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Abstract

Among wheat storage proteins, omega-gliadins display a singular amino acid sequence only composed by repetitive sequences. They have been described as a major wheat allergen and also contain T-cell epitopes implicated in coeliac disease. To study the structural and biophysical properties of omega-gliadins and other gliadins (e.g., gluten network formation, allergic response), highly purified and concentrated fractions are needed. In the present work, we used chromatography media screening to improve their fractionation. A S Ceramic Hyper D (Pall) resin was selected for its ability to concentrate omega-gliadins in the first elution peaks. Gamma- and beta-gliadins were then separated by hydrophobic interaction chromatography and eluted with an ethanol gradient. Alpha-gliadins were purified by gel filtration. Each fraction was characterized by electrophoresis and reverse phase HPLC. The developed preparative protocol enabled the purification of several grams of highly purified gliadins to the detriment of the yield.

Keywords

Wheat protein fractionation, gluten proteins, omega-gliadins, cation exchange chromatography

1. INTRODUCTION

Wheat is overwhelmingly present in the diets of Western civilizations. Unfortunately, wheat-derived products are also responsible for adverse reactions such as coeliac disease and food allergies. The largest number of allergenic plant proteins is found among the prolamin super-family, which notably contains cereal storage proteins such as gliadins (Tatham and Shewry, 2008). Gliadins are monomeric proteins characterized by their solubility in alcohol-water media, with molecular weights (MW) ranging from 30 to 45 kDa (major fraction) and up to 75 kDa (minor fraction) and isolelectric points (pI) from 5 to 8 (Shewry, 2019). Gliadins consist of highly polymorphic polypeptides divided into four groups: alpha, beta, gamma, and omega, according to their decreasing electrophoretic mobility at low pH. Because of their molecular weight, their low sulfur content and their particular amino acid sequence based on only repetitive motives, omega-gliadin differ from other gliadins (Shewry, 2019). According to their N-terminal and repetitive sequences, to their elution order in reverse phase high performance liquid chromatography (RP-HPLC) and to their electrophoretic mobility, they are separated into 2 subgroups: omega 5-gliadin, also called fast omega-gliadins (eluted first in RP-HPLC), and omega 1,2-gliadins, slow omega-gliadins (eluted second in RP-HPLC) (Schalk et al., 2017; van Eckert et al., 2006). Omega 5 gliadins have been reported as a major allergen in Wheat-Dependent Exercise-Induced Anaphylaxis (WDEIA) (Morita et al., 2003), while omega 1,2 gliadins have been identified as one of the dominant allergens in allergic reactions to deamidated gluten (Denery-Papini et al., 2012). Gliadins are the main triggers of coeliac disease. In particular, T-cells from coeliac patients recognize epitopes containing the repetitive sequences present in omega-gliadins (Sollid et al., 2012). Yet, omega-gliadins are only a minor group among prolamins, amount-wise, and have been until recently less well characterized than other prolamin classes for their polymorphism and contribution to gluten properties (Altenbach et al., 2018).

The storage proteins of wheat grains have the unique property of forming a viscoelastic network called gluten, which is essential for the preparation of a wide diversity of wheat-based foods. Gliadins are essential in the formation of the gluten network in combination with glutenins: in dough, hydrated gliadins are commonly thought to contribute to the viscosity and extensibility of the network (Cornec et al., 1994; Shewry, 2019). Baking quality has been correlated positively with the contents in glutenin polymeric proteins and omega-gliadin (Malalgoda et al., 2018). The repeated sequences present in omega-gliadins and glutenins are believed to form inter-protein hydrogen bonds resulting in the particular viscoelastic properties of the gluten network (Belton, 1999). Besides, omega-gliadins were recently found to form supramolecular assemblies with glutenin through non-covalent interactions (Morel et al., 2020).

To study the structural, biophysical or immunochemical properties of omega-gliadins, highly purified and concentrated fractions are needed. Gliadins comprise several groups of proteins encoded by multigene families responsible for heterogeneity within each group. This complexity and the variable amount of each gliadin within wheat varieties makes their fractionation and study difficult. For example, the proportion of omega-gliadin may vary from 10 to 20 % of the total gliadins as recently reviewed by Shewry (Shewry, 2019). Their proportion is increased under increased nitrogen availability or sulfur deficiency in the cultivation conditions (Wan et al., 2014). Several methods have been described to obtain more or less purified gliadins but, generally, they yielded fractions comprising several components or only small quantities of individual proteins.

Recombinant proteins or peptides have been produced to study the role of the immune-dominant part of gliadins (Denery-Papini et al., 2000; Mameri et al., 2015; Matsuo et al., 2005; Tamás and Shewry, 2006). Such a strategy ensures the authenticity of the expressed proteins but masks the heterogeneity of the group and yields only low amounts of proteins. Moreover,

differences may be observed between purified and recombinant gliadin fractions in their secondary structure contents and antigenic activities (Mameri et al., 2015).

Classically, purification of gliadins starts with obtaining gluten by extensive washing of hydrated flour with water followed by successive extraction according to Osborne fractionation (Marsh et al., 2003; Schalk et al., 2017). These conditions of extraction separate gliadins from glutenins, even though gliadin fractions may still contain traces of glutenins (Shewry, 2019). Usually, omega-gliadins were fractionated with a combination of cation exchange chromatography at acidic pH (Charbonnier, 1974). All cation exchange chromatography resins are effective in separating omega-gliadins from other groups of gliadins (Charbonier and Mossé, 1980; Larre et al., 1991; Patey and Evans, 1973; Popineau et al., 1986). A low salt concentration makes it possible to selectively elute omega-gliadins. They are thus obtained in the first eluted fractions, but many authors noted that some of these omega-gliadins were eluted later, thus scattering the omega fraction and contaminating the other fractions (Charbonier and Mossé, 1980; Larré et al., 1991). The yield is often not specified in literature but is generally around 60 % for all gliadins groups corresponding to a few hundreds mg for omega-gliadins (Patey and Evans, 1973; Schalk et al., 2017). To produce more than one gram of pure omega-gliadins, the fractionation should run over several hours up to several days when large gliadin amounts are treated (Patey and Evans, 1973). A regeneration step in harsh conditions is necessary to eliminate proteins clogged onto the column to keep a good yield and reproducibility (Larre et al., 1991; Popineau et al., 1986).

This work aims to identify a cation exchange chromatography medium able to separate omega-gliadins from other gliadins, with a high yield. Different chromatography media were compared: strong (sulfoethyl, S and sulfopropyl, SP) and weak cation (carboxymethyl, CM) exchanger resins and the following parameters were explored: resolution, yield and duration. Several chemical features for the support matrix were tested to evaluate possible interactions

with the resin. A resin was selected and applied at a preparative scale to obtain highly purified fractions. This procedure helped us to purify omega-gliadins and all other gliadin groups for future physico-chemical investigations.

2. MATERIALS AND METHODS

2.1. Materials

Bread wheat grains (7 kg) were obtained from INRAE, Le Rheu, France (cv. Récital harvested in 2018) and were grounded into flour (5 kg) by Livrac (SA) (France). Protein content of wheat flour were 13.6 % on a dry basis.

2.2. Gliadins extraction

The flour was hydrated, kneaded into a dough and washed with water using a Martin-type process on a preparative scale (Popineau and Pineau, 1985). The recovered gluten was freeze-dried, ground with a Waring blender and defatted with dichloromethane during 2 hours at room temperature (gluten/solvent ratio (m/v) : 1/4). After filtration (Whatman filter paper n°3), two rinsing steps with the same gluten/solvent ratio were performed. Then, the solvent was evaporated under vacuum and defatted gluten was freeze-dried.

Total gliadins were extracted from dried gluten powder (514 g) with ethanol 70% (v/v) (gluten/solvent ratio (m/v): 1/6) during 3 hours with a propeller mixer at 850 rpm. The suspension was centrifuged at 10 000 g during for 30 min (Avanti J-26 XP, Beckman Coulter) to pellet glutenins not soluble in alcohol. The pellet was discarded and the supernatant was extensively dialyzed against water and then acetic acid 0.01 M and freeze-dried. It contained total gliadins and glutenins in a small amount.

2.3. Chromatography media screening

Different ion exchange chromatography resins were tested under fixed conditions: Toyopearl CM 650 M (Tosoh), Cellufine CM C500 (Amicon), CM Ceramic hyper D, S Ceramic Hyper D and SP Trisacryl M (Pall). Each resin (30 mL) was packed in XK 16/15 support following the manufacturer's instructions. Gliadins (1 g) were dispersed in 50 mL buffer (Na lactate 0.01 M pH 3.6, urea 2 M) overnight on a rotary shaker. The extract was centrifuged 30 min at 20 000

g (SR 12.22, Jouan Centrifuge, France) and 5 mL supernatant were injected onto the column at 1 mL/min. First, the column was equilibrated with the extraction buffer and washed with 60 mL (corresponding to twice the column volume). Proteins were eluted in a 420ml gradient from 0 to 30% of the same buffer pH 3.6 with NaCl 0.5 M using a total volume corresponding to 14 times the column volume. Protein absorbance was recorded at 280 nm. Fractions of 5 mL were collected and analyzed with polyacrylamide gel electrophoresis.

2.4. Preparative fractionation

Total gliadins (10 g) per run were dissolved in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M) and stirred for 2 hours at room temperature on a rotary shaker. They were then centrifuged for 30 min at 17 000 g (Beckam, Avanti JSP26, rotor JSP F500). The supernatant was injected on an S Ceramic Hyper D column (5 x 15 cm) using a preparative purification system (Pharmacia biotech, Sweden). The column was first equilibrated with buffer (Na lactate 0.01 M, pH 3.6, urea 2 M) and gliadins were eluted with a step gradient of NaCl 0.5 M. The absorbance of proteins was recorded at 280 nm. This chromatography step allowed for separating the different groups of gliadins. Omega-gliadins were eluted in the first peaks. They were dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

Eluted fractions containing alpha gliadins (2 g) were purified on a Sephacryl S100 HR column (5 x 100 cm) equilibrated in buffer (Na lactate 0.01 M, pH 3.6, urea 0.5 M). Different fractions were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

The other eluted fractions containing beta and gamma gliadins (10 g) were loaded on SP Sepharose FF (5 x 15cm) in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M). Proteins were eluted using a gradient from 0 to 25 % of the same buffer with NaCl 0.5 M. Different peaks were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried. This

chromatography step separated gamma- 44 and 46 gliadins forms, and removed traces of alpha/beta gliadins.

Gamma gliadins were last purified on a phenyl sepharose FF column (5 x 15cm). The column was equilibrated in buffer (Na lactate 0.01 M, pH 3.6, urea 2 M), then the sample (1.5 g) dissolved in the same buffer was injected. The column was washed with buffer (ammonia 0.02 M pH 11), and proteins were eluted in a gradient from 0 to 100 % of the same buffer with ethanol 70 % (v/v). Different fractions were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

Beta gliadins were purified from the flowthrough peak of phenyl sepharose step. Different fractions were pooled, dialyzed first in water, then in acetic acid 0.01 M and freeze-dried.

2.5. Analytical characterization

2.5.1. Determination of water, ash, and protein contents

Prior to analysis, protein powders were stored at 20 °C in a desiccator containing K_2CO_3 saturated salt to ensure constant moisture content. The water content was measured on equilibrated powders using thermogravimetric analysis under nitrogen atmosphere (TGA 2050, TA-Instrument). About 10 mg of powder were heated from 20 to 105 °C at 3 K/min and at 105 °C for at least 80 min until constant weight was achieved.

Protein content was determined according to the Dumas method with an elemental analyzer for nitrogen (Vario Micro Cube, Elementar, Frankfurt, Germany) with nitrogen-to-protein conversion factor of 5.4 as recommended for wheat proteins (Mariotti et al., 2008).

2.5.2. Polyacrylamide gel electrophoresis at acid pH (A-PAGE)

Gliadins were separated onto acid polyacrylamide gel according to the method described by Morel (1994). Briefly protein solutions were loaded onto a 12 % polyacrylamide gel composed

of urea 2 M, ascorbic acid 0.1 %, ferrous sulfate 7 H_2O 0.0014% (w/v) and acetic acid 1 N 13 %, pH 3.1 polymerized by 60 µL H_2O_2 0.6 % (v/v). Migration in acetic acid buffer pH 3.1 was run during 1 h 40 under 250 V. Then gels were fixed in trichloroacetic acid 10 % and stained with Coomassie Brilliant blue R250 0.4 % (w/v).

2.5.3. Polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions. Proteins were dissolved at 1 mg/mL in a pH 6.8-buffer constituted of Tris 0.5 M, SDS 10%, glycerol 30%, β-mercaptoethanol 8% and bromophenol blue buffer. Samples were incubated at room temperature overnight and heated at 95 °C for 5 min before being loaded onto 4–12% Bis–Tris precast gels Novex Nupage™ (Invitrogen, Thermo Fisher Scientific) with MES SDS running buffer (Invitrogen, Thermo Fisher Scientific) and Recombinant Precision Plus unstained protein standards (Biorad). Electrophoresis was performed at constant voltage (150 V) and the gels were stained with a colloidal Coomassie blue solution. Gels were washed with water and scanned.

2.5.4. RP-HPLC

Purity of gliadin fractions was probed by RP-HPLC (Bietz and Simpson, 1992). Briefly, 120 µg of proteins were dissolved in acetonitrile 25 % TFA 0.1 % and injected onto a C18 analytical column (Nucleosil C18, 4 x 250 mm, 5µm, 300 Å). They were separated at 50 °C using a gradient running from acetonitrile 25 % TFA 0.1 % to acetonitrile 55 % TFA 0.1 % during 70 min at 0.8 mL/min. Monitoring the protein was carried out using the absorbance at 214 nm.

3. RESULTS AND DISCUSSION

3.1. Total gliadins extraction

First, 514 g of gluten were obtained from the wheat dough prepared from 5 kg of flour extensively washed with water to remove starch and water-soluble constituants. This process concentrated proteins: the resulting dry gluten contained 73 % proteins of the flour and corresponds to 10.2 % of the flour and 79% of the flour proteins. This quantitative value was in good agreement with the isolation protocol from Popineau et al. (1985) wich yielded routinely 10.8 % of gluten from flour. Then, gliadins were separated from glutenins following Osborne fractionation by solubilisation in 70% v/v ethanol. 84 g of total gliadins from 5 kg of flour were produced with 86 % protein content. The extracted gliadins represent 1.7 % of the initial flour. In comparison with the yield of 2.8% for the preparation of Prolamin Working Group (PWG) gliadin reference (van Eckert et al., 2006), a large amount of gliadins may be lost in the pellet during alcohol extraction or the initial gliadin content of our flour was lower. The total gliadins (TG) extract was composed by the four types of gliadins : alpha-, beta-, gamma-, omega-gliadins and small amount of glutenins as revealed by electrophoresis at low pH (Figure 1 A, B, C, D bottom, lines TG).

3.2. Chromatography media screening

The different groups of gliadins were fractionated by cation exchange chromatography according to their charge at acidic pH (pH 3.6) under denaturing conditions (urea 2 M). Five different cation exchanger chromatography resins varying in their electrostatic and chemical characteristics were tested under the same conditions to separate the different groups of wheat gliadins. The fractionation of omega-gliadin and the loading capacity were compared for each resin.

Peak area is used to evaluate the protein amount bound to the resin. The largest area under the peaks eluted between between 0 and 600 min was obtained with with Toyopearl CM 650 M, S Ceramic Hyper D and Cellufine C-500 resin, respectively 36, 34 and 34 AU*min. CM Ceramic

Hyper D and SP Trisacryl M had the smallest peak area (respectively 27 and 30 AU*min), indicating that they bind fewer proteins than the other resins. Moreover, CM Ceramic Hyper D did not lead to any gliadin separation (Figure 1A). All gliadins were eluted in the first unbound peak during the initial washing step and few proteins were eluted by the NaCl gradient. This resin was therefore eliminated. SP Trisacryl M (Figure S1) having the smallest peaks area under curve was not retained.

Omega-gliadins were eluted in the first peaks of the chromatograms. They were either unbound or eluted at the very beginning of the gradient, which is in line with other studies (Patey and Evans, 1973; Popineau et al., 1986). Then, the salt gradient induced the joint elution of gamma and beta-gliadins, followed by alpha-gliadins (Figure 1B, C, D). Only the resin S Ceramic Hyper D (Figure 1B, fractions 2 - 40) allowed for a clear split of the omega gliadins from other gliadins, all of them were eluted before 7 mS/cm conductivity in three separated peaks. The well-resolved peaks containing omega-gliadins for Cellufine C500 (Figure 1C fractions 4 - 6) and for Toyopearl CM 650 M (Figure 1D fractions 4 - 6) were smaller than for S Ceramic Hyper D. Furthermore, it can be noticed that omega-gliadins were not separated (Figure 1C, fractions 25 - 29) and partly co-eluted with gamma gliadins with Cellufine C500 (Figure 1C, fractions 33 - 35). The same observation applies to Toyopearl CM 650 M (Figure 1D, fractions 29 - 35). Since S Ceramic Hyper D (Figure 1 B) had a good loading capacity (34 AU*min) and concentrated omega-gliadins in the first peaks of elution, this resin was selected.

Omega-gliadins are mainly composed of glutamine (40 - 50 % mol) and proline (20 - 30% mol) (Shewry, 2019). Therefore, they have few charges on their surface, and their binding capacity to cation exchanger resin is expected to be low. S Ceramic Hyper D has particular properties: it is a hybrid resin called “gel-in-a-shell”. The functionalized hydrogel is entrapped in a rigid ceramic bead (Boschetti, 1994). Large pores at the surface of the beads allow proteins to diffuse rapidly and bind to the hydrogel throughout its volume, not just on the surface (Rendueles De

La Vega et al., 1998). The pore size of S Ceramic Hyper D is the largest among the tested resins (Table 1). Its binding sites would be more accessible than in the other resins, thus improving the sorption capacity. Moreover, omega-gliadins could have a compact structure even in 8 M urea while the other gliadins are unfolded in 2 M urea (Paananen et al., 2006), it could be speculated that omega-gliadins could diffuse faster into the ceramic bead. Altogether, this could explain the better performance of S Ceramic Hyper D resin.

3.3. Preparative fractionation

Following the analytical screening, the S Ceramic Hyper D column was retained for the first step of gliadin fractionation to reach a good separation of omega-gliadins. The process scaled-up involved the same extraction and purification buffers as at analytical scale. The general purification process is presented in Figure 2 A. S Ceramic Hyper D could fractionate roughly the four gliadins groups, and concentrate omega-gliadins in the first peaks of elution with the same efficiency as in the optimization step. At preparative scale, a stepwise elution of increasing NaCl concentration was preferred to speed up the separation time and reduce buffer consumption while retaining the required purity level. An extension of the washing step at 5% of elution buffer corresponding to 25 mM NaCl was necessary to avoid contamination of gliadin peaks by omega-gliadins. Two fractions of omega-gliadins were produced according to the salt elution gradient. They contained both a mixture of omega 5- and omega 1,2-gliadins. The fraction of omega-gliadins eluted with 25 mM NaCl was further called “Omega NaCl25” and the fraction eluted with 50 mM NaCl “Omega NaCl50”. Then gamma and beta gliadins were eluted with 100 mM NaCl and finally alpha-gliadins and traces of glutenins with 500 mM NaCl (Figure 2 B). The preparative fractionation allowed to produce very concentrated protein fractions with a protein content greater then 92% for each gliadin fraction (Table 2).

286 Eighty four grams of total gliadins were applied to the column in nine runs. A wash with NaOH
287 1 M was necessary after five runs to avoid loss of capacity. Omega-gliadin recovery including
288 chromatography, dialysis and freeze-drying was 1.5 g of Omega NaCl25 and 3 g of Omega
289 NaCl50 (Table 2). This recovery was in accordance with large scale fractionation of gliadins
290 which produced from 4 g to 6 g of omega-gliadins from a greater amount of total gliadins (Patey
291 and Evans, 1973; Popineau et al., 1986). It was noticed that omega gliadin powders were highly
292 sensitive to water content. A change in relative humidity induced a solid-to-liquid transition at
293 ambient temperature in a few hours after removal from the freeze dryer. To avoid such a
294 transition, powders were stored in a desiccator containing K_2CO_3 saturated salt to avoid an
295 increase of moisture content during storage. Such a transition was not observed with other
296 gliadin powders.
297 The next separation step was another cation exchanger chromatography (Figure 2B). A SP
298 sepharose FF resin had previously been shown to fractionate different isoforms of gamma
299 gliadins according to charge (Popineau and Pineau, 1985). The fraction obtained from the S
300 Ceramic Hyper D column, containing gamma and beta gliadins, was injected onto SP FF with
301 the same buffers as the first step in four runs. A NaCl gradient from 25 mM to 125 mM was
302 used to separate gamma 46 and gamma 44 (Figure 2B). These isoforms could be detected in A-
303 PAGE and correspond to the two major bands at the level of gamma-gliadin migration in the
304 total gliadin lane (Figure 1B). They were not well separated with S Ceramic Hyper D column
305 because of step elution. Gamma and beta gliadins were separated by hydrophobic interaction
306 chromatography (HIC) with a Phenyl Sepharose FF column and eluted with an ethanol gradient
307 (Popineau and Pineau, 1985). Briefly, the whole gamma fraction solubilized in urea lactate
308 buffer was injected on the Phenyl sepharose FF column, then was eluted by a linear gradient of
309 ethanol in ammonia buffer pH 11. Beta-gliadins were eluted by 10 % (v/v) ethanol and gamma
310 46 and 44 were eluted respectively with 39% and 46% (v/v) ethanol (Figure 2B). They were

311 separated according to their surface hydrophobicity. A similar elution pattern was observed in
312 literature, confirming that separation by HIC is highly reproducible (Popineau and Pineau,
313 1985). The gamma gliadin recovery was 1.6 g of gamma 46, 0.6 g of gamma 44 and 0.2 g of
314 gamma 44 and gamma 46 mixture (Table 2). Three grams of beta-gliadin were purified (Table
315 2). The overall recovery for gamma and beta was 3 and 4 % repectively. Such very low
316 recovery, which are encountered when targeting fractions with high purity (Figure 3 - 4) were
317 due to the numerous steps and a technical problem with one batch at the freeze-drying step
318 which led to losses. From batch to batch, the mean recovery for HIC was around 65% which
319 gave a final yield of 7 %.
320 Alpha-gliadins were last purified by gel filtration. They represent the major protein in total
321 gliadins. Whole alpha gliadins were collected in the first chromatographic step and
322 corresponded to the last peak eluted with 500 mM NaCl. This fraction contained traces of
323 glutenins eliminated by size exclusion chromatography (Charbonier and Mossé, 1980; Patey
324 and Evans, 1973). Glutenins are composed of sub-units stabilized by disulphide bonds. Their
325 apparent molecular weight is much larger than that of gliadins in non-reducing conditions
326 (Shewry, 2019). The sephacryl S100-HR column had a separation range adapted to the size of
327 the targeted proteins, and was equilibrated with the same buffers as in the first step. Glutenins
328 were excluded from the gel (27 % peak area at 280 nm) and alpha-gliadins were eluted at elution
329 volume of 1000 mL in the main peak (62% peak area at 280 nm) (Figure 2B). More than 4 g of
330 alpha gliadin were purified (Table 2) with a final recovery of 11 %. Only 11.8 g over 22 g of
331 fraction was purified by Sephacryl S100-HR. This purification step had a 40 % yield. If the
332 entire fraction containing alpha-gliadins had been processed, 9 g of alpha could have been
333 purified in total.
334 The overall procedure was rather long but highly purified fractions (over 85% for each gliadin)
335 were recovered in the gram-range. Due to the purification step series, the final recovery was

relatively low (< 25%). Protein losses may also be due to protein bonding onto the columns. Indeed, regular cleaning of the columns with sodium hydroxide was necessary to keep reproducible results.

3.4. Analysis of the purified fractions

The purity of each gliadin fraction was evaluated by electrophoresis and analytical reversed phase HPLC. In SDS-PAGE, proteins are only separated according to their size, as negatively charged SDS masks the protein charges. The characteristic bands for each gliadin are expected at the corresponding MW ranges of 60 000-80 000 Da for omega-gliadins, and 32 000 to 45 000 Da for alpha, beta and gamma-gliadins (Shewry, 2019). In the present work, purified gliadins showed a typical electrophoretic profile with only one band for highly pure proteins. Omega-gliadins showed one band at 70 kDa in SDS-PAGE (Figure 3A). Omega-gliadins showed a weak Coomassie staining, probably due to the repetitive pattern of their sequence and lack of basic amino acids as reviewed by Shewry (Shewry, 2019). Beta-gliadins (Figure 3A) and Gamma (Figure 3A) migrated around 40 kDa. Alpha-gliadins (Figure 3A) had an intense band at 37 kDa with minor bands at lower MW. These bands, around 10 – 15 kDa, could correspond to alpha-amylase/trypsin inhibitors (ATIs) (Shewry, 2019).

Gliadins could be subclassified as omega, gamma, beta and alpha based on increasing mobility in electrophoresis at acidic pH (Shewry, 2019). In A-PAGE, proteins are separated according to their molecular weight and their apparent charge at pH below 3 (Bietz and Simpson, 1992). Migration profiles showed characteristic bands for each gliadin according to the expected profiles, with only traces of contaminants (Figure 3B) (Bietz and Simpson, 1992). Omega NaCl25 fraction showed two bands with low electrophoretic mobility in A-PAGE corresponding to omega1,2-gliadins, whereas Omega NaCl50 fraction presented three bands corresponding to a mixture of omega1,2 and omega5-gliadins (Figure 3B). Gamma-gliadins

(Figure 3B) had a similar profile. They migrated to the middle of the gel with an intense band and another weak band just above, meaning that these fractions had homogeneous charges. The beta-gliadin fraction (Figure 3B) migrated between gamma- and alpha-gliadins. Alpha-gliadins (Figure 3B) migrated to the migration front with several bands. It could be different isoforms of proteins (Mameri et al., 2015).

Analytical C18 columns have also been widely used to characterize gluten proteins since 1980's (Bietz and Simpson, 1992). Proteins are separated by surface hydrophobicity and are eluted with an acetonitrile gradient. Figure 4 presents the elution pattern of purified gliadins on C18 column. Each fraction was eluted in a single major peak which highlights a high protein purity rarely obtained because of gliadin polymorphism. Omega5-gliadins were eluted between 30 min and 35 min, omega1,2-gliadins around 38 min (Figure 4). Both omega-gliadin fractions are composed of a mixture of omega5 and omega1,2. Based on the respective peak areas, Omega NaCl25 fraction contained 71% of omega1,2 and 29% of omega5-gliadin, while Omega NaCl50 fraction contained 69% of omega1,2 and 31% of omega5-gliadin . When both types of omega 1,2 and omega 5 need to be separated from the omega-gliadin fraction, preparative RP-HPLC is the most appropriate technique. Beta gliadins were eluted around 42 min (Figure 4) in a major peak, which confirms the purity (88 % of total peak area). Only a slight peak was visible at 45 min, which corresponds to gamma gliadins and represents 7 % of the fraction. Alpha-gliadins were eluted between 44 min and 49 min (Figure 4). Two main peaks were eluted at 46 min and 47.5 min (76% peak area) meaning that alpha-gliadins do not have a homogeneous composition in terms of polarity. Gamma44-gliadins (Figure 4) were eluted at 50 min (93 % peak area). Gamma46-gliadins (Figure 4) were eluted at 51 min (92 % peak area) and a more polar fraction is eluted at 59 min (8 % peak area). These gamma-gliadin fractions are highly pure.

4. CONCLUSION

386 The developed preparative protocol enabled the purification of several grams of highly purified
387 gliadins. Analytical techniques confirmed that each gliadin fraction was purified near
388 electrophoretic and HPLC homogeneity. Gliadins with high purity at the level of grams are
389 rarely obtained because of the considerable polymorphism of gliadins. We choose a S Ceramic
390 Hyper D (Pall) column for its ability to concentrate omega-gliadins in the first elution peaks
391 and to separate different groups of gliadins. Its specific chemistry “gel in a-shell” could explain
392 the improved resolution of this resin compared to the other tested. The mass transfer mechanism
393 in Ceramic hyper D resin is described as intraparticle convection rather than diffusion which
394 allows larger proteins as omega gliadins to enter the pores of the gel.
395

ABBREVIATIONS

A-PAGE, acidic polyacrylamide gel electrophoresis

CM, carboxy methyl

CV coefficient variation

FF fast flow

HIC hydrophobic interaction chromatography

HMW high molecular weight

HPLC, high performance liquid chromatography,

RP, reversed phase,

RP-HPLC reversed phase high performance liquid chromatography

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SP sulfo propyl

TFA, trifluoroacetic acid,

TG total gliadin

TGA thermogravimetric analysis

UV ultraviolet

DECLARATION OF COMPETING INTEREST

The authors declare to have no conflicts of interest.

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AUTHORS CONTRIBUTIONS

Véronique Solé-Jamault: conceptualization, formal analysis, original draft, review & editing; **Joëlle Davy:** investigation; **Rémy Cochereau:** investigation review & editing; **Adeline Boire:** supervision, funding acquisition review & editing; **Colette Larré**: supervision, review & editing; **Sandra Denery-Papini:** supervision, review & editing

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522
523

524 **Table 1 List of tested cation exchanger chromatography resins**

Functional group	Commercial name and manufacturer	Particle size µm	Pore size Å	Resin chemistry
Carboxy methyl (CM)	Toyopearl CM 650 M, Tosoh	65	1000	hydroxylated methacrylic polymer
	Cellufine C-500, Chisso	53-125	/	cross-linked cellulose
	CM Ceramic Hyper D, Pall	50	2000	ceramic, gel-in-a-shell
Sulfo ethyl (S)	S Ceramic Hyper D, Pall	50	2000	ceramic, gel-in-a-shell
SulfoPropyl (SP)	SP Trisacryl M, Pall	40-80	/	acrylic copolymer

525

526 **Table 2 Recoveries of purified gliadins at the end of the optimized process.**

Gliadins fraction	Protein recovered weight (g)	Protein content % (dry mass)	Chromatography steps number	Final yield (%) From 84 g of total gliadins treated
Omega NaCl25	1.5	92.3 ± 1.3	1	2
Omega NaCl50	3.0	94.1 ± 1.8	1	4
Gamma	2.4	93.4 ± 1.3	3	3
Beta	3.0	96.6 ± 1.0	3	4
Alpha	4.7 *	96.7 ± 1.1	2	11

527 *. If the entire fraction containing alpha-gliadins had been processed, 9 g of alpha could have
528 been purified in total

LIST OF FIGURE CAPTIONS

Figure 1 Gliadins fractionation on (A) CM Ceramic Hyper D, (B) S Ceramic Hyper D (C) Cellufine C 500 and (D) Toyopearl CM 650 M with associated A-PAGE analysis (bottom). Numbers above the peaks correspond to numbered lanes in A-PAGE. TG stands for total gliadins. Fractions signalized by an arrow on the chromatogram (≈10 µg) were analyzed with A-PAGE stained by Coomassie Brilliant blue. Chromatogram of SP Trisacryl M is presented in supplementary S1.

Figure 2 : Purification process and associated representative chromatograms

A. Chromatography series are represented with operating conditions and the subsequent purified fractions numbered in the elution order (SCHD for S Ceramic HyperD fractions, SP for Sepharose FF fractions, Phen for Phenyl Sepharode FF fractions and S100 for Sephacryl S100 fractions). B. Representative chromatograms are presented. The absorbance of proteins is recorded at 280 nm (black line). Proteins are eluted in a elution of buffer B (concentration B %, grey line) and real gradient is measured by conductivity (grey dashed line). Please note the differences in the range of the UV axes between chromatograms. The peaks of interest are colored in blue for omega-gliadin, green for gamma-gliadin, yellow for alpha-gliadin and red for beta gliadin. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Figure 3 SDS-PAGE and A-PAGE of purified gliadins fractions.

Each fraction (10 µg) is analyzed by electrophoresis and purity is check by Coomassie Brilliant blue staining: (M) indicates molecular weight marker, (TG) total gliadins, (1) omega NaCl25 fraction, (2) omega NaCl50 fraction, (3) gamma46-gliadins, (4) gamma44-gliadins, (5) beta-gliadins, (6) alpha-gliadins. Two images of gels are combined to present all of the fractions in a single figure.

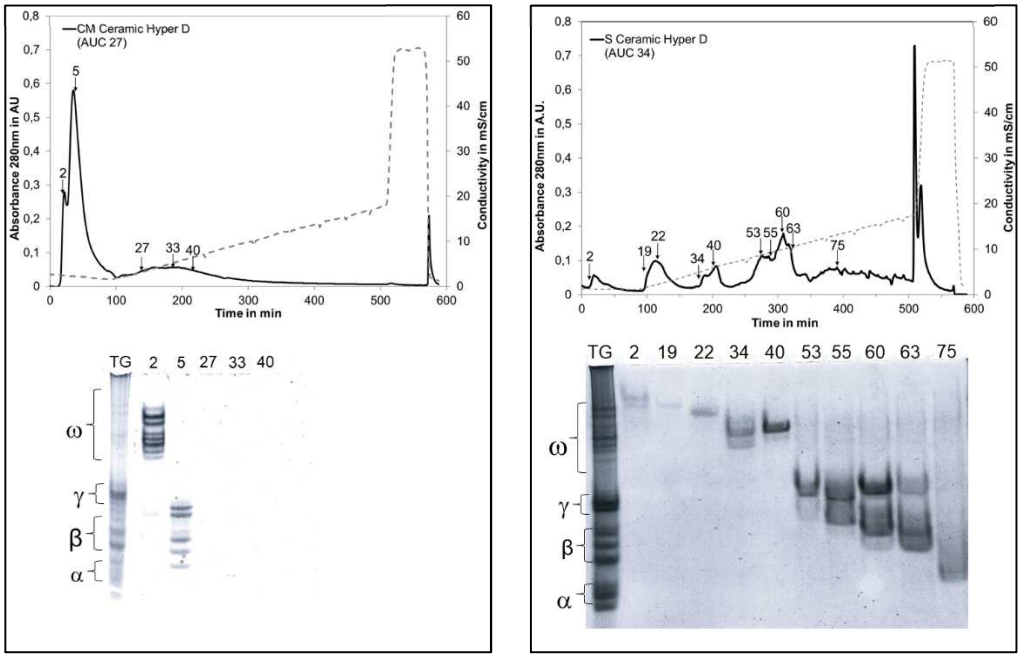
Figure 4 RP-HPLC chromatograms of purified gliadins fractions.

Each fraction (120 µg) is injected on analytical C18 column. Proteins are eluted in a gradient from 25 to 55% acetonitrile-TFA 0.1% in 58 min at 0.8 mL/min. The absorbance of proteins recorded at 214 nm is represented in light blue for omega NaCl25, dark blue for omega NaCl50, red for beta-gliadins, yellow for alpha-gliadins, light green for gamma44-gliadins and dark green for gamma46-gliadins. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

FIGURE 1

A. CM Ceramic Hyper D

B. S Ceramic Hyper D



C. Cellufine C 500

D. Toyopearl CM 650

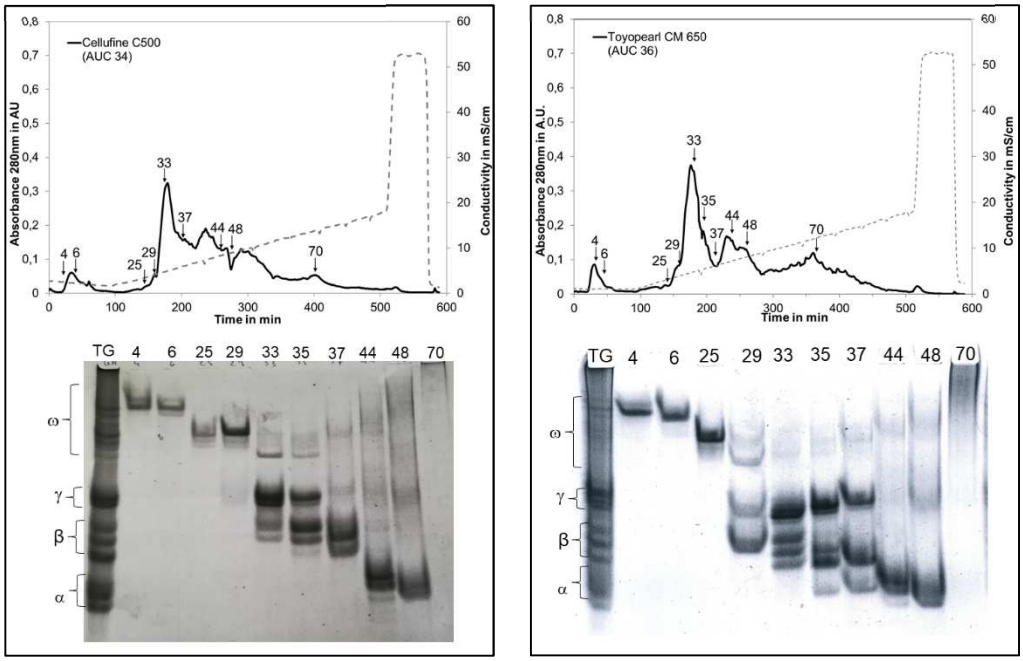


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FIGURE 2

A. B.

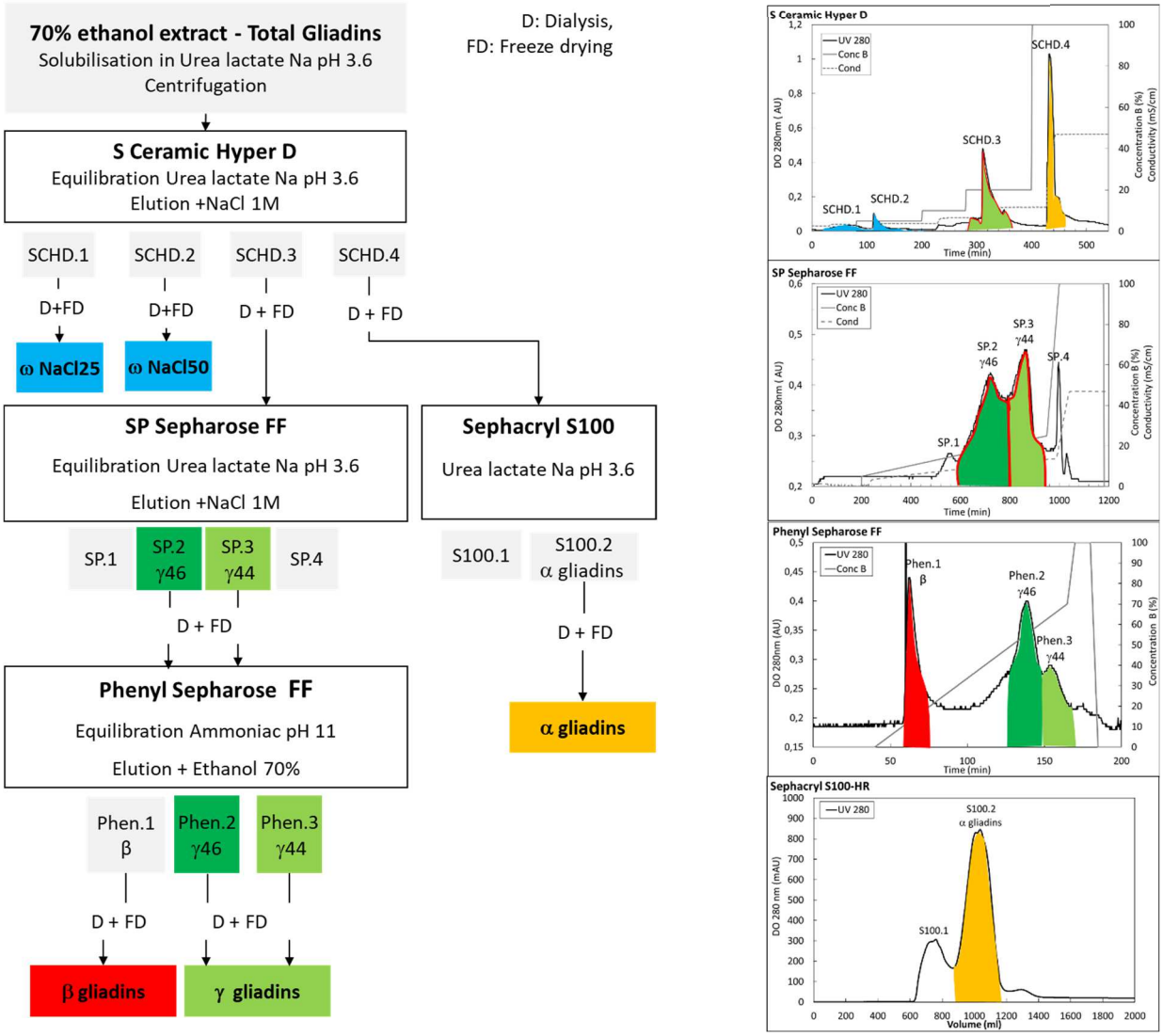

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FIGURE 3

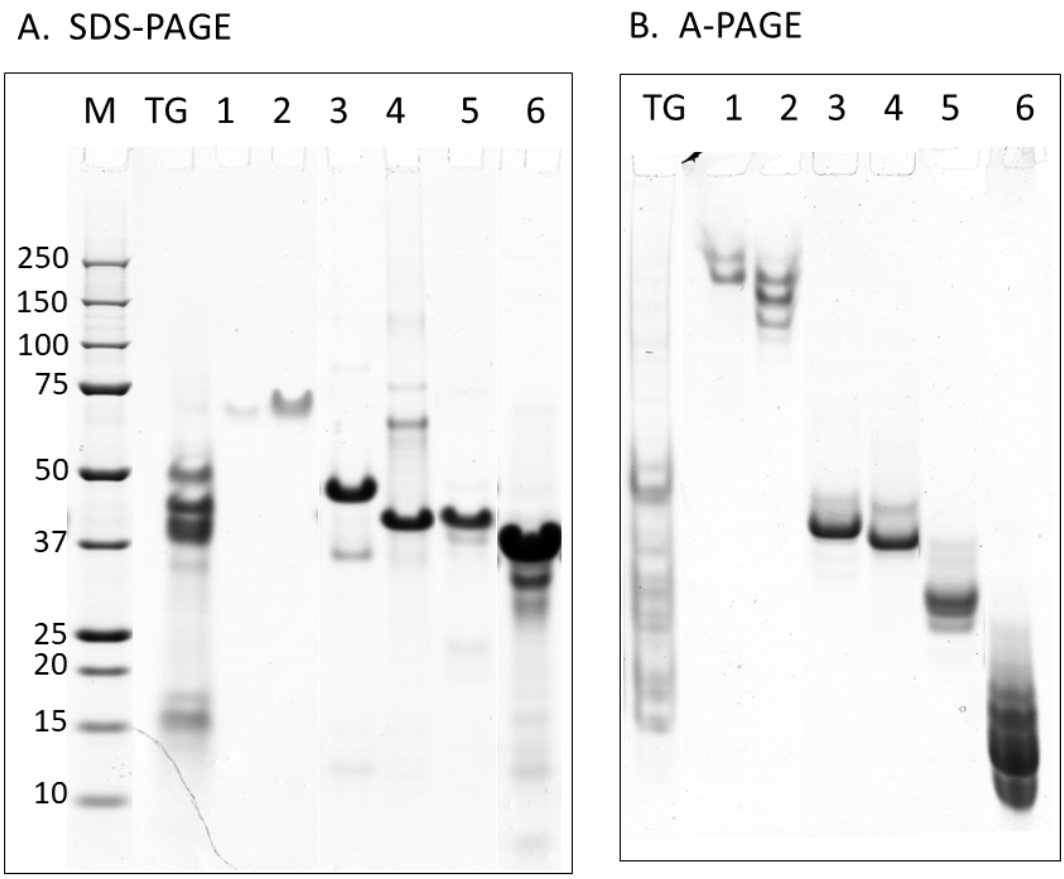

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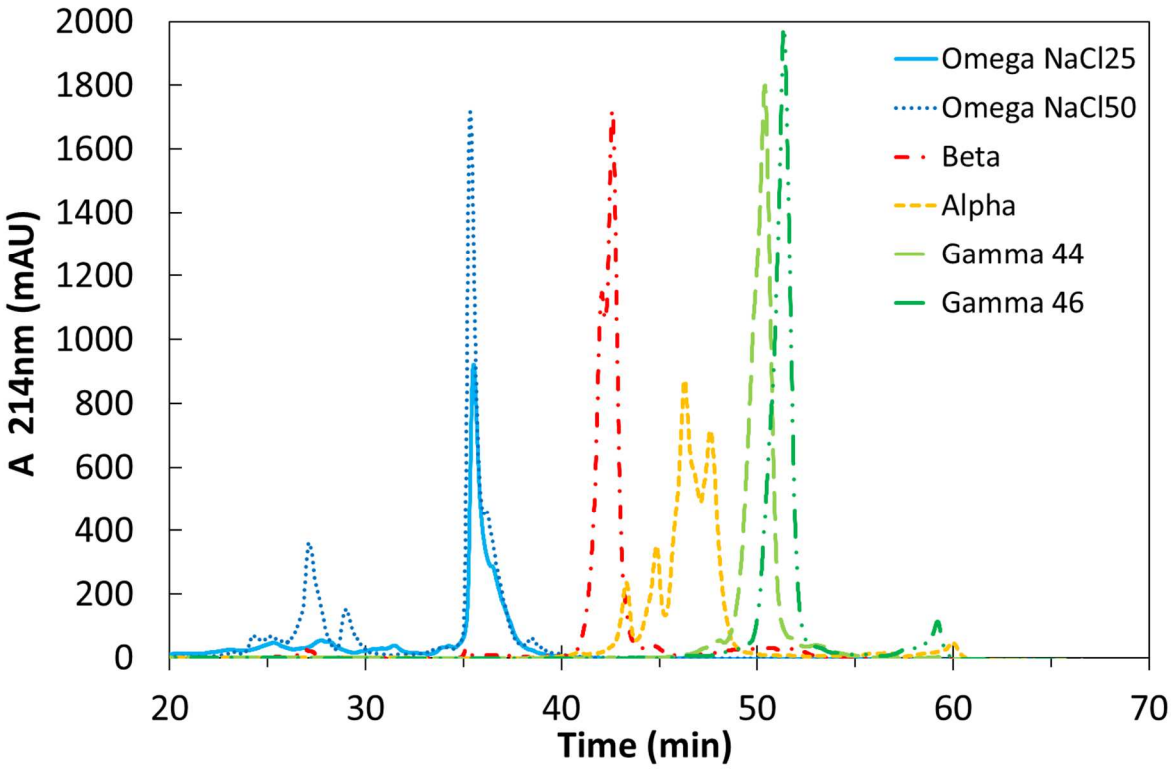
FIGURE 4



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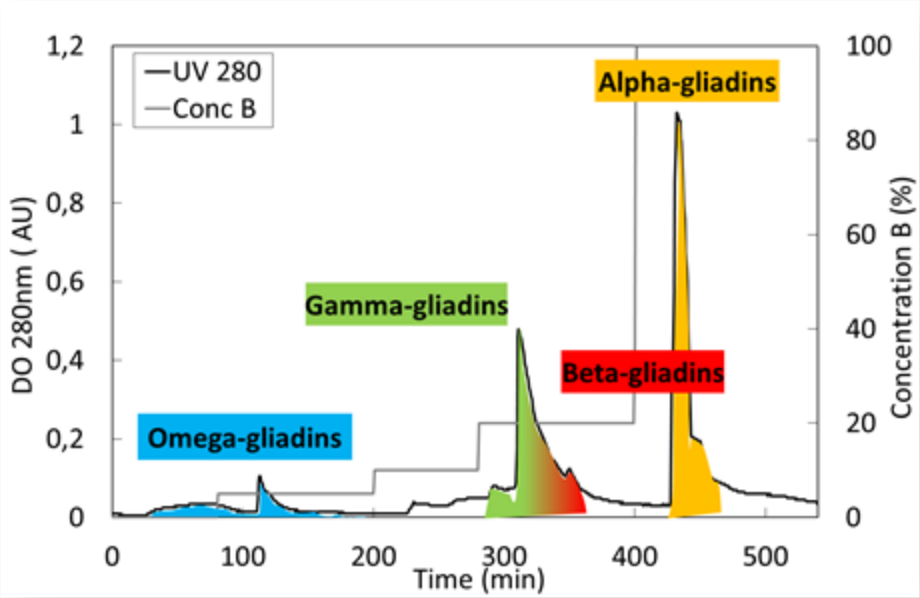
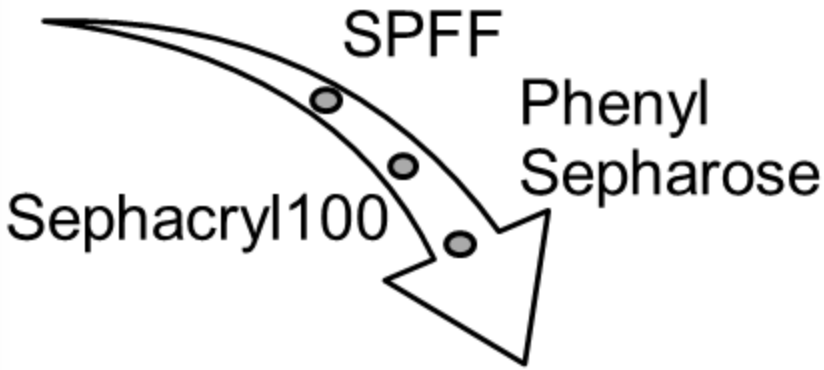
Optimized separation of gliadins on S Ceramic Hyper D - Pall





Final Purification

Gliadins fraction	Chromatography steps number	Final recovery g
Omega	1	4.5
Gamma	3	2.4
Beta	3	3
Alpha	2	9*