is the major protein of leaf protein concentrates. RuBisCO is a water-soluble chloroplast protein and is a key enzyme for photosynthesis (Trown, 1965). Several reviews relate the history of the discovery of RuBisCO and the numerous technological developments to extract it (Portis & Parry, 2007; Santamaria-Fernandez, Ambye-Jensen, Damborg, & Lübeck, 2019; Wildman, 2002). Its nutritional interest for

human food and animal feed has long been highlighted (Barbeau & Kinsella, 1988; Pirie, 1942), in particular thanks to its equilibrated amino acid profile (Gerloff et al., 1965). In the same way as legumes, addition of RuBisCO to wheat-based foods could therefore increase their protein content and improve their amino acid profile. Only few researches are available on the nutritional interest of RuBisCO/wheat mixtures. In particular, it has been shown that mixes of cereals with leaf protein concentrates had beneficial effects on the chemical scores and biological value of the diets (Carlsson & Hanczakowski, 1985) and on plasma and liver protein levels in rats (Shukla & Sur, 1978). However, these studies were carried out on simple mixtures of cereal flour and leaf protein concentrate powders and not on food matrices resulting from several process steps. Recently, we studied the effect of the RuBisCO enrichment of wheat doughs on their protein structures (Ducrocq, Boire, Anton, Micard, & Morel, 2020). It has been shown that RuBisCO behaves differently than legume proteins when introduced in wheat dough, notably by its capacity to form both weak and covalent bonds during dough hydration and mixing. RuBisCO integrates the water-insoluble gluten protein network and increases the concentration of large polymers during dough thermal treatment.

The aim of this study was thus to investigate the effect of a RuBisCO enrichment on the nutritional quality of proteins in wheat-based foods in relation to the protein aggregation state. Thermally treated micro wheat semolina doughs were used as a model food system to this purpose. Protein aggregation state was investigated by protein sequential extraction followed by size-exclusion chromatography. The nutritional interest of a RuBisCO enrichment has been evaluated by its capacity to improve the composition of wheat dough in essential amino acid and by its effect on the *in vitro* digestibility of dough proteins. The matrices were digested using the standardised static *in vitro* digestion model developed by the COST INFOGEST network (Brodkorb et al., 2019). The proteolysis was monitored both by the degree of hydrolysis and the rate of nitrogen solubilisation during the digestion. The *in vitro* digestibility of proteins was estimated from the soluble nitrogen content of digesta with a molecular weight below 10 kDa. The effect of RuBisCO enrichment of wheat doughs was compared with those of two others plant proteins, gluten and pea proteins.

2 Material and methods

2.1 Material

The raw materials used for the study were the same as those used in (Ducrocq et al., 2020). The protein content of all raw materials was analysed according to the Kjeldahl method (NF V 03-050, 1970). A nitrogen to protein conversion factor of 5.7 was used for wheat semolina, pea proteins and gluten concentrates. A conversion factor of 5.8 was used for the leaf protein concentrate (LPC) based on the amino acid profile of its proteins (Ducrocq et al., under submission). As RuBisCO is the major protein of LPC, we will use the term RuBisCO concentrate in this study.

Alpha-Amylase from human saliva (A0521), Pepsin from porcine gastric mucosa (P7125), pancreatin from porcine pancreas (P7545), trypsin from bovine pancreas (T9201), bile extract porcine (B8631), pepstatin A (P5318), pefabloc® SC (76307) and N α -Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPA, B4875) were purchased from Sigma-Aldrich (Saint Louis, MO 63103, USA). All other chemicals were of analytical grade and Milli-Q water was used (Millipore Systems, Guyancourt, Molsheim, France).

2.2 Amino acid profile and chemical score calculations

Amino acid analysis was performed on raw materials at UNH (Clermont-Ferrand, INRAE, France) as described in (Margier et al., 2018) using an amino acid analyser (L-8900, Hitachi, Paris, France). Briefly, four different hydrolysis of proteins were carried out prior to analysis. An acid hydrolysis with 6 N HCl was carried out for 24 h at 110 °C to determine the amount of the majority of the amino acids. The acid hydrolysis has been prolonged to 48 h to quantify leucine, isoleucine and valine. To assay the sulphur-amino acids, oxidation with performic acid was carried out prior to hydrolysis. The amount of tryptophan was measured after basic hydrolysis with 4 N Ba(OH)₂ for 16 h at 110 °C. For each hydrolysis, norleucine was used as an internal standard.

The chemical score (CS) of each amino acid was determined using the reference profile proposed by (AFSSA, 2007) for adults and was computed as follows :

$$CS_i (\%) = \frac{m_{i \text{ sample}}}{m_{i \text{ reference}}} * 100$$

Where CS_i corresponds to the chemical score of the amino acid *i* in the sample, m_{i sample} corresponds to the content of this amino acid in the sample and m_{i reference} corresponds to its content in the reference profile, both contents expressed in grams per 100 grams of total amino acids. When the CS of an amino acid is below 100, then this amino acid is deficient in the protein. The CS of a protein or a product correspond to the lowest amino acid CS (AFSSA, 2007).

2.3 Trypsin inhibitory activity (TIA) measurement

Trypsin inhibitory activity (TIA) was determined on raw materials in triplicate based on the standardised method ISO14902 (2009) with minor modifications. N α -Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPNA) was used as trypsin substrate. The enzymatic activity of trypsin was measured alone and in the presence of the raw materials (semolina and protein concentrates). The p-nitroaniline produced by DL-BAPNA hydrolysis by trypsin was detected at 410 nm using microplate reader (Spark®, Tecan Trading, Ltd., Switzerland). The TIA was expressed as mg of trypsin

inhibited per gram of sample. In the standardize method, a conversion factor f_2 is used to calculate the quantity of trypsin contained in the reactive medium. Its value ($2.8 \cdot 10^{-4}$) is given in the method but it is based on a trypsin purity of 56% (Smith, van Megen, Twaalfhoven, & Hitchcock, 1980) and is calculated from the dilutions performed during the experiment. This conversion factor has been modified in our study to adapt to the purity of our trypsin and to our dilutions: we used a factor of $5.5 \cdot 10^{-4}$.

2.4 Protein-enriched dough preparation

The dough formulations are described in **Table 1**. The control dough corresponds to a 100% wheat semolina dough, without any protein enrichment. Wheat doughs were enriched with either RuBisCO, gluten or pea protein concentrates by substituting 10% (w/w) of wheat semolina by each of the protein concentrate. This substitution rate leads to doughs with a total protein content of about 20% (db) from which 35-39% were originated from the protein concentrate (extrinsic proteins) and 61-65% from native semolina protein. A standard water content of 67% was used (db) to obtain a homogeneous cohesive dough. Dough samples were prepared as described in (Ducrocq et al., 2020) using a “2-g Mixograph” (TMC, Lincoln, NE, USA) equipped with a double-walled jacket coupled with a water-regulated bath to control the temperature of the bowl. Dough were then thermally treated using a hermetically sealed aluminium container made to contain 1.2 g of dough, immersed in a water bath at 80°C for three minutes. It was then cooled down on ice. The dough sample was then removed and immersed in liquid nitrogen before freeze drying and grinding.

Table 1. Formulation and protein composition of control and protein-enriched doughs

Dough sample	Semolina substitution	Total protein content	Wheat proteins	Extrinsic proteins
	% wb	% db	% total protein	
Control dough	0.0	14.0	100.0	0.0
RuBisCO-enriched dough	10.0	20.1	62.6	37.4
Gluten-enriched dough	10.0	19.4	65.0	35.0
Pea protein-enriched dough	10.0	20.5	61.3	38.7

2.5 Protein aggregation state determination

Dough proteins were extracted as described in (Morel, Dehlon, Autran, Leygue, & Bar-L’Helgouac’H, 2000) with some modifications. Proteins were first extracted in 0.1M sodium phosphate buffer pH 6.8 with 1% SDS and with a solid to liquid ratio of 8. Extraction was performed on a rotary shaker set at 60 rpm at 60 °C for 80 min. SDS-soluble proteins were recovered in the supernatant after centrifugation ($39191 \times g$, 30 min, 20 °C). A second extraction was performed on pellets at 60 °C for 60 min, in the same buffer including 20 mM of thiol reducing agent, dithioerythritol (DTE). The pellets were then sonicated (Vibracell 72434, Bioblock Scientific, Illkirch, France) at 50% power setting 20 kHz for 3 min. Supernatants were recovered after centrifugation as described previously. Supernatants were diluted twice with 0.1 M phosphate, 1% SDS buffer including 40 mM of iodoacetamide (IAM), to prevent reformation of the disulphide bonds. For readability reasons, proteins insoluble in SDS but soluble in SDS and DTE are hereafter called “SDS + DTE soluble proteins”. The SE-HPLC apparatus (Waters model

LC Module1 plus) was equipped with an analytical column, TSK G4000-SW (7.5 x 300 mm) and a guard column, TSK G3000-SW (7.5 x 75 mm) (both from Merck, Darmstadt, Germany).

2.6 *In vitro static digestion*

In vitro static digestion of dough was performed according to the standardised simulated gastrointestinal digestion model developed by the INFOGEST consortium (Brodkorb et al., 2019). This method consists of sequential oral, gastric and intestinal digestions of the sample. Parameters relative to electrolytes, enzymes, bile, pH and digestion times are determined from the available physiological data (Brodkorb et al., 2019). The quantities of acid or base added to the digesta to adjust the pH were determined for each dough composition in a preliminary study involving higher quantity of dough (200 mg). Enzyme activities and bile salt concentration were measured as described in the supplementary materials of the standardised protocol. Pancreatin was used for the intestinal phase and its amount was determined from its measured trypsin activity. Considering the low lipid content of semolina (about 1-3% dry matter) (MacRitchie, 1984; Sissons, 2008), no gastric lipase was used. Inhibition of salivary amylase activities was achieved by rapidly transferring the samples on ice after the oral phase. Pepstatin was used to inhibit pepsin (5 µg/mL of digesta) and pefabloc was used to inhibit intestinal enzymes (1.2 mg/mL of digesta).

Digestion was performed on 100 mg of dough powder in Eppendorf tubes of 5 mL. Water was added to the powder to reach a powder:liquid ratio of 0.2:1 at the end of the oral phase. This leads to a swallowable bolus with a paste-like consistency at the end of oral phase as suggested by (Brodkorb et al., 2019). Each kinetic point corresponded to one sample so that no material was collected during the progress of digestion. Each kinetic point was done in triplicate. Digesta were centrifuged at 5000xg for 15 min at 4°C to separate and recover the digestion supernatant and the pellet. Digestion supernatant and pellet were frozen in liquid nitrogen before further analysis. In addition to sample digestion, “enzyme blanks” were obtained in the same way except that the 100 mg samples were replaced by 100 µL milliQ water. “Enzyme-free” were obtained by replacing the volumes of amylase and pepsin by water and the volumes of pancreatin and bile salts by simulated intestinal fluids.

2.7 *Analysis of digestion supernatants*

Soluble nitrogen content

The soluble nitrogen resulting from the gastric and intestinal digestions were analysed using a Dumas protein analyser system (rapid MAX N exceed, Elementar, Germany). About 1 mL of the digesta soluble fraction was placed in a steel crucible and injected into an oven under a stream of oxygen and helium. The combustion was performed at 900°C for 120 s with an oxygen flow rate of 100 mL/min. The gas mixture was then reduced in a reduction tube and separated on selective trap columns. Glycine was used as a standard for nitrogen content. Measurements were done in triplicates. The measurement was also performed on the “enzyme-free” and “enzyme blank” samples. A mass balance was carried out on the soluble and insoluble fractions after centrifugation of the digesta to determine the proportion of the total soluble fraction.

Degree of hydrolysis (DH)

The DH was calculated from the assay of primary amino groups released during the digestion phases. Primary amino groups of the soluble fraction were assayed using the ninhydrin method (Moore, 1968). Briefly, samples were mixed with 2% ninhydrin reagent (sigma, N 7285) in a ratio 1:1. Tubes were placed in boiling water for 25 min and then let to cool for 5 min. Ethanol 50% (v:v) was added in a ratio 1:0.4 and tubes were vortexed. Absorbance reading was performed at 570 nm using a plate reader (Spark®, Tecan Trading, Ltd., Switzerland). Ninhydrin-reactive amino groups content was calculated in mmol per 100 grams of sample using Leucine as a standard in a range from 0 to 0.6 mM. The primary amino group content was measured in the soluble fraction of sample digesta after a time *t* of digestion ($[NH_2]_t$), in the “enzyme-free” sample ($[NH_2]_{t0}$), in the “enzyme blank” sample ($[NH_2]_{enzymes}$) and after a total chemical hydrolysis of dough powder (HCl 6 M, 24 hours, 105 °C) ($[NH_2]_{tot}$). The DH was computed according to the following equation:

$$DH (\%) = \frac{[NH_2]_t - [NH_2]_{t0} - [NH_2]_{enzymes}}{[NH_2]_{tot} - [NH_2]_{t0}}$$

Apparent in vitro digestibility

The apparent *in vitro* digestibility was estimated from the part of soluble nitrogen recovered in the fraction of molecules with molecular weight below 10 kDa as suggested by (Cave, 1988). To do so, 500 µL of supernatant of intestinal digestion were diluted twice in ultrapure water before being ultrafiltrated through a 10 kDa Vivaspin® Polyethersulfone centrifugal filter (Vivaspin® 500, 10 kDa MWCO, PES, Sartorius Stedim Biotech GmbH, Goettigen, Germany) for 15 minutes at 12 000 g. The filtrate was removed and saved. The ultrafiltration was performed two additional times after the retentate was diluted with 300 µL additional ultrapure water. The three filtrates were combined and frozen. The apparent *in vitro* digestibility was computed as follow:

$$\text{App. in vitro Digestibility (\%)} = \frac{N_{<10 \text{ kDa}}}{N_{\text{Total}}} * 100$$

Where $N_{<10 \text{ kDa}}$ corresponds to the amount of nitrogen assayed in the ultrafiltrated fraction (in gram per 100 grams of dough) and N_{Total} correspond to the total amount of nitrogen introduced in the tube (in gram per 100 grams of dough).

PDCAAS like calculation

A PDCAAS (Protein digestibility-corrected amino acid score) like score was calculated from this apparent *in vitro* digestibility according to (Le Roux et al., 2020). This method replaces the true faecal protein digestibility (usually determined using *in vivo* digestion) by the apparent *in vitro* digestibility in the standardised PDCAAS calculation (FAO/WHO, 1990). The PDCAAS corresponds thus to the apparent *in vitro* digestibility multiply by the CS of the protein fraction of the dough.

2.8 Statistical analyses

All values were the mean of three independent replicates and were subjected to one-way ANOVA using RStudio software (RStudio™, Boston, MA, USA). The significant difference between the values was estimated by Tukey's HSD test ($p < 0.05$).

3 Results and discussion

3.1 Protein content and amino acid composition of protein-enriched doughs

The chemical scores (CS) of essential amino acids (EAA) of the doughs were estimated from the amino acid profiles of raw materials and are given in **Table 2**. The gluten enrichment at 35.0% of total proteins leads to a 4% decrease of dough essential amino acid content. RuBisCO and pea protein enrichments of dough at 37.4% and 38.7% of total proteins respectively, lead to an increase of their essential amino acid content of 11.8% and 5.1%, respectively. Both RuBisCO and pea proteins have an amino acid profile complementary to that of wheat, especially regarding the lysine score. The incorporation of RuBisCO and pea proteins results in a lysine CS of 82% and 98%, respectively, to be compared to 49% for the control dough. The lysine CS is not increased by the addition of gluten which is deficient in lysine like wheat semolina. This estimate of the CS from the amino acid composition of raw materials is reasonable for most amino acids. However, it has been shown that the processing of pasta could reduce the CS for lysine, histidine and sulphur amino acids by 17, 11 and 6%, respectively on durum wheat pasta (Laleg et al., 2016). Considering this negative effect of the process, the potential CS of lysine could be reduced to 68% in the RuBisCO-enriched dough and to 81% in pea protein-enriched doughs. The CS of the other EAAs would all remain above 100%.

Table 2. Essential amino acid (EAA) chemical scores of control and protein-enriched doughs calculated from the amino acid profiles of raw materials as compared to AFSSA recommendations for adults (AFSSA, 2007).

	AFSSA recommendations <i>g.100g⁻¹ protein</i>	Chemical score (% of AFSSA recommendations)			
		Control dough	RuBisCO- enriched dough	Gluten- enriched dough	Pea protein- enriched dough
Methionine + Cysteine	2.3	202	247	159	156
Histidine	1.7	183	187	178	181
Isoleucine	2.7	172	170	169	181
Leucine	5.9	116	130	122	127
Lysine	4.5	49	82	47	98
Phenylalanine + Tyrosine	4.1	197	215	205	210
Threonine	2.5	122	160	124	140
Tryptophan	0.6	679	551	487	473
Valine	2.5	186	210	188	200
Total EEA content (g.100g⁻¹ protein)	26.8	41.3	46.2	39.7	43.4

3.2 Trypsin inhibitor activity

Trypsin inhibitors are major anti-nutritional factors in plant proteins (Richardson, 1977) and many studies report an increase of the trypsin inhibitor activity in wheat-based food after their enrichment in legume proteins (Anton, Gary Fulcher, & Arntfield, 2009; Berrazaga et al., 2020; Laleg et al., 2017). Therefore, the trypsin inhibitory activity was measured on wheat semolina and protein concentrates. Results are given in **Table 3** in mg of inhibited trypsin per gram of sample. The TIA of durum wheat semolina was below the limit of detection but gluten exhibits a TIA of 1.18 mg.g⁻¹ of concentrate. Wheat grains contain trypsin inhibitors (reviewed in Shewry, 2019) that may be concentrated in gluten concentrate as compared to semolina. The TIA of pea protein concentrate and RuBisCO concentrate are 3.5 and 2.3 fold higher than that of gluten, respectively. The TIA of RuBisCO could be related to phenolic compounds that are known to regularly occur in LPC (McNabb, Peters, Foo, Waghorn, & Jackson, 1998).

Table 3. Trypsin Inhibitors Activity (TIA) of the durum wheat semolina and the protein concentrates. "LOD" stands for "limit of detection".

	TIA (mg.g ⁻¹ of sample)
Durum wheat semolina	< LOD
Gluten	1.18 ± 0.20
RuBisCO concentrate	2.66 ± 0.45
Pea protein concentrate	4.08 ± 0.21

3.3 Protein aggregation state in doughs

The aggregation state of proteins in the control and protein-enriched doughs was estimated from the amount of SDS-soluble and SDS+DTE soluble proteins. These last two protein extracts represent respectively the small and weakly bound polymers and the large disulphide linked polymers. Following sequential extraction in SDS and SDS+DTE, 100% of the proteins was extracted for all doughs. The results are presented in **Table 4** in proportion of the total extractable proteins. The proteins of control dough are 79% soluble in SDS. This value is slightly higher than the 70% of SDS-soluble proteins reported for durum wheat pasta dried at low temperature (55°C) (Laleg et al., 2017), likely due to the additional processing steps for pasta as compared to our dough. Pea protein-enriched dough presents similar values of SDS-soluble proteins than the control dough. These similar values can be explained by the similar amount of SDS-Soluble protein between pea protein concentrate and the control dough (77% vs 79%). In addition, it has been previously shown that pea protein enrichment does not modify intrinsic wheat protein (proteins from semolina) polymerisation (Ducrocq et al., 2020). In contrast, gluten-enriched dough presents a slight decreased SDS-soluble protein content, whereas gluten concentrate contains more SDS-soluble proteins than control dough (87% vs 79%). Although the gluten enrichment does not modify the intrinsic wheat protein polymerisation, the heat treatment results in the partial aggregation of the added gluten (Ducrocq et al., 2020), which explains the decrease in SDS-soluble proteins as compared to the control.

Considering the high SDS solubility of RuBisCO (96.4% in SDS; result not shown), the RuBisCO enriched doughs at 37.4% of total proteins presents an unexpectedly low SDS-soluble proteins content. It is 1.2 fold lower than that of the control dough. The increase of the proportion in large-size disulphide linked polymers (SDS+DTE soluble proteins) is related to the ability of RuBisCO to form disulphide-linked polymers after dough thermal treatment as previously observed on doughs enriched at 16.3% (Ducrocq et al., 2020).

Table 4. Proportion of SDS-soluble and SDS+DTE soluble proteins in control and protein-enriched doughs at 37.4% RuBisCO, 35.0% gluten and 38.7% pea proteins. Results are means of duplicates, the variation coefficient of this method is below 5%.

	SDS soluble proteins	SDS+DTE soluble proteins
	<i>% of extractable proteins</i>	
Control dough	79%	21%
RuBisCO-enriched	65%	35%
Gluten-enriched	75%	25%
Pea protein-enriched	76%	24%

3.4 Evolution of the soluble nitrogen content and of the protein hydrolysis degree during in vitro digestion

Both the assay of soluble nitrogen and the analysis of the protein hydrolysis degree (DH) give information about protein degradation during digestion. The content of soluble nitrogen was measured at the end of gastric and intestinal phases, after removing of the contribution of nitrogen from digestive enzymes. It is expressed as a proportion of the total nitrogen in dough in **Figure 1**. Protein hydrolysis degree (DH) was evaluated by assaying the free amino group content in the soluble fractions of digesta. **Table 5** gives the DH of proteins at the end of gastric and intestinal phases, the DH at the end of the oral phase being close to zero for all samples (result not shown).

General trends are observed in the evolution of soluble nitrogen content and DH. **Figure 1** shows that before any action of digestive enzymes, all the doughs display similar proportion of soluble nitrogen. For the control dough, the major part of the final soluble nitrogen is solubilised during the gastric phase. In addition, little or even no nitrogen is solubilised during the intestinal phase and the final soluble nitrogen content corresponds to about 62% of the total dough nitrogen content. Similar results were previously reported on wheat dough and bread crumb and on other dietary proteins (legumes and milk proteins) (Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001; Santos-Hernández et al., 2020). Protein enrichment slightly modifies this trend as some nitrogen is also solubilised during the intestinal digestion of all protein-enriched doughs. In addition, the proportion of soluble nitrogen at the end of digestion tends to be increased (up to 71-88%). At the end of the gastric phase, the DH remains low (~2%) and it increases markedly during the intestinal phase, reaching values around 20% (**Table 5**). This evolution of the DH is largely reported in the literature for wheat-based food digestion (Berrazaga et al., 2020; De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007; Laleg et al., 2016;

Petitot et al., 2009) and other types of food (Baugreet et al., 2019; Lorieau et al., 2018). Despite these general trends, there are differences between the samples during the two digestion phases.

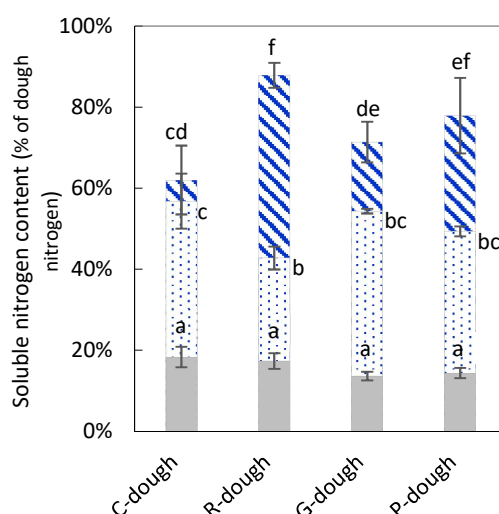


Figure 1. Amount of soluble nitrogen released from doughs before digestion (grey area), during the gastric phase (pointed area) and during the intestinal phase (dashed area) in proportion of total dough nitrogen content. “C-dough” stands for control dough; “R-dough”, “G-dough” and “P-dough” stand for protein-enriched doughs at 37.4% RuBisCO, 35.0% gluten and 38.7% pea proteins, respectively. Statistical analyses were performed on the data from all samples simultaneously. Values with the same letters are not significantly different ($p > 0.05$).

Table 5. Degree of hydrolysis at the end of gastric and intestinal phases for control and protein-enriched doughs at 37.4% RuBisCO, 35.0% gluten and 38.7% pea proteins.

Doughs	Degree of hydrolysis*					
	% of total -NH ₂ in sample					
	Gastric phase			Intestinal phase		
Control	2.2 ^{ab}	±	0.6	17.5 ^a	±	2.2
RuBisCO-enriched	2.1 ^{ab}	±	0.3	15.5 ^a	±	3.5
Gluten-enriched	1.6 ^a	±	0.3	24.9 ^b	±	1.5
Pea protein-enriched	3.0 ^b	±	0.4	22.0 ^{ab}	±	3.2

*Values with the same upper case letters in the same column are not significantly different ($p > 0.05$). DH of gastric and intestinal phases were analysed separately.

Gastric phase

At the end of gastric phase, sole the RuBisCO-enriched dough shows a proportion of soluble nitrogen significantly lower than control dough. The gastric digestion was performed at pH 3 and RuBisCO is known to have a low solubility ($< 20\%$) at this pH (Ducrocq et al., under submission; Van de Velde, Alting, & Pouvreau, 2011). In addition, RuBisCO-enriched dough presents the highest protein aggregation state. Both the low solubility of RuBisCO and the high aggregation state of proteins in dough could reduce the accessibility of proteases to the cleavage sites (Domenek, Brendel, Morel, & Guilbert, 2004), leading to lower nitrogen release (Berrazaga et al., 2020; Laguna, Picouet, Guardia, Renard, & Sarkar, 2017). Despite a lower proportion of soluble nitrogen, the introduction of RuBisCO does not alter the DH at the end of gastric digestion (Table 5). Similar trends are observed when

comparing RuBisCO- to gluten-enriched dough digesta, despite non-statistically significant differences. These results suggest that a RuBisCO enrichment favours the release of smaller peptides during the gastric phase. This is evidenced by the low ratio of soluble nitrogen to primary amine group content (N:NH₂ ratio) for RuBisCO-enriched dough at the end of the gastric phase (8.6 for RuBisCO-enriched dough vs 15.2 for control dough, **Supplementary 1**). The smaller size of peptides may be related to a different number of cleavage sites for digestive enzymes driven by the protein primary sequence. Pepsin preferentially cleaves peptide bonds after aromatic residues or leucine (Keil, 1992). Given the larger content of aromatic residues or leucine of RuBisCO as compared to gluten (212 vs 176 of aromatic amino acids and leucine per gram of total amino acids), the solubilised nitrogen in the digesta of RuBisCO-enriched dough may correspond to RuBisCO-derived peptides. This result is consistent with previous study that showed that RuBisCO is no longer detectable on SDS-PAGE gels after 30 seconds of peptic hydrolysis (Fu, Abbott, & Hatzos, 2002).

In contrast, the proportion of soluble nitrogen at the end of gastric phase is similar between the control, the gluten- and the pea protein-enriched doughs. However, DH of pea protein-enriched dough is higher than that of gluten-enriched dough. These results are consistent with a previous study that showed a higher DH for pea protein concentrate than for gluten at the end of the gastric digestion (Reynaud, Lopez, Riaublanc, Souchon, & Dupont, 2020). Our data suggest that pea protein-enriched dough digestion releases peptides of smaller size as compared to gluten-enriched doughs during gastric digestion. This is illustrated by a significantly lower N:NH₂ ratio for pea protein-enriched dough than for gluten-enriched dough (**Supplementary 1**). As for RuBisCO, this might be explained by the slightly higher number of potential cleavage sites of pea proteins as compared to gluten proteins (189 vs 176 mg of aromatic amino acids and leucine per gram of total amino acids).

Intestinal phase

RuBisCO-enriched dough presents the highest proportion of soluble nitrogen at the end of intestinal digestion (**Figure 1**). RuBisCO is known to be highly soluble at pH 7 (>93%) (Ducrocq et al., under submission.; Van de Velde et al., 2011). However, RuBisCO forms SDS-insoluble disulphide-linked polymers during dough thermal treatment (**Table 4**). Therefore, the high solubility of RuBisCO at pH 7 may not explain the strong increase in soluble nitrogen during the intestinal phase. As observed at the end of gastric phase, the DH is similar between RuBisCO-enriched dough and control dough (**Table 5**). In addition, the final DH of RuBisCO-enriched dough is significantly lower than that of gluten-enriched dough. These variations result in a much higher N:NH₂ ratio for the RuBisCO-enriched dough (5.1) than for the control and gluten-enriched doughs (~2.9). The RuBisCO enrichment of dough thus favours the release of larger peptides as compared to the control and gluten enriched-doughs. Similarly to pepsin, pancreatic enzymes (trypsin and chymotrypsin) have preferential cleavage sites. Chymotrypsin catalysed the hydrolysis of peptides bonds formed by tryptophan, tyrosine and phenylalanine and to a lower extent by methionine, leucine and histidine residues. Trypsin preferentially cleaves after arginine and lysine but not before proline which cause steric hindrance (Olsen, Ong, & Mann, 2004). However, RuBisCO displays a higher content of amino acid that would be sensitive to pancreatic enzymes than gluten. A larger spacing of cleavage sites on the protein may also result in the release of peptides with larger sizes. The ExPASy tool PeptideMass was used to compared the predicted peptide sizes of RuBisCO and of a high-molecular-weight glutenin subunit (HMW-GS) after hydrolysis by trypsin and chymotrypsin. To do so, the sequence of a lettuce RuBisCO (UniProt accession number: P48706

and Q40250 for the large and small chains, respectively) and a sequence of wheat HMW-GS (UniProt accession number: P10388) were used. Results suggest that RuBisCO hydrolysis by trypsin and chymotrypsin would lead to peptides of similar or even smaller mean size than that obtained on HMW-GS (data not shown). In addition, the median molecular weight of peptides of 6 amino acids minimum and with a maximum molecular weight of 4000 Da, are predicted around 1200 and 1237 Da after the trypsin digestion for RuBisCO and HMW-GS, respectively. These values are closed to the value of 1202 Da experimentally measured on pea protein-enriched pasta after gastro-intestinal digestion (Berrazaga et al., 2020). Therefore, neither the amino acid composition nor the sequence of RuBisCO seems to explain the low sensitivity of RuBisCO-enriched dough soluble fraction to intestinal hydrolysis. However, these results can be correlated with previous studies that showed high stability of RuBisCO to hydrolysis by pancreatic enzymes, with RuBisCO subunits still identifiable on SDS-PAGE gels after 120 minutes of hydrolysis (Fu et al., 2002).

The DH and the proportion of soluble nitrogen are similar between gluten- and pea protein-enriched doughs at the end of the intestinal phase. In addition, both the DH and the soluble nitrogen proportion tend to be higher in these two latter doughs than in control dough. It is interesting to note that pea proteins, despite their high TIA, do not affect intestinal hydrolysis. This is probably related to the dough thermal treatment which is known to decrease the TIA of proteins (Berrazaga et al., 2020; Laleg et al., 2017). These results cannot be explained by the aggregation state of proteins since the introduction of gluten or pea proteins does not modify it much, neither modify wheat protein polymerisation. However, it is possible that the network geometry may be modified due to dilution of the intrinsic protein network. Such modifications could result in a less compact structure leading to an enhancement of protease accessibility to the proteins as demonstrated on starch digestion (Zou, Sissons, Warren, Gidley, & Gilbert, 2016). Also, larger pore sizes may enhance protease diffusion in the matrix as demonstrated on egg-white or casein gels for pepsin diffusion (Somaratne et al., 2020; Thévenot, Cauty, Legland, Dupont, & Floury, 2017). Overall, the digesta of control, gluten- and pea protein-enriched doughs to have similar mean peptide size, as estimated by the similar N:NH₂ ratio of the three doughs (**Supplementary 1**).

3.5 *In vitro* apparent protein digestibility

Apparent digestibility is usually calculated from a nitrogen balance measured during *in vivo* measurements (AFSSA, 2007). It has been shown that a filtration of the *in vitro* digestion products with a 10 kDa molecular weight cut-off gave a good estimation of the *in vivo* digestibility of amino acid (Cave, 1988). In our study, the proportion of the soluble fraction of digesta which passed the 10 kDa threshold during microfiltration was used to evaluate the apparent *in vitro* digestibility of proteins, as similarly performed in (Huang, Tan, Xing, Pan, & Li, 2000; Le Roux et al., 2020). Results are presented in **Table 6**. Apparent *in vitro* digestibility of doughs is about 58% with no significant difference between the samples. Although the introduction of RuBisCO in dough may increase the average size of peptides at the end of digestion, it does not change the proportion of peptides with molecular weight below 10 kDa.

PDCAAS-like scores were calculated on control and protein-enriched doughs (**Table 6**) based on the *in vitro* digestibility measurement as suggested by (Le Roux et al., 2020). PDCAAS-like of control and gluten-enriched doughs are of 0.27, which is close of the PDCAAS of gluten protein experimentally

measured at 0.25 (Tarté, 2009). However, these scores are much lower than the PDCAAS of 0.42 measured from the *in vivo* digestibility on rats fed with 28% gluten-enriched pasta (Laleg, 2016). The improved CS of RuBisCO- and pea protein-enriched doughs lead respectively to 1.8 and 2.1 fold higher PDCAAS-like, as compared to control and gluten-enriched doughs. As a comparison the PDCAAS-like values of the doughs enriched in RuBisCO or pea proteins are close to the PDCASS measured on a pea emulsion (0.64) (Reynaud, 2020) but remain lower than those obtained on pea protein concentrate (0.73), soy concentrates (0.99) or on casein (1) (reviewed in Tarté, 2009).

Table 6. Protein *in vitro* digestibility, chemical score and PDCAAS-like of control and protein-enriched doughs at 37.4% RuBisCO, 35.0% gluten and 38.7% pea proteins. *In vitro* digestibility corresponds to the nitrogen content of the fraction of molecules with molecular weights below 10 kDa as a proportion of the total nitrogen content in the digesta.

Dough formulation	<i>In vitro</i> digestibility*	Chemical score	PDCAAS-like		
	%	%			
Control	55.1 ^a	49	0.27	±	0.02
RuBisCO-enriched	60.2 ^a	82	0.49	±	0.04
Gluten-enriched	57.3 ^a	47	0.27	±	0.04
Pea protein-enriched	58.9 ^a	98	0.58	±	0.11

*Values with the same upper case letters in the same column are not significantly different ($p > 0.05$).

4 Conclusion

This study aimed at evaluating the effect of a RuBisCO enrichment on the nutritional quality of proteins in wheat dough, in comparison to gluten- and pea protein-enriched doughs. *In vitro* digestibility was evaluated from the proportion of nitrogen contained in the soluble fraction lower than 10 kDa. We have shown that the dough protein enrichment does not affect *in vitro* digestibility, regardless of the protein used. In addition, the enrichment with RuBisCO and pea proteins lead to higher PDCAAS-like as compared to control and gluten-enriched doughs related to the improved amino acid profile of dough. In this study, the higher lysine content of the pea protein concentrate makes it more interesting than RuBisCO to improve the amino acid profile of wheat-based foods. The results underline that there is no clear relation between the protein aggregation state and the proteolysis in our matrixes. Unlike the effect of adding pea or gluten protein, the degree of hydrolysis is not altered by the addition of RuBisCO. However, the rate of nitrogen solubilisation is increased, suggesting that the average peptide size is increased at the end of digestion. Although this does not alter the *in vitro* digestibility that we measured, these modifications deserve further investigation, especially when studying digestibility on real food matrixes enriched with RuBisCO. The modification of the peptide size in the intestinal soluble fraction could be further verified by peptidome analysis.

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Supplementary material

Supplementary 1. Ratio between soluble nitrogen content ($\text{mmol} \cdot 100\text{g}^{-1}$ of dough) and the free amino group content ($\text{mmol} \cdot 100\text{g}^{-1}$ of dough) contained in the soluble part of digesta after gastric and intestinal digestions of control and protein-enriched doughs at 37.4% RuBisCO, 35.0% gluten and 38.7% pea proteins. Both nitrogen and free amino groups are from food as the contribution of digestive enzymes was removed.

Dough formulation	Ratio of Nitrogen to primary amino group content*	
	<i>Gastric phase</i>	<i>Intestinal phase</i>
Control	15.2 ^b	3.0 ^a
RuBisCO-enriched	8.6 ^c	5.1 ^b
Gluten-enriched	22.1 ^a	2.7 ^a
Pea protein-enriched	12.6 ^{bc}	3.5 ^{ab}

*Values with the same upper case letters in a same column are not significantly different ($p > 0.05$). Data of gastric and intestinal phases were analysed separately.

The ratio of Nitrogen to primary amino group content gives a rough estimate of the length of peptides contained in digestion supernatants. Rather than estimating an absolute size, these ratios allow a relative comparison of the samples. Result should be taken with care because the side chains of the additional amine groups of amide some amino acids (Asn, Gln, Arg, Lys) are included in the primary amino group content

Chapter 6. Discussion, conclusions and perspectives

Part 1. General discussion

The objectives of this PhD were to assess the impact of RuBisCO introduction on dough mechanical properties and protein structures and on the protein *in vitro* digestibility. We used a multidisciplinary approach combining biochemical, physical-chemical and physical analysis techniques to probe the protein structures, their assembly and their digestibility in a complex food system.

The result chapters of this manuscript present our research findings and discuss them in relation to the literature. The purpose of this section is to discuss the whole results together to provide a more comprehensive and integrated view on the thesis results. First, we will address the modification of RuBisCO properties, in particular the solubility, induced by its introduction in wheat dough. Then, the effect of the protein enrichment on the mechanical properties of the dough during thermal treatment will be discussed. Finally, we will discuss the factors that may influence the protein *in vitro* digestibility in our matrices.

1 RuBisCO, a singular and reactive dietary plant protein

1.1 *The solubility of RuBisCO as an indicator of its reactivity in wheat doughs*

The biochemical and physical-chemical characterisation of the leaf protein concentrate (LPC) in dispersion (Chapter 3) provided the main characteristics of RuBisCO, the major protein of the LPC. We identified the large-chain (LC, $\sim 45 \text{ kg.mol}^{-1}$) and the small-chain (SC, $\sim 15 \text{ kg.mol}^{-1}$) of the hexadecameric form of RuBisCO. In addition, we identified a disulphide-linked dimer of LC (LC-dimer, $\sim 100 \text{ kg.mol}^{-1}$) which has already been observed in LPC (Mehta, Fawcett, Porath, & Mattoo, 1992; Rintamaki, 1989). The formation of LC dimer is probably due to oxidative conditions during the extraction process of LPC, as previously suggested (Mehta et al., 1992). The solubility of RuBisCO is minimal at pH 4 ($\sim 17\%$) which roughly corresponds to its minimum net charge (pH 3.8). Above pH 5, RuBisCO solubility increases to reach 93% for pH higher than 7. This solubility profile is regularly observed on RuBisCO concentrates or isolates (Kiskini, 2017; Van de Velde, Alting, & Pouvreau, 2011).

After mixing of the RuBisCO-enriched dough, part of the RuBisCO is recovered in the gluten-like fraction (GLF), corresponding to the water-insoluble protein fraction. To understand this loss of solubility, proteins from the RuBisCO-enriched dough and the GLF were extracted in denaturing buffer (SDS-containing buffer) and analysed by steric exclusion chromatography. The elution profile analysis showed that both weak and covalent bonds are involved in the loss of RuBisCO solubility. Despite the modification of its aggregation state, RuBisCO is homogeneously distributed in the protein network at the micron scale, as visualised on micrographs of RuBisCO-enriched dough after the specific labelling of RuBisCO.

The thermal treatment of the dough was shown to affect more drastically RuBisCO solubility than mixing. During dough thermal treatment at 80°C for 3 minutes, both RuBisCO LC and SC subunits establish covalent bonds and form large-size covalently linked polymers. In addition, the SDS-soluble wheat proteins decrease unexpectedly after the thermal treatment of RuBisCO-enriched dough. This suggests that RuBisCO introduction also affects wheat protein aggregation state. As a result, the SDS-soluble protein content of dough decreases and most of the RuBisCO becomes insoluble in SDS at pH 6.8. The SDS-insoluble proteins were extracted using a thiol reducing agent (DTE), showing that disulphide bridges are, at least in part, responsible for the formation of these aggregates. The ability of RuBisCO to form disulphide bonds under the effect of heating can be related to its high amount of cysteine (45 mg.g⁻¹ of total amino acids) and its high content of free thiols (67.2 µmol.g⁻¹ of protein). In comparison to pea proteins, RuBisCO contains 4.5 fold higher cysteine and 6 fold higher content in free thiols groups, which may explain that the extensive formation of covalent bonds was not observed in pea protein-enriched dough. Berrazaga (2018) reported a positive correlation between the initial total amount of cysteine in pasta and the SDS+DTE soluble protein content of cooked pasta. This correlation was unique for different protein compositions of pasta (100% wheat pasta or pasta enriched with faba, pea or lentil flours). This underlines that the protein aggregation state in pasta is influenced by the amount of total cysteine regardless their specific origin. Such a correlation could not be established in our study. This may be due to the small range of variation in SDS+DTE soluble protein content (from 20 to 35% vs from 20 to 80% in (Berrazaga, 2018)) related to the low thermal processing of our doughs.

In addition to give a global picture of the protein solubility in SDS, the originality of this PhD was to accurately analyse the elution profile of protein extracts. By comparing the experimental elution profiles with theoretical profiles, we identified which protein types were affected by covalent aggregation changes. The results suggest the formation of a co-protein network between RuBisCO and gluten proteins. It was not possible to prove the existence of such a co-protein network with the methodology used in this PhD. This constitutes one of the major perspectives of this project. In order to prove the covalent interaction between gluten and RuBisCO, the Fluorescence Resonance Energy Transfer (or Förster resonance energy transfer; FRET) could be used. It is a process by which energy is transferred from a fluorophore in the excited state (the donor) to a second fluorophore in close proximity (the acceptor) (Jares-Erijman & Jovin, 2003). The energy transfer is manifested by the extinction of the donor's fluorescence and the increase in the fluorescence emission of the acceptor. Energy transfer occurs if the distance between the two chromophores is less than about 10 nanometres. Thus, close spatial association of labelled proteins may be identified measuring the resonance energy transfer effect. For example, the use of FRET has enabled the identification of interactions between some antibiotics and allergenic caseins in milk, susceptible to reinforce the allergenic effect of this food by altering the protein structure (Dantas et al., 2020).

1.2 RuBisCO in wheat dough, a singular dietary plant protein

In our study, the consequences of enriching wheat dough with RuBisCO were compared to those of adding gluten or pea proteins. The two latter also form disulphide linked aggregates during dough thermal treatment. Their reactivity remained limited compared with RuBisCO, as illustrated by the minor reduction of peaks in the elution profile of gluten- and pea protein-enriched doughs in comparison to RuBisCO-enriched dough. The low thermal reactivity of gluten could be attributed to

the relatively low temperature (80°C) and short time (3 min) of the heat treatment used in our study. Heat treatment at higher temperature was demonstrated to induce a strong polymerisation of gluten proteins in pasta with SDS-soluble proteins decreasing from 70% to 20% when the drying temperature was increased from 55°C to 95°C (Laleg et al., 2017). Similarly, the thermal denaturation temperature of pea proteins is above 80°C (Mession, Sok, Assifaoui, & Saurel, 2013; Osen, Toelstede, Wild, Eisner, & Schweiggert-Weisz, 2014; Shand, Ya, Pietrasik, & Wanasundara, 2007). However, pea proteins are highly denatured during their extraction process (Osen et al., 2014) and contain few cysteine, so it is unlikely that an increase in heat treatment temperature of dough would result in much higher aggregation of the proteins via disulphide bridges. The incorporation of pea proteins in the dough weakens the protein network through the dilution of gluten proteins. Adding our pea protein concentrate to wheat-based food would probably lead to the deterioration of food quality. Unlike pea proteins, RuBisCO introduction enhances the polymerisation of proteins under thermal treatment. The reactivity of RuBisCO could compensate for the decrease in gluten content and prevent the deterioration of food quality. RuBisCO therefore differs from legume proteins and could constitute an alternative protein to enrich wheat-based products.

RuBisCO behaviour in a dense cereal matrix seems to be similar to that of egg proteins. The incorporation of egg white powder at a level of 27% of the total protein was shown to reduce the amount of SDS-soluble protein of cooked pasta by about 29% (Laleg, Barron, Santé-Lhoutellier, Walrand, & Micard, 2016). In our study, the incorporation of RuBisCO at 37% of total proteins leads to a decrease of SDS-soluble proteins by about 18%. In contrast, faba-flour enrichment of pasta leads to twice higher SDS-soluble protein content (Laleg et al., 2016). Although all three proteins are initially highly soluble in SDS (>96%), only the enrichments in egg white proteins or RuBisCO induce a decrease of SDS-soluble proteins (Ducrocq, Boire, Anton, Micard, & Morel, 2020; Laleg et al., 2016). Despite different heat treatment conditions, the behaviour of RuBisCO in wheat dough is closer to that of egg white proteins in pasta rather than legume proteins. In gluten/egg protein blends, Lambrecht, Rombouts, Ketelaere, & Delcour (2017) showed that the heat-induced polymerisation of the protein mixture was more intense than that of isolated proteins. In addition, they showed that both egg protein and gluten protein polymerisation were enhanced. Based on results obtained on gluten mixtures with other dietary globular proteins, they developed a model that predict the heat induced polymerisation of the protein mixture. They suggested that initial accessible free thiol content and surface hydrophobicity of the heated globular proteins were the main factors affecting the protein polymerisation in the mixture. To deepen the understanding of the RuBisCO reactivity, we could determine the surface hydrophobicity of RuBisCO and verify if the model can be applied to RuBisCO/gluten mixtures.

2 RuBisCO, an enhancer of dough thermal sensitivity

The dough mechanical properties were evaluated during a temperature ramp from 25°C to 100°C. The results (Chapter 4) show that, unlike gluten or pea proteins, RuBisCO is able to preserve the increase in dough elasticity between 50°C and 75°C. This increase corresponds to the cumulative effect of starch swelling and protein aggregation. We hypothesised that a gluten or a pea protein enrichment may compromise starch gelatinisation, which would decrease the elastic modulus rise during heating. In contrast, we suggested that the thermal reactivity of RuBisCO and its possible low competition with starch for water allowed the preservation of the increase of the elastic modulus. The dough mechanical

properties were analysed from 50°C to 75°C, as the major physical-chemical changes of starch and wheat proteins occur in this temperature range (Cuq, Abecassis, & Guilbert, 2003). However, the biochemical analyses demonstrate that RuBisCO also modifies the protein aggregation before heating, during dough mixing. It would therefore be worthy to discuss the effect of RuBisCO on mechanical properties below 50°C. To this end, the complex uniaxial viscosity (η^*) can be analysed, as it includes both the evolution of the elastic (E') and storage modulus (E'') at a given frequency (ω):

$$\eta' = \frac{E'}{\omega}$$

$$\eta'' = \frac{E''}{\omega}$$

$$\eta^* = \eta' - i\eta''$$

The magnitude of the complex viscosity ($|\eta^*|$) was computed as followed and will be referred as “complex viscosity” hereafter:

$$|\eta^*| = \sqrt{(\eta')^2 + (\eta'')^2}$$

It should be noted that our experiments were designed to probe the elastic and storage moduli of dough during heating, not viscosity by itself. In future work, we should check that the Cox-Merz law, which relates complex viscosity to steady state viscosity (conventionally measured with flow or creep-recovery experiments), is verified.

Figure 1A represents the evolution of the complex viscosity from 25° to 100°C for a control dough and for RuBisCO-, gluten- and pea protein- enriched doughs at 31.9%, 29.6% and 33.0% enrichment rates, respectively. The complex viscosity evolution with temperature is similar to that of the elastic modulus as described in chapter 4.

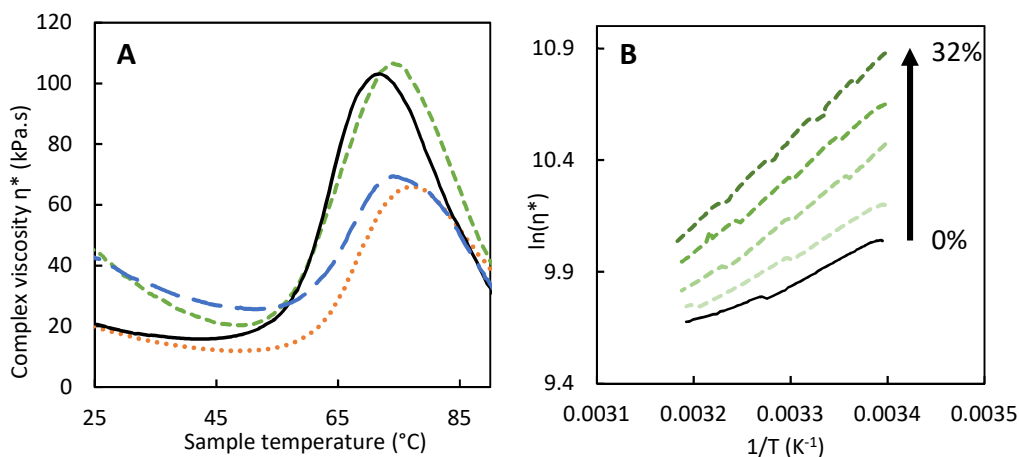


Figure 1. A. Thermal variation of the complex viscosity in control (black full line) dough and in 31.9% RuBisCO- (green short dashed line), 29.6% gluten- (orange dotted line) and 33.0% pea protein- (blue large dashed line) enriched doughs. **B.** Linear dependence of the natural logarithm of the complex viscosity to the reciprocal temperature for control (black full line) and RuBisCO enriched doughs (green dashed lines) with increasing enrichment rate up to 32% of total proteins between 20°C and 40°C.

At temperatures far from phase transition temperatures, the influence of temperature on dough viscosity can be described by an Arrhenius-type equation involving an activation energy for viscous flow (Bloksma, 1975; Chandler, 2014; Eyring, 1936):

$$\eta^* = \alpha \cdot \exp\left(\frac{E_a}{RT}\right)$$

where η^* is the complex viscosity (Pa.s), α is a prefactor (Pa.s), E_a is the activation energy of the viscous flow ($\text{J} \cdot \text{mol}^{-1}$), R is the universal gas constant ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) and T is the temperature (K). **Figure 1B** shows an example of the linear dependence of the natural logarithm of viscosity to reciprocal temperature for control and RuBisCO enriched doughs between 25°C and 40°C. This linear dependence was found for all samples. Curves were thus fitted with a linear function ($r^2 > 0.97$) and a viscosity at 25°C and an E_a were therefore determined. The evolution of the viscosity and of E_a are represented as a function of the protein enrichment rate in **Figure 2**.

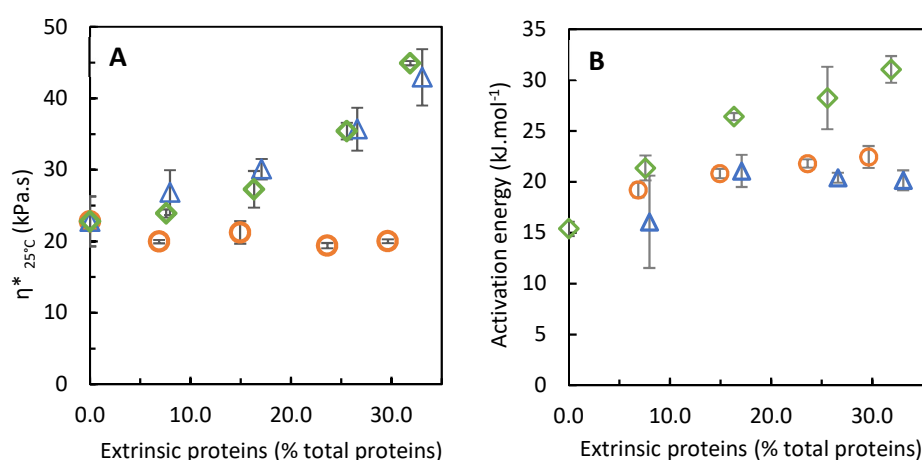


Figure 2. Viscosity at 25°C (**A**) and activation energy of the viscous flow (**B**) of RuBisCO- (green diamonds), gluten- (orange circles) and pea protein- (blue triangles) enriched doughs according to the content in extrinsic proteins in proportion of total proteins.

Figure 2A shows that the addition of gluten up to 30% of the total proteins does not alter the viscosity of the dough at 25°C. This result suggests that, in the protein enrichment ranges studied, a decrease in the starch:gluten ratio does not modify the viscosity at 25°C. In contrast to gluten, RuBisCO and pea protein enrichments above 16% of total proteins increase the dough viscosity. The dough viscosity at 25°C seems thus to be dominated by the nature of the protein phase. Additional experiments have shown that a decrease in water content from 72.6% to 61.5% (db) in a control dough increases its viscosity at 25°C from 16 to 37 kPa (data not shown). It can be supposed that the enrichments in pea proteins and RuBisCO increase the dough viscosity due to the modification of the water distribution in the system. Indeed, the water-binding capacity of pea proteins are higher compared to gluten proteins (Peters, Vergeldt, Boom, & van der Goot, 2017). The pea protein phase could absorb more water than gluten, which would modify the mechanical properties of the dough (Dekkers et al., 2016). This hypothesis is consistent with the results we obtained on the mechanical properties of pea protein-enriched dough during thermal treatment (chapter 4). We showed that the enrichment of wheat dough with pea proteins reduced the elastic modulus rise during heating with no major modification of wheat protein crosslinking. These results led us to the hypothesis that pea proteins act as a water trap, leading to a decrease in starch gelatinisation during heating. A competition for water between

the RuBisCO and the wheat components could also influence the viscosity of the dough at 25°C. However, we had shown that the addition of RuBisCO maintained a notable rise in the elastic modulus during heating in a similar way to control dough. Contrary to pea protein, the thermal reactivity of the RuBisCO and its contribution to the mechanical properties of the system during heating would counterbalance the effects of the change in water distribution.

The evolution of E_a as a function of extrinsic protein content is represented in **Figure 2B**. The dough enrichment with gluten and pea proteins slightly increases the E_a but it reaches a plateau above 7% of protein enrichment. Above this enrichment rate, a common E_a of about 21 kJ.mol⁻¹ characterizes gluten- and pea protein-enriched doughs. Slightly higher E_a have been determined in the same temperature ranges on bread doughs (Bloksma, 1975; Launay & Buré, 1973). It has also been shown that bread dough E_a was not affected by a variation in water content between 66 and 76% (db) (Launay & Buré, 1973). We obtained similar results on our dough with water content between 63 to 67% (not shown). In contrast to gluten and pea proteins, RuBisCO enrichment increases the E_a with increasing enrichment rate from 8 to 32% of total proteins, meaning that RuBisCO increases the temperature sensitivity of the system between 25°C and 40°C. This increase in the thermal sensitivity of the dough may be related to the lower denaturation temperature of the RuBisCO as compared to the temperatures of starch gelatinisation and gluten reticulation that occur around 70°C at 67% water content (db) (Cuq et al., 2003). Indeed, conformational changes of RuBisCO occur around 45°C (Li et al., 2002) and its denaturation occurs around 65°C (Libouga, Aguié-Béghin, & Douillard, 1996; Martin, Nieuwland, & De Jong, 2014). These structural modifications of RuBisCO may increase the protein-protein or protein-water interactions, as suggested by (Woldeyes, Qi, Razinkov, Furst, & Roberts, 2020) for the temperature dependence of antibodies solution viscosities.

These results obtained on the mechanical properties underline that the introduction of RuBisCO increases the sensitivity of the dough to temperature. Similarly, the influence of RuBisCO on the thermal sensitivity of secondary protein structures was demonstrated in chapter 3. The thermal treatment was shown to increase the β -sheet structures at the expense of α -helices in all protein-enriched doughs, as regularly observed on pasta (Laleg et al., 2016) or gluten (Georget & Belton, 2006). In our systems, these changes were enhanced in RuBisCO-enriched doughs and were intensified with the increase of RuBisCO enrichment rate.

3 RuBisCO, a potential ingredient to improve the protein nutritional quality of wheat-based food?

The results presented in the chapter 5 showed that RuBisCO allowed an improvement of the amino acid profile of wheat dough, notably by increasing the lysine chemical score. Protein *in vitro* digestibility was evaluated by determining the nitrogen content of the fraction of soluble molecules with a molecular weight below 10 kDa. It was shown that *in vitro* digestibility remained unchanged after a protein enrichment regardless of the protein used, RuBisCO, pea proteins or gluten. This result suggests that overall, the protein enrichment of dough (with ~20% total protein content in dry basis) allows the release of a higher amount of peptide or free AA lower than 10 kDa than the control dough (14% protein content in dry basis). Altogether, the RuBisCO enrichment resulted in a 1.8 fold higher PDCAAS (protein digestibility-corrected amino acid score) like as compared to a control dough. These results highlight the potential benefits of RuBisCO enrichment of wheat products. Despite the

unchanged protein digestibility, the study highlighted changes in proteolysis during digestion of RuBisCO, pea protein and gluten enriched doughs.

3.1 Factors influencing proteolysis in protein-enriched doughs

To evaluate the proteolysis rate, we measured the degree of protein hydrolysis (DH). DH is often reported to be increased as a result of the incorporation of non-wheat proteins in wheat-based foods (Laleg et al., 2017, 2016; Mubarak, 2001; Rayas-Duarte, Mock, & D., 1996). The authors frequently related the increased DH to the decrease of protein aggregation state, assessed by the increase of the SDS-soluble protein content. In our study, neither the addition of gluten nor the addition of pea proteins decreases the protein aggregation state of dough. However, pea protein and gluten introduction in wheat dough both tend to increase the DH at the end of digestion. In addition, the incorporation of RuBisCO increases the protein aggregation state whereas it does not alter the DH after the gastric or the intestinal. The protein aggregation state of the protein-enriched doughs thus does not explain the changes in the degree of DH. Nevertheless, the DH corresponds to the amount of soluble free amino groups ($-NH_2$) after digestion in proportion of the total $-NH_2$ in the food after a total chemical hydrolysis. DH reflects the number of cleavage sites among the soluble protein/peptide recovered in the soluble fraction. It provides no information on their average size after enzymatic hydrolysis. For this reason, we have combined the DH measurement with the assay of nitrogen in the soluble fraction of digesta. The combination of these two techniques is not commonly performed in the published literature, probably due to the little change of soluble nitrogen content observed between the gastric and intestinal phases for many dietary proteins (Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001; Santos-Hernández et al., 2020).

The results highlight major variations in the content of soluble nitrogen during digestion following the incorporation of RuBisCO into the dough. Nitrogen solubilisation is limited during gastric digestion of RuBisCO-enriched dough as compared to the other doughs. We suggest that this may be related to the higher degree of protein aggregation in RuBisCO-enriched dough. Although the protein aggregation state does not seem to affect DH, it could reduce the nitrogen solubilisation by limiting the diffusion of enzymes into the matrix (Domenek, Brendel, Morel, & Guilbert, 2004). The DH remaining unchanged, the RuBisCO enrichment would result in the decrease of peptide mean size at the end of gastric digestion. On the contrary, during the intestinal phase, RuBisCO enrichment leads to a higher release of nitrogen as compared to the control dough. As the final DH remains unchanged, this suggests that RuBisCO enrichment tends to increase the average size of soluble peptides at the end of digestion. However, we have shown that the potential enzyme cleavage sites in RuBisCO do not explain this decrease in soluble peptide hydrolysis. In contrast, both the gluten and the pea protein enrichments tend to increase the soluble nitrogen and the DH at the end of digestion, while both doughs have similar protein aggregation state than control dough. Overall, the study showed that the link between the protein aggregation state in the matrices (estimated via the proportion of SDS-insoluble protein), the protein DH and the amount of solubilised nitrogen during digestion remains unclear.

Numerous factors other than the protein aggregation state could have affected the proteolysis in our matrices. Carbonaro et al. (2012) highlighted a strong negative correlation between the proportion of β -sheet structures in legume proteins and their *in vitro* digestibility (Carbonaro, Maselli, & Nucara, 2012). We have shown that the RuBisCO-enrichment of dough resulted in an increase in the proportion of β -sheets after heat treatment of the dough. RuBisCO-induced secondary structure changes could

have therefore influenced the proteolysis of dough protein. However, it has been suggested on faba-enriched pasta that the effect of the protein aggregation state predominates over the effect of secondary structures on the proteolysis rate during *in vitro* digestion (Laleg et al., 2017).

These results also raise the question of the interplay between the nitrogen release and the hydrolysis of peptide bonds. Indeed, the solubilisation of proteins, peptides and free amino acids is related to the physical-chemical conditions of digestion, such as pH or ionic strength, but also to the effect of enzymatic hydrolysis. In addition, it is usually reported that the DH remains limited during the gastric digestion of food while most of the nitrogen is solubilised (Pasini et al., 2001; Santos-Hernández et al., 2020). On the contrary, little nitrogen is released during the intestinal phase whereas the DH increases sharply at the beginning of the intestinal phase. The strong increase of DH during the intestinal phase is thus mainly due to the hydrolysis of proteins and peptides solubilised during the gastric phase. In our matrices, the introduction of RuBisCO reduces the nitrogen release during gastric phase. This may limit the proteolysis during intestinal phase. It would then be interesting to monitor the release kinetics of nitrogen and DH during the intestinal phase.

3.2 *Factors that may influence the nutritional value of RuBisCO enriched wheat-based foods*

In this study, the digestion experiments were performed on matrices in powder form. It is highly probable that the influence of the microstructure of the protein network is minimised compared to the effect it could have in a whole food. Indeed, the food microstructural characteristics, and in particular the microstructure of protein network, is an important factor that may affect enzyme accessibility to the substrate. As an example, Fardet et al. (1998) has shown that the microstructure of the gluten protein network, and especially the tortuosity, may restrict the pepsin diffusion throughout the matrix (Fardet, Hoebler, Djelveh, & Barry, 1998). The microstructure of the protein network formed by the coagulation of egg proteins was also shown to influence the diffusion of the pepsin, in particular by the pore size and the inter-particle aggregate distance (Somaratne et al., 2020). In our study, we showed that gluten and RuBisCO enrichment of dough do not modify the structure of the protein network at micrometer scale prior to dough heat treatment, as visualised by confocal microscopy. It would be then necessary to perform the microscopic observations on thermally treated dough and to evaluate its effect on the protein digestibility of non-freeze-dried dough.

Finally, the aim of this study was to evaluate the effect of protein enrichment on protein digestion. However, the nutritional value of wheat-based foods is also related on their high starch content. Besides, pasta are considered as healthy carbohydrate-based foods due to the slow digestion of starch in pasta. In pasta, both the compact structure of pasta and the presence of the gluten network reduce the accessibility of amylase to the starch granules (Zou, Sissons, Gidley, Gilbert, & Warren, 2015). The literature reports variable effects of protein enrichment on starch digestibility. Indeed, the *in vitro* starch digestibility were reported to remain unchanged after pasta enrichment with 35-100% faba bean or split pea flour (Greffeuille et al., 2015; Petitot, Boyer, Minier, & Micard, 2010). In contrast, *in vitro* starch digestibility was significantly reduced in pasta enriched with 19% protein from pea flour (Padalino et al., 2014) and in spaghetti fortified with 25% of chickpea flour (Goñi & Valentín-Gamazo, 2003). Regarding the use of RuBisCO as an ingredient in the formulation of wheat-based foodstuffs, especially pasta, it will be necessary to evaluate its influence on the digestibility of starch.

Part 2. General conclusion and perspectives

This PhD thesis is the first study on the behaviour of RuBisCO, the major protein of leaf protein concentrates, within a dense cereal matrix. The global aim of the study was to investigate the possible consequences of RuBisCO enrichment on the quality of wheat-based products. This study was conducted in three major axes:

- * The biochemical and physical-chemical characterisation of RuBisCO in leaf protein concentrate
- * The characterisation of the dough mechanical properties and protein structures after the RuBisCO enrichment
- * The evaluation of the *in vitro* digestibility of proteins in RuBisCO-enriched doughs

RuBisCO is not yet a protein ingredient for human consumption and its properties may depend on the extraction process. It was therefore necessary to carry out a detailed characterisation of the RuBisCO biochemical and physical-chemical properties to better understand its behaviour in wheat matrices. In addition to the characteristics related to RuBisCO, this study also highlighted the unconventional values of some physical-chemical parameters of RuBisCO in dispersion. These characteristics can alter the accuracy of conventional methods of protein detection or assay. The major hypothesis of this part is that these non-classical values arise from protein-phenolic interactions. The presence of phenolic compounds in LPC can have multiple negative consequences on the functionality, organoleptic qualities and nutritional properties of the protein concentrate. During this study, we faced the experimental difficulty of identifying protein/polyphenol interaction markers. Thus, a long-term perspective of this work would be to deepen the methods of identification of RuBisCO/contaminant interactions.

The second part of this study focused on the effect of RuBisCO enrichment on the gluten network, through the study of the mechanical properties and protein structures of a model matrix. We showed that RuBisCO differs from legume proteins, commonly introduced in wheat-based products. During hydration and mixing, RuBisCO becomes insoluble and contributes to the water-insoluble protein fraction. Moreover, because of its high content in cysteine and free thiols, RuBisCO forms disulphide-linked aggregates. In addition, the major effect of the introduction of RuBisCO is the increased sensitivity of the dough to heat treatment. Thus, overall covalent interactions between proteins during thermal treatment are promoted by the introduction of RuBisCO and the changes in protein secondary structures are enhanced. The reactivity and ability of RuBisCO to form weak and covalent interactions could prevent the negative consequences of a weakening of the gluten network in real wheat-based foods.

Finally, in a third part we showed the potential nutritional benefits of fortifying wheat-based products with RuBisCO. The *in vitro* digestibility of the proteins is unchanged and the amino acid profile is improved following the RuBisCO enrichment. Thus, RuBisCO may be a promising protein to enhance the protein nutritional quality of wheat-based products. Nevertheless, proteolysis seems to be affected, as illustrated by the modified peptide/protein solubilisation and hydrolysis of peptide bonds. In future studies, the origin of these modifications, which may have an effect on the assimilation of peptides *in vivo*, will have to be explored more precisely.

The major perspective of this work will be to compare the results obtained with our micro-scale approach with those obtained in real food systems, such as durum wheat pasta. The shear and heat treatment conditions will be different than in our study and the protein structures will certainly be modified. Finally, protein digestibility studies will have to be coupled with a study of the digestibility of starch.

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Appendix

Detailed summary of the thesis in French

Résumé détaillé de la thèse en français

1 Contexte de l'étude

L'apport moyen en protéines des adultes en Europe atteint ou dépasse souvent l'apport moyen de référence de 0,83 g par kg de poids corporel par jour (apport moyen de 0,8 à 1,25 g/kg) (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). De plus, dans la plupart des pays européens, plus de 60 % des protéines alimentaires proviennent de sources animales. Cette surconsommation de produits d'origine animale pose des problèmes environnementaux et de santé publique (Aiking, 2014; Friel et al., 2009; John Reynolds, David Buckley, Weinstein, & Boland, 2014). Un groupe d'experts a récemment souligné la nécessité urgente de transformer nos régimes et nos systèmes alimentaires. Ils ont mis en évidence le besoin d'augmenter la part de protéines d'origine végétale dans nos régimes alimentaires (Willett et al., 2019). Dans ce contexte, le projet GreenProtein a reçu un financement du programme de recherche et d'innovation Horizon 2020 de l'Union européenne (accord de subvention n° 720728). Il vise à revaloriser les déchets verts agro-industriels en ingrédients protéiques destinés à la consommation humaine. L'objectif principal du projet GreenProtein est d'établir un pilote d'extraction à l'échelle semi-industrielle pour la purification de concentré de protéines foliaires (CPF) de qualité alimentaire.

Les protéines foliaires constituent entre 15 et 35% de la masse sèche des feuilles (Aramrueang, Zicari, & Zhang, 2017; Yeoh & Wee, 1994). Les protéines de feuilles sont souvent classées en fonction de leur affinité pour l'eau. Les protéines insolubles dans l'eau sont principalement situées dans les membranes des organites végétaux. Elles sont généralement considérées comme impropres à la consommation humaine en raison de leur couleur verte et de leur astringence due à leur association avec des pigments lipophiles. Au contraire, les protéines solubles dans l'eau sont appelées "protéines blanches" et sont composées jusqu'à 50% de RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygénase (Fiorentini & Galoppini, 1983). La RuBisCO est une enzyme clé de la photosynthèse qui est présente en grandes quantités dans les feuilles en croissance. Cela fait d'elle la protéine la plus abondante sur Terre (Kung, 1976). Outre son abondance, l'intérêt de la RuBisCO provient également de son profil équilibré en acides aminés. Cette protéine présente des quantités d'acides aminés essentiels souvent supérieures à celles des profils de référence (Barbeau & Kinsella, 1988; Gerloff, Lima, & Stahmann, 1965). De plus, des études *in vivo* et *in vitro* révèlent que la valeur nutritionnelle des CPF est généralement supérieure à 95% (revue dans Fiorentini & Galoppini, 1983). La RuBisCO présente également des propriétés fonctionnelles intéressantes. La solubilité de la RuBisCO a été estimée plus élevée que celle d'autres isolats de protéines végétales et comparable à la solubilité des protéines animales, comme les protéines de lactosérum (Martin, Castellani, de Jong, Bovetto, & Schmitt, 2019). La RuBisCO est également reconnue pour ses propriétés gélifiantes à faible concentration (Martin, Nieuwland, & De

Jong, 2014). La fonctionnalité des protéines dans les matrices alimentaires et leur valeur nutritionnelle sont deux paramètres importants pour estimer leur intérêt à être utilisées dans l'alimentation humaine. La RuBisCO est donc un ingrédient protéique potentiel pour la consommation humaine. Bien que plusieurs procédés d'extraction aient été décrits (Edwards et al., 1975; Knuckles, Bickoff, & Kohler, 1972), la RuBisCO n'est pas encore utilisée comme ingrédient dans l'alimentation humaine. Ceci est lié à la difficulté d'extraire un CPF qui réponde aux exigences des industriels et des consommateurs. Cependant, l'amélioration des procédés d'extraction et les études récentes sur les fonctionnalités de la RuBisCO encouragent la recherche sur l'utilisation des CPF dans l'alimentation humaine (Firdaous et al., 2017; Hadidi, Khaksar, Pagan, & Ibarz, 2020; Tamayo Tenorio, Gieteling, De Jong, Boom, & Van Der Goot, 2016).

Contrairement à de nombreuses protéines alimentaires animales, les protéines végétales ont des déficiences en certains acides aminés essentiels. Cette caractéristique est un inconvénient majeur du remplacement partiel des protéines animales de nos régimes alimentaires par les protéines végétales. Une stratégie pour pallier à ce problème consiste alors à associer différentes protéines végétales ayant des profils d'acides aminés complémentaires. Par exemple, les carences en lysine des produits à base de blé peuvent être compensées par l'ajout de protéines plus riches en lysine, telles que les protéines de légumineuses. De même, la RuBisCO a une teneur en lysine plus élevée que celle des protéines de blé (Gerloff et al., 1965). Son incorporation dans les produits à base de blé améliorerait donc leur profil d'acides aminés. Cependant, l'enrichissement en protéines des produits à base de blé entraîne souvent l'altération de leurs qualités culinaires et organoleptiques. Par exemple, l'enrichissement des pâtes de blé dur avec des farines de légumineuses peut augmenter les pertes à la cuisson et modifier la texture des pâtes (Laleg et al., 2017; Petitot, Boyer, Minier, & Micard, 2010). De même, l'ajout de protéines de légumineuses à la pâte à pain peut affecter le volume du pain en réduisant sa capacité de rétention des gaz (Boukid, Zannini, Carini, & Vittadini, 2019). Ces changements de qualité résultent en partie de la modification de la structure des protéines du blé. Sous l'effet de l'hydratation et de l'apport d'énergie mécanique ou thermique, les protéines de réserve du blé sont capables de former un réseau protéique viscoélastique: le réseau de gluten. Les propriétés viscoélastiques uniques du réseau de gluten sont responsables des propriétés texturales des aliments à base de blé et sont essentielles pour leur qualité. Ainsi, l'introduction de protéines autres que les protéines de blé dilue souvent le réseau de gluten, voire empêche son bon développement. Cela entraîne une détérioration de la qualité de l'aliment. Il est donc difficile d'enrichir en protéines des aliments à base de blé avec une altération minimale, voire nulle, du gluten.

2 Stratégie de l'étude

2.1 Objectifs de l'étude et approche expérimentale

Cette thèse a consisté en l'étude des conséquences possibles d'un enrichissement en RuBisCO sur la qualité des produits à base de blé. Les faibles quantités de CPF disponibles au début de l'étude n'ont pas permis de produire des matrices alimentaires réelles. L'étude a ainsi été réalisée sur un système modèle : une pâte de blé composée de semoule, d'eau et de concentré de protéines. Les effets de l'enrichissement de la pâte avec des protéines de pois ou du gluten ont également été étudiés à titre de comparaison avec la RuBisCO. Les objectifs de l'étude étaient d'évaluer l'impact de l'introduction de RuBisCO sur les propriétés mécaniques et les structures protéiques de la pâte ainsi que sur la digestibilité *in vitro* des protéines.

L'approche expérimentale a été divisée en trois parties, comme représenté sur la **Figure 1**.

- * La première partie a consisté en la caractérisation détaillée des propriétés biochimiques et physico-chimiques du CPF. L'objectif était de décrire la composition du CPF et les principales caractéristiques de la RuBisCO.
- * La deuxième partie a été consacrée à l'étude des changements des propriétés mécaniques et des structures protéiques de la pâte de blé induits par un enrichissement en protéines. L'impact de l'enrichissement en protéines sur la structuration de la pâte pendant le chauffage, sur la polymérisation des protéines et sur la microstructure du réseau a été étudié.
- * La troisième partie a traité de l'effet de l'enrichissement en RuBisCO sur la digestion *in vitro* des protéines de la pâte de blé enrichie. La sensibilité des protéines à l'hydrolyse enzymatique a été évaluée par la mesure du degré d'hydrolyse et du taux de solubilisation de l'azote pendant la digestion. La digestibilité *in vitro* des protéines a été estimée à partir de la teneur en azote de la fraction soluble des digesta de poids moléculaires inférieurs à 10 kDa.

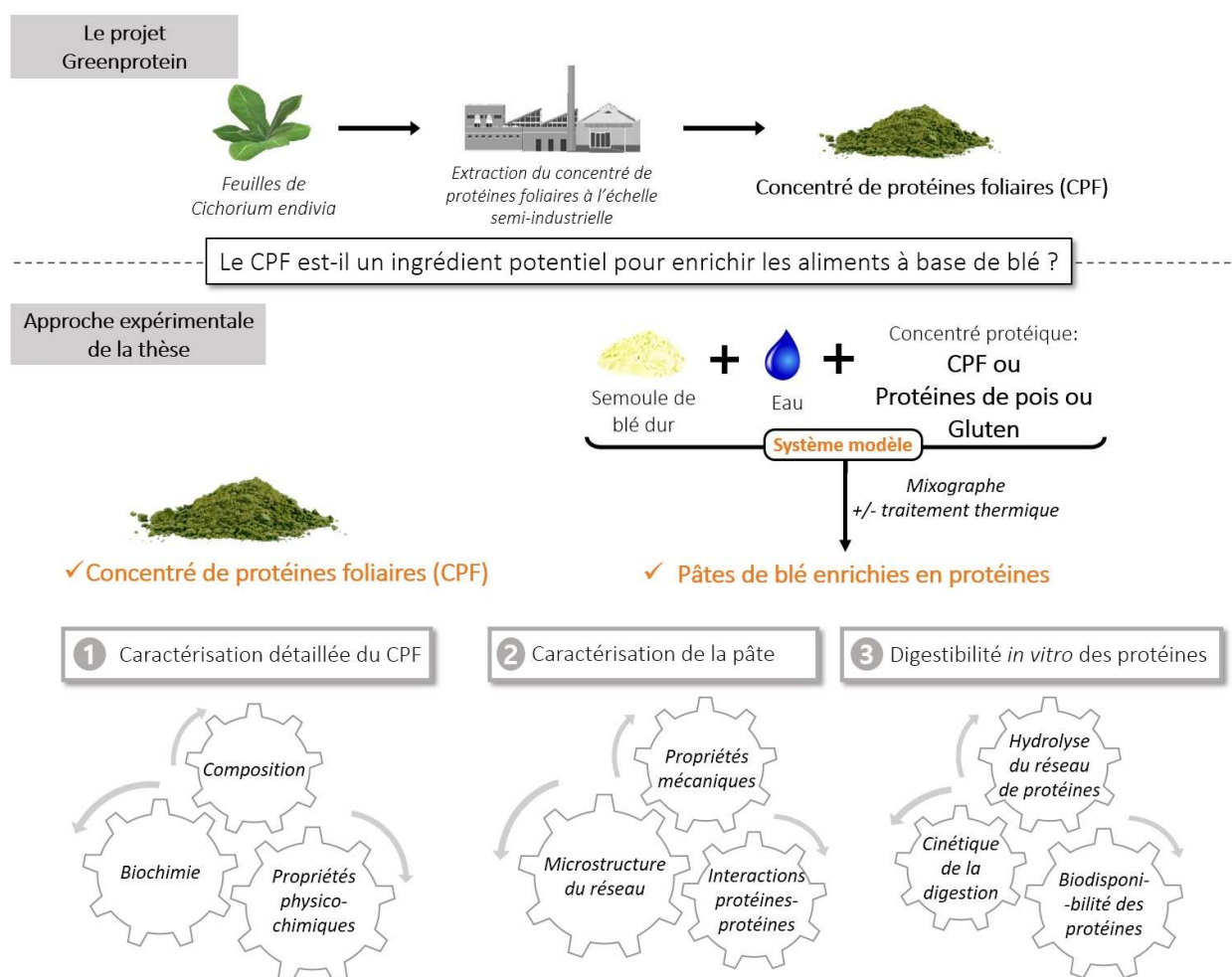


Figure 1. Schéma de l'approche expérimentale de la thèse

2.2 Fabrication des pâtes enrichies en protéines

La pâte fabriquée uniquement à partir de semoule et d'eau a été considérée comme la pâte témoin. Pour la fabrication des pâtes enrichies en protéines, de 1,5 à 10 % du poids de la semoule a été remplacé par un des concentrés de protéines (**Tableau 1**). Pour un même taux de substitution de la semoule, toutes les pâtes enrichies en protéines avaient la même teneur en protéines de blé provenant de la semoule, également appelées protéines intrinsèques. La composition des pâtes enrichies a été exprimée en teneur en protéines extrinsèques (provenant des concentrés protéiques) en pourcentage des protéines totales.

Les pâtes ont été fabriquées en utilisant un pétrin de laboratoire, le mixographe. Le mixographe que nous avons utilisé permet de produire de la pâte avec seulement 2 g de semoule ou de mélange semoule/concentré protéique. Brièvement, le protocole de fabrication consistait en deux étapes successives d'hydratation des ingrédients au repos puis de mixage. Lors de l'étude de l'effet de la température sur la structure protéique de la pâte, un traitement thermique a été appliqué. Directement après le mélange, un récipient en aluminium hermétiquement fermé a été rempli de 1,2 g de pâte et immergé dans un bain d'eau à 80 °C pendant 3 minutes. Les analyses biochimiques,

spectroscopiques et de digestibilité ont été réalisées sur des pâtes congelées à l'azote liquide puis lyophilisées.

Tableau 1. Exemple de la composition protéique des pâtes en fonction du taux de substitution de la semoule et du type de concentré protéique utilisé. Les "protéines intrinsèques" correspondent aux protéines provenant de la semoule, les "protéines extrinsèques" correspondent aux protéines provenant du concentré de protéines.

Composition de la pâte	Taux de substitution de la semoule (% bh)	Protéines intrinsèques	Protéines extrinsèques	Protéines totales	Protéines intrinsèques	Protéines extrinsèques
		g / 100g bs	g / 100g bs	g / 100g bs	% protéines totales	
Témoin	0.0	14.0	0.0	14.0	100.0	0.0
Enrichies en RuBisCO	3.5	13.5	2.6	16.2	83.7	16.3
	10.0	12.6	7.5	20.1	62.6	37.4
Enrichies en gluten	3.5	13.5	2.4	15.9	85.1	14.9
	10.0	12.6	6.8	19.4	65.0	35.0
Enrichies en protéines de pois	3.5	13.5	2.8	16.3	82.9	17.1
	10.0	12.6	7.9	20.5	61.3	38.7

3 Résultats

3.1 Caractérisation du concentré de protéines foliaires

Dans le domaine des sciences et des technologies alimentaires, beaucoup d'études portent sur les techniques d'extraction en relation avec les propriétés fonctionnelles du CPF. Cependant, la caractérisation du CPF résultant de ces extractions est fragmentée et, à notre connaissance, aucune étude ne caractérise en détail les propriétés biochimiques et physico-chimiques d'un CPF. Le comportement des protéines dans les systèmes alimentaires est étroitement lié à leurs caractéristiques biochimiques et physico-chimiques. Le but de cette partie a donc été d'étudier les propriétés biochimiques et physico-chimiques du CPF que nous avons utilisé.

Le CPF a été extrait à partir de feuilles de *Cichorium endivia* par un partenaire du projet GreenProtein (Florette ; Lessay, France). Le procédé d'extraction utilisé a été celui du brevet WO 2014/104880 en excluant l'étape d'adsorption sur colonne hydrophobe. Plusieurs techniques basées sur des analyses biochimiques, de la chromatographie et de la spectroscopie ont été utilisées pour étudier la composition du CPF et ses principales propriétés physico-chimiques. Les paramètres mesurés expérimentalement ont été comparés à des valeurs issues de la littérature ainsi qu'à des valeurs théoriques calculées à partir de séquences de RuBisCO connues.

Le CPF contenait 74,1 % de protéines (base sèche) et les polysaccharides représentaient le principal contaminant non protéique (9,6 % du poids du CPF en base sèche). Bien que la pureté en RuBisCO du CPF n'ait pas été quantifiée, les analyses en électrophorèses ont souligné que la RuBisCO était la protéine majeure du CPF. Nous avons identifié la grande et la petite sous-unité de la RuBisCO (**Figure**

2). Un dimère de grande sous-unité a également été identifié par Western Blot (**Figure 2**). Les teneurs en acides aminés essentiels étaient toutes supérieures à celles d'une protéine de référence ayant un profil d'acide aminé équilibré (AFSSA, 2007). Cela signifie que le CPF comprend tous les acides aminés essentiels nécessaires à l'alimentation humaine. Les analyses ont montré des teneurs en cystéines 2,5 fois plus élevées dans le CPF que celles calculées à partir de séquences de RuBisCO connues.

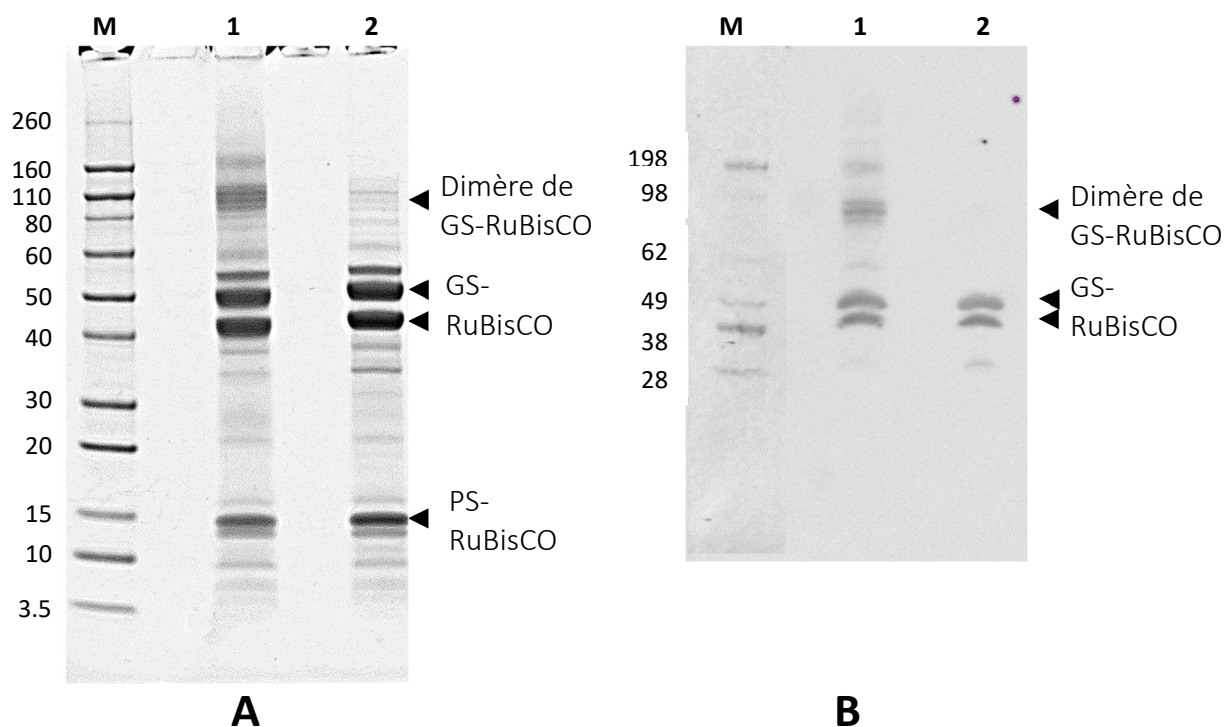


Figure 2. Gels SDS-PAGE (A) et Western blot (B) de CPF dans des conditions non réductrices (voie 1) et réductrices (voie 2). Les flèches indiquent les sous-unités RuBisCO. GS-RuBisCO et PS-RuBisCO correspondent à la grande et la petite sous-unité de la RuBisCO respectivement. Le western blot a été fait avec des anticorps anti grande sous-unité de RuBisCO. La voie M correspond aux étalons de masse moléculaire en kg.mol⁻¹.

L'étude des propriétés physico-chimiques du CPF en dispersion a mis en évidence des valeurs non conventionnelles de certains paramètres. Le spectre UV-visible du CPF atteint un maximum, au-dessus de 245 nm, à 258 nm et présente une absorption résiduelle entre 300 et 420 nm. Au contraire, le spectre théorique calculé à partir de la composition en acides aminés du CPF montre qu'un maximum d'absorbance, au-delà de 245 nm, est attendu à 275 nm. De plus, nous avons montré que le coefficient d'extinction spécifique du CPF est jusqu'à 4.2 fois plus élevé que ceux rapportés dans la littérature sur d'autres RuBisCO. Ces résultats suggèrent la présence d'espèces non protéiques absorbant les UV. Des expériences de chromatographie dans différentes conditions physico-chimiques suggèrent que ces composés sont liés aux protéines du CPF à la fois par des interactions covalentes et des liaisons faibles. De même, les valeurs d'incrément d'indice de réfraction mesurées sur des dispersions de CPF sont élevées par rapport aux valeurs calculées à partir de la composition en acides aminés du CPF. Des composés phénoliques ou des polysaccharides pourraient être à l'origine de ces valeurs non conventionnelles pour des protéines pures.

3.2 Caractérisation des propriétés mécaniques et des structures protéiques des pâtes de blé enrichies en RuBisCO

La qualité des produits à base de blé dépend principalement de la capacité des protéines de réserve du blé, les gliadines et les gluténines, à s'associer via des ponts disulfures et des liaisons faibles conduisant à la formation du réseau de gluten. Au cours du procédé, le réseau de gluten commence à se former lors de l'hydratation et de l'apport d'énergie mécanique à la pâte. Les interactions entre les protéines sont ensuite renforcées par la température grâce à des échanges thiols/disulfures. Dans de nombreux cas, l'enrichissement en protéines des aliments à base de blé dilue le réseau de gluten. Cela provoque une modification des propriétés texturales et de la qualité des aliments. L'objectif de cette partie était d'évaluer les changements des propriétés mécaniques de la pâte et des structures protéiques provoqués par un enrichissement en RuBisCO de la pâte de blé.

L'effet d'un enrichissement en RuBisCO, en gluten ou en protéines de pois sur la structuration de la pâte pendant le chauffage a été étudié par analyse thermo-mécanique dynamique (DMTA) à plusieurs taux d'enrichissement allant de 0 à 33% des protéines totales. La polymérisation des protéines dans les pâtes a été étudiée par extraction séquentielle des protéines en tampon dénaturant suivie d'une chromatographie d'exclusion stérique. La structure globale du réseau de protéines a été visualisée par microscopie confocale à balayage lumineux (CSLM). Enfin, les structures secondaires des protéines des pâtes enrichies en protéines ont été étudiées par spectroscopie infrarouge à transformée de Fourier (FTIR).

Nos résultats ont montré que le comportement de la RuBisCO dans la pâte était très différent de celui des protéines de légumineuses ou de gluten. Nous avons montré que la RuBisCO est hautement soluble (>90%) dans des tampons aqueux pour des pH supérieurs à 7. Or, lors du mixage de la pâte, la RuBisCO intègre le réseau de protéines insolubles dans l'eau via l'établissement de ponts disulfures et de liaisons faibles. Après traitement thermique de la pâte, l'enrichissement en RuBisCO diminue la teneur en protéines solubles dans le SDS de la pâte. De plus, les sous-unités de RuBisCO forment des liaisons disulfures intermoléculaires et intègrent les polymères de hauts poids moléculaires insolubles dans le SDS (**Figure 3**). Au contraire, l'enrichissement en protéines de pois ou en gluten ne modifie pas la polymérisation des protéines, même après un traitement thermique (**Figure 3**). Les analyses en microscopie confocale montrent que le gluten intrinsèque et la RuBisCO sont colocalisés à l'échelle microscopique dans les pâtes enrichies en RuBisCO. Ces résultats suggèrent la formation d'un réseau protéique entre la RuBisCO et les protéines du gluten.

Au cours du traitement thermique, le module élastique de la pâte augmente fortement entre 50°C et 75°C. Cela reflète le durcissement de la pâte lié aux effets cumulés de la polymérisation des protéines et de la gélatinisation de l'amidon. Contrairement aux protéines de pois et au gluten, l'ajout de RuBisCO dans la pâte n'affecte pas cette augmentation du module élastique pendant la cuisson. Cela pourrait être relié à son effet sur la polymérisation des protéines ou à une éventuelle faible compétition avec l'amidon pour l'eau.

Ces résultats soulignent que l'introduction de RuBisCO augmente la sensibilité de la pâte à la température. L'influence de la RuBisCO sur les structures secondaires des protéines a été étudiée. Il a été démontré que le traitement thermique augmentait la proportion de feuillets β au détriment des hélices α dans toutes les pâtes enrichies en protéines. Dans nos systèmes, ces changements ont été renforcés dans les pâtes enrichies en RuBisCO et ont été intensifiés avec l'augmentation du taux d'enrichissement en RuBisCO.

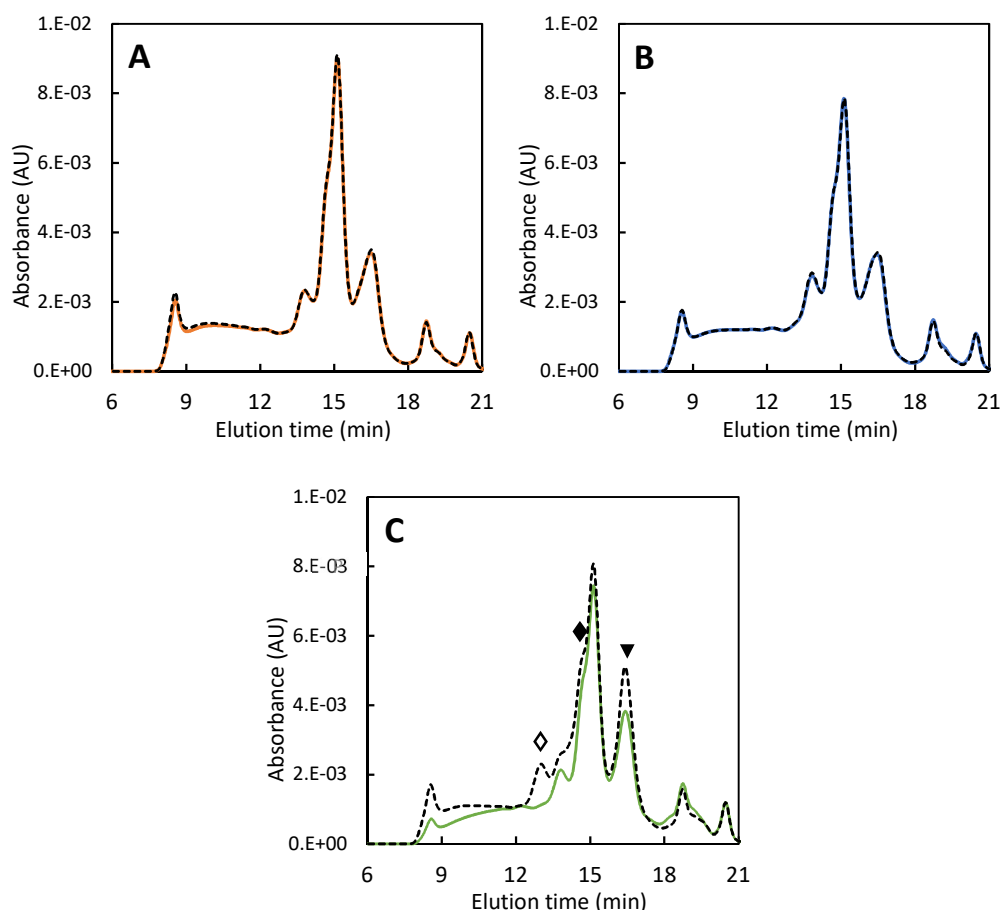


Figure 3. Profils expérimentaux (traits pleins en couleur) et théoriques (tirets noirs) de distribution en taille des protéines des pâtes solubles dans le SDS. Les protéines ont été extraites à partir de pâtes traitées thermiquement enrichies à 14,9% en gluten (**A**), à 17,1% en protéines de pois (**B**) et à 16,3% en RuBisCO (**C**). Les profils d'élution théoriques correspondent à la somme du profil d'élution de la pâte témoin cuite et du profil d'élution du concentré protéique seul. Les symboles (triangle et losanges) indiquent le temps d'élution des sous-unités de RuBisCO. La superposition des profils expérimentaux et théoriques indique que l'enrichissement en protéines de la pâte ne provoque pas de modifications de la polymérisation ni des protéines de la semoule ni des protéines du concentré protéique.

3.3 Digestibilité *in vitro* des protéines des pâtes de blé enrichies en RuBisCO

Le degré d'hydrolyse des protéines des aliments à base de blé est généralement augmenté suite à un enrichissement en protéines de légumineuses. Un lien entre l'augmentation de l'hydrolyse des protéines et la dilution du réseau de gluten dans l'aliment est souvent évoqué dans la littérature (Laleg et al., 2017; Patil, Brennan, Mason, & Brennan, 2016; Rayas-Duarte, Mock, & D., 1996). Nous avons

montré que l'ajout de RuBisCO à un taux d'enrichissement de 16,3 % modifie l'état d'agrégation des protéines dans les pâtes traitées thermiquement. L'objectif de cette partie a été d'étudier l'impact de ces modifications de la structure des protéines sur la digestibilité des protéines *in vitro*.

Des pâtes de semoule de blé traitées thermiquement et enrichies 37,4 % de RuBisCO, 35,0 % de gluten et 38,7 % de protéines de pois ont été utilisées. L'intérêt nutritionnel d'un enrichissement en RuBisCO a été évalué par sa capacité à améliorer la composition en acides aminés essentiels de la pâte de blé et par son effet sur la digestibilité *in vitro* des protéines de la pâte. Les matrices ont été digérées en utilisant le modèle statique de digestion *in vitro* développé par le réseau COST INFOGEST (Brodkorb et al., 2019). La protéolyse a été suivie à la fois par la mesure du degré d'hydrolyse et le taux de solubilisation de l'azote pendant la digestion. La digestibilité *in vitro* des protéines a été estimée à partir de la teneur en azote de la fraction soluble des molécules ayant un poids moléculaire inférieur à 10 kDa. Le PDCAAS (Protein digestibility-corrected amino acid score) est un index standardisé permettant d'évaluer la qualité nutritionnelle des protéines alimentaires. Il est normalement calculé à partir de la composition en acides aminés de l'aliment et des données de digestibilité *in vivo*. Un score de type PDCAAS a été calculé à partir de la digestibilité *in vitro* mesurée dans notre étude.

Les résultats ont montré que l'enrichissement en protéines ne modifiait pas la digestibilité *in vitro* des protéines de la pâte, quel que soit le concentré protéique utilisé (**Tableau 2**). De plus, les enrichissements en protéines de pois et en RuBisCO augmentent les teneurs en lysine de la pâte. C'est pourquoi les enrichissements en protéines de pois et en RuBisCO induisent une augmentation du PDCAAS par rapport à la pâte témoin et à la pâte enrichie en gluten.

Bien que la digestibilité *in vitro* ne soit pas modifiée, l'enrichissement en protéines de la pâte affecte la protéolyse pendant les phases gastrique et intestinale. Le degré d'hydrolyse finale n'est pas modifié par l'ajout de RuBisCO. Cependant, la proportion d'azote solubilisée est augmentée, ce qui suggère que la taille moyenne des peptides est augmentée à la fin de la digestion. L'ajout de protéines de pois ou de gluten tend à augmenter le degré d'hydrolyse et la solubilisation de l'azote en fin de digestion. Cependant, l'introduction de gluten et de protéines de pois ne semble pas affecter la taille moyenne des peptides en fin de digestion. Malgré ces tendances générales qui se dessinent en fin de digestion, le degré d'hydrolyse et la solubilisation de l'azote en fin de phase gastrique sont affectés différemment par les trois concentrés protéiques. Nous n'avons pas pu établir de relation claire entre l'état d'agrégation des protéines de la pâte et le degré d'hydrolyse ou le taux de solubilisation de l'azote au cours de la digestion.

Tableau 2. Digestibilité *in vitro* des protéines, score chimique et score PDCAAS de la pâte témoin et des pâtes enrichies en protéines à 37,4 % de RuBisCO, 35,0 % de gluten et 38,7 % de protéines de pois. La digestibilité *in vitro* correspond à la teneur en azote de la fraction des molécules de poids moléculaire inférieur à 10 kDa par rapport à la teneur totale en azote des digesta.

Formulation de la pâte	Digestibilité <i>in vitro</i> des protéines	PDCAAS-type		
	%			
Control	55.1 ^a	0.27	±	0.02
Enrichie en RuBisCO	60.2 ^a	0.49	±	0.04
Enrichie en gluten	57.3 ^a	0.27	±	0.04
Enrichie en protéines de pois	58.9 ^a	0.58	±	0.11

4 Conclusion et perspectives majeures

Cette thèse de doctorat est la première étude évaluant le comportement de la RuBisCO, la principale protéine des concentrés de protéines foliaires, au sein d'une matrice céréalière dense. L'objectif de l'étude était d'évaluer les conséquences possibles de l'enrichissement en RuBisCO sur la qualité des produits à base de blé.

Le RuBisCO n'est pas encore un ingrédient protéique destiné à la consommation humaine et ses propriétés peuvent dépendre de son processus d'extraction. Il a donc été nécessaire de procéder à une caractérisation détaillée de ses propriétés biochimiques et physico-chimiques pour mieux comprendre son comportement dans les matrices de blé. Cette étude a également mis en évidence les valeurs non conventionnelles de certains paramètres physico-chimiques de la RuBisCO en dispersion. Ces caractéristiques peuvent altérer la fiabilité des méthodes conventionnelles de détection ou de dosage des protéines. L'hypothèse majeure de cette partie est que ces valeurs non classiques proviennent d'interactions entre les protéines et des composés phénoliques. La présence de composés phénoliques dans le LPC peut avoir de multiples conséquences négatives sur la fonctionnalité, les qualités organoleptiques et les propriétés nutritionnelles du concentré de protéines. Au cours de cette étude, nous avons été confrontés à la difficulté expérimentale d'identifier les marqueurs d'interactions protéines/polyphénols. Ainsi, une perspective à long terme de ce travail serait d'approfondir les méthodes d'identification des interactions RuBisCo/contaminant.

La deuxième partie de l'approche abordait l'effet de l'enrichissement en RuBisCO sur le réseau de gluten, par l'étude des propriétés mécaniques et des structures protéiques d'une matrice modèle. Nous avons montré que la RuBisCO adoptait un comportement singulier par rapport aux protéines de légumineuses, couramment introduites dans les produits à base de blé. L'effet majeur de l'introduction du RuBisCO est l'augmentation de la sensibilité de la pâte au traitement thermique. Ainsi, les interactions covalentes de toutes les protéines pendant le traitement thermique sont favorisées par l'introduction de RuBisCO et les modifications des structures secondaires des protéines sont renforcées. Ces propriétés permettraient notamment de compenser l'effet de la teneur plus faible en

gluten sur les propriétés mécaniques de la pâte pendant le traitement thermique. La réactivité et la capacité du RuBisCO à former des interactions faibles et covalentes pourraient prévenir les conséquences négatives d'un affaiblissement du réseau de gluten dans les véritables aliments à base de blé. Les résultats suggèrent la formation d'un réseau commun entre la RuBisCO et les protéines du gluten. Il n'a pas été possible de prouver l'existence d'interactions entre la RuBisCO et les protéines du gluten avec la méthodologie utilisée dans ce doctorat. Ceci constitue l'une des perspectives majeures de ce projet.

Enfin, dans une troisième partie, nous avons montré les avantages nutritionnels potentiels de l'enrichissement des produits à base de blé avec de la RuBisCO. La digestibilité *in vitro* des protéines est inchangée et le profil des acides aminés est amélioré suite à l'enrichissement. La RuBisCO pourrait donc être une protéine prometteuse pour améliorer la qualité nutritionnelle des produits à base de blé. Néanmoins, la protéolyse semble être affectée, comme l'illustre la cinétique modifiée de la solubilisation des peptides et de l'hydrolyse des liaisons peptidiques. L'origine de ces modifications, qui pourraient avoir un effet sur l'assimilation des peptides *in vivo*, devra être explorée plus précisément dans de futures études.

La perspective majeure de ce travail est d'évaluer le comportement de la RuBisCO dans un vrai produit alimentaire. Les conditions de cisaillement et de traitement thermique seront différentes de celles de notre étude et les structures protéiques seront certainement modifiées. Enfin, les études de digestibilité des protéines devront être couplées à une étude de la digestibilité de l'amidon.

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Title: RuBisCO: a potential protein ingredient to enrich wheat-based food?**Understanding the impact of RuBisCO on wheat dough structure, protein-protein interactions and protein *in vitro* digestibility**

Keywords: RuBisCO, protein-enriched food, food structure, protein network, protein-protein interactions, protein *in vitro* digestibility

Abstract: Dietary proteins from plants often display an unbalanced amino acid profile in comparison to animal proteins. A way to overcome this problem is to combine plant proteins from different sources with complementary amino acid compositions. RuBisCO is the main protein of leaf protein concentrates. Because of its high content in lysine, RuBisCO could improve the amino acid profile of wheat-based products. However, the introduction of non-wheat proteins in wheat-based foods often dilutes the gluten network or even impede its proper formation. This leads to poorer textural and culinary properties of the food. In this context, we have studied the possible consequences of RuBisCO enrichment on the quality of wheat-based products. The study was performed on a model system, a wheat dough. For comparison, enrichments with gluten and pea proteins were also

studied. The main output of this work is that RuBisCO behaviour differs from that of legume proteins when introduced in dough. RuBisCO subunits establish weak bonds and form disulphide bridges when introduced in wheat dough. In addition, RuBisCO enrichment enhances the formation of disulphide bonded large size polymers during the dough thermal treatment. This may be related to its high content of cysteines and free thiols. This could prevent the negative consequences of gluten network weakening in wheat-based food. The enrichment of dough with RuBisCO modifies the kinetics of proteolysis during *in vitro* digestion. However, the global *in vitro* digestibility of proteins remains unchanged. These promising results confirm the potential of RuBisCO to be used for enriching wheat-based food.

Titre: La RuBisCO : un ingrédient protéique pour enrichir les aliments à base de blé ?**Etude de la structure, des interactions protéine-protéine et de la digestibilité *in vitro* des protéines de la pâte de blé enrichie en RuBisCO**

Mots-clés : RuBisCO, aliments enrichis en protéines, structure des aliments, réseau protéique, interactions protéine-protéine, digestibilité *in vitro* des protéines

Résumé : Les protéines végétales ont généralement un profil d'acides aminés déséquilibré par rapport aux protéines animales. Une façon de pallier ce problème est de combiner des protéines végétales avec des compositions en acides aminés complémentaires. La RuBisCO est la principale protéine des concentrés de protéines de feuilles. En raison de sa teneur élevée en lysine, la RuBisCO pourrait améliorer le profil en acides aminés des produits à base de blé. Cependant, l'introduction de nouvelles protéines dans les aliments à base de blé dilue le réseau de gluten et peut empêcher son développement. Cela provoque la détérioration des propriétés texturales et culinaires de l'aliment. Dans ce contexte, nous avons étudié les conséquences de l'enrichissement en RuBisCO sur la qualité des produits à base de blé. L'étude a été réalisée sur un système modèle, une pâte à base de blé. À titre de comparaison, l'enrichissement de la

pâte avec du gluten et des protéines de pois a également été étudié. Nous avons montré que le comportement de la RuBisCO diffère de celui des protéines de légumineuses lorsqu'elle est introduite dans la pâte. Les sous-unités de la RuBisCO forment des liaisons faibles et des ponts disulfures lorsqu'elles sont introduites dans la pâte de blé. De plus, la RuBisCO favorise la formation de polymères de grande taille liés par des ponts disulfures lors du traitement thermique de la pâte. Cela pourrait limiter les conséquences négatives d'un affaiblissement du réseau de gluten dans les véritables aliments à base de blé. L'enrichissement de la pâte avec de la RuBisCO modifie la cinétique de la protéolyse pendant la digestion *in vitro*. Cependant, la digestibilité globale des protéines *in vitro* reste inchangée. Ces résultats prometteurs confirment le potentiel de la RuBisCO à être utilisée pour enrichir les aliments à base de blé.