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Ultrafiltration to fractionate wheat polypeptides

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Abstract

An ultrafiltration process allowing the fractionation of two kinds of polypeptides issued from limited chymotryptic hydrolysis of wheat gliadins was applied to wheat gluten hydrolysates. Hydrophilic and poorly charged polypeptides were well transmitted through an inorganic ZrO_2-based membrane at acidic pH, whereas hydrophobic and positively charged polypeptides were highly retained. By combining reversed-phase and cation-exchange chromatography (CEC), it was proved that the fractionation of the polypeptides was based on electrostatic repulsion of the charged polypeptides by the positively charged membrane. After a continuous diafiltration process, retentates containing 75 to 88% of hydrophobic polypeptide and permeates containing 84 to 90% of hydrophilic polypeptides were recovered, depending on the size of membrane used. Even if the ultrafiltration fractions were less purified than fractions issued from CEC, it was shown that they exhibited very different foaming properties: permeate did not produce nor stabilize foams, whereas retentate was more efficient than the whole hydrolysates and BSA.

Keywords: Ultrafiltration; Wheat; Gluten; Polypeptides

1. Introduction

Wheat gluten uses can be largely diversified through modification of its major proteins: gliadins and glutenins. Limited chymotryptic hydrolysis is shown to enhance gluten solubility over a large range of pHs. When applied to gliadins, this hydrolysis generates essentially two types of polypeptides, corresponding to the repetitive (glutamine and proline rich) and the non-repetitive sequence domains of the proteins, respectively [1]. Only the latter ones adsorb at the polar/apolar interface and stabilize the emulsions, due to their amphiphilic behaviour [2]. This difference makes valuable the fractionation of hydrolysates.

This fractionation has been made first by ultrafiltration with ZrO_2-made inorganic membranes [3], although polypeptides have similar molecular masses, near 17000 g/mol. In fact, repetitive polypeptides, more hydrophilic than the non-repetitive polypeptides, are also poorly positively charged in the range pH 3 to pH 8; conversely, the non-repetitive polypeptides are more positively charged at acidic pH, charges being determined by the use of a prediction software (CHARGPRO in PC-GENE™). The possibility in ultrafiltration process to exploit electrostatic interactions occurring between fouled membranes and solutes to retain them has been demonstrated [3–9]: the fouled membrane becomes similarly-charged as the fouling component (protein) whatever the initial membrane charge before fouling [7]. In a 80:20 (v:v) ethanol–water mixture, repeti-

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tive polypeptides are more transmitted through inorganic membranes than the non-repetitive ones. The best separation condition is obtained when the non-repetitive polypeptides are co-ion of the membranes, at pH 3 and the ionic strength minimum [3].

Here, fractions of gliadin hydrolysates obtained by ultrafiltration in the absence of ethanol were characterised by cation-exchange chromatography, in order to better understand the behaviour of each kind of polypeptides. Then the process was applied to hydrolysates of whole gluten. In fact, glutenins are composed of subunits linked by intermolecular SS bonds. These subunits resemble to the gliadins except for the proportion of repetitive and non-repetitive domains and the extent of repetitive domain which can reach more than 500 amino acids in some of them [10].

2. Experimental

2.1. Materials

Partially defatted gluten was extracted from wheat flour (variety Soissons), with a non-ionic detergent, Triton X 114 [11].

Gliadins were prepared by extraction from gluten powder with a 70% (v/v) ethanol–water mixture. Bovine serum albumin (BSA) was from Sigma.

2.2. Enzymatic hydrolysis

Gliadins (15 g) were mixed with 0.1 *M* acetic acid solution (1.5 L). Gluten (15 g) was dispersed 10 min with deionised water (27.9 g) containing cysteine (5 mg/g of gluten) to break intermolecular S–S bonds of glutenins, in a ''Minor pin'' mixer (Henry Simon). It was then diluted with 1.5 L of 0.1 *M* acetic acid.

Hydrolysis of 10 g/l gliadins or reduced gluten was performed with Protease mixture 2500 S (Novo Nordisk, with tryptic and chymotryptic activities) at pH 5 and 20°C with an enzyme/substrate ratio of 1/150 (w/w). Reaction was stopped after 24 h by heating the suspension at 80°C during 5 min. Suspension was adjusted at pH 3 by 1 *M* HCl then centrifuged at 12 000×*g* for 20 min at 20°C. Consequently, the medium was composed of 0.065 *M* NaCl and 0.1 *M* acetic acid. Supernatant was collected and submitted to ultrafiltration. The degree of hydrolysis (DH) was determined by the *o*-phthaldialdehyde (OPA) method.

2.3. Ultrafiltration process

The ultrafiltration rig comprised essentially a thermostatted 2 L feed tank, a volumetric feed pump, a single tubular membrane (length 0.60 m, inner diameter 6 mm, membrane area $A=1.09\ 10^{-2}\ m^2$) and gauges to control process course [3]. The rig was real time monitored with signal transducers (6B, Analog Devices, Norwood, MA, USA) and software (Labtech Notebook, Laboratory Technologies Corp., Wilmington, MA, USA). The rig volume was measured as 0.35 L.

Inorganic membranes Carbosep (Orelis, Miribel, France) made of porous carbon with a thin layer of ZrO_2 were used: M4 and M1 (molecular mass cut-off *MWCO* 50 and 150 kg/mol, water permeance 49 and 52 L $h^{-1}\ m^{-2}\ bar^{-1}$ at 20°C, water hydraulic resistance *Rm* 8.6 and 7.8 $10^{12}\ m^{-1}$ at 20°C, pore radius 6.2 and 10.3 nm, respectively). These membranes were positively charged at pH 3 [3].

The hydrolysates (0.5 L) were diafiltered at constant volume by up to 2 L of 0.067 *M* acetic acid solution at pH 3 (transmembrane pressure 3 bars, 20°C, tangential velocity 4 m/s). Total permeate and retentate were then lyophilised.

Ultrafiltration performances were measured using two variables: the transmission T_A of a solute A, and a selectivity factor *Sf*.

T_A reveals the part of a solute ''A'' crossing the membrane:

$$T_A = Cp_A / Cr_A$$

where the concentrations of the solute are *Cp* in the permeate and *Cr* in the retentate.

The precision of measurements was estimated at 4% for *T* [3].

Sf is the ratio of the transmission T_A of the solute A (which is expected to cross the membrane) to the transmission T_B of the solute B (which is expected to be retained by the membrane):

$$Sf = T_A / T_B$$

Sf values must be carefully discussed, taking into account the accuracy of *T* values.

In continuous diafiltration mode, concentrations of retentate and permeate can be deduced from initial concentration *Co* from the mass balance, as:

$$Cr = Co.\exp(-T.V^*) \quad \text{and} \quad Cp = (Co-Cr)/V^*$$

with V^* (diafiltration volume) is equal to the ratio of the volume of solvent added, at volume constant, to the volume of feed to separate [3,12].

2.4. Biochemical characterization of hydrolysates and their fractions

Size exclusion chromatography (SEC) of polypeptides was carried out on Hiload Superdex 75 16/60 column (Pharmacia) equilibrated with 0.0125 *M* borate buffer, 0.1% SDS, pH 8.5.

Gliadin and reduced gluten hydrolysates were analysed by cation-exchange chromatography (CEC): lyophilised products were diluted in buffer A (0.01 *M* sodium lactate containing 2 *M* urea adjusted at pH 3.6 by addition of lactic acid) to make 10 mg/ml solutions. Solution (1 ml) was applied on a mono S column (Pharmacia, 1 ml) equilibrated with buffer A. Elution was achieved at 1 ml/min by a gradient from buffer A to buffer B (buffer A containing 1 *M* NaCl) in steps: 0% buffer B for 3 min, from 0 to 50% buffer B over 40 min, from 50 to 100% over 1 min and a step at 100% B over 3 min. Elution was monitored by UV absorbance at 280 nm.

Polypeptides and fractions issued from CEC were analysed by reversed-phase high-performance liquid chromatography (RPC) on a column of Nucleosil C_{18} (Macherey-Nagel, 300 Å, 5 μm, 250×4.6 mm) [3]. Elution was performed using a gradient of acetonitrile: 15–60% over 60 min, 60–100% over 1 min and 100% for 4 min. Polypeptide elution was monitored by UV absorbance at 220 nm.

2.5. Foaming properties

The polypeptide solutions (1 mg/ml) were prepared in dilute acetic acid then adjusted to pH 6.5 with sodium hydroxide.

Foam formation and stability were analysed with the automated conductimetric/optic instrument described previously [13]. Foam body was created in a column by sparging air through the test liquid. During the test, conductivity, volume of foam and volume of air incorporated were recorded. Conductivity measurements as a function of time (C_t) and with reference to the conductivity of the buffered test solution (C_{init}) were used to calculate the volume of liquid in the foam (V_L) [13]:

$$V_L = V_{init}[1 - (C_t/C_{init})],$$

where V_{init} is the volume of sample solution (8 ml) introduced into the sparging chamber.

All determinations were performed at least in duplicate.

3. Results

3.1. Liquid chromatographic analysis of hydrolysates

Limited enzymatic hydrolysis of gliadins produced hydrolysates with DH = 2.6. SEC pattern of hydrolysates showed a major peak around 17 000 and a small one at lower molecular masses. This was consistent with the limited hydrolysis of proteins with initial molecular masses around 35 000. RPC patterns demonstrated that gliadin hydrolysates contained essentially two groups of polypeptide components (Fig. 1A) [3]. The first group, eluting between 20 and 38 min, was termed ''hydrophilic'' and corresponded roughly to the repetitive sequence domain of the gliadins; the second group eluted after 38 min, ''hydrophobic'', corresponded to the non repetitive sequence domain. They were accounting for 51 and 49% of total hydrolysate, respectively [1].

As gliadin hydrolysates, hydrolysates of whole gluten reduced by cysteine contained essentially two groups of polypeptide components, hydrophilic and more hydrophobic (Fig. 1B). RPC retention time and proportions of the two groups were very similar to those of gliadin hydrolysates.

There was very weak difference between CEC patterns of gliadin and gluten hydrolysates. Hydrolysates were separated in two main fractions (Fig. 1C and D): a first fraction not retained and eluted for less than 0.08 *M* NaCl, and a second one eluted between 0.12 and 0.40 *M* NaCl. RPC patterns of these fractions showed that the first one was very

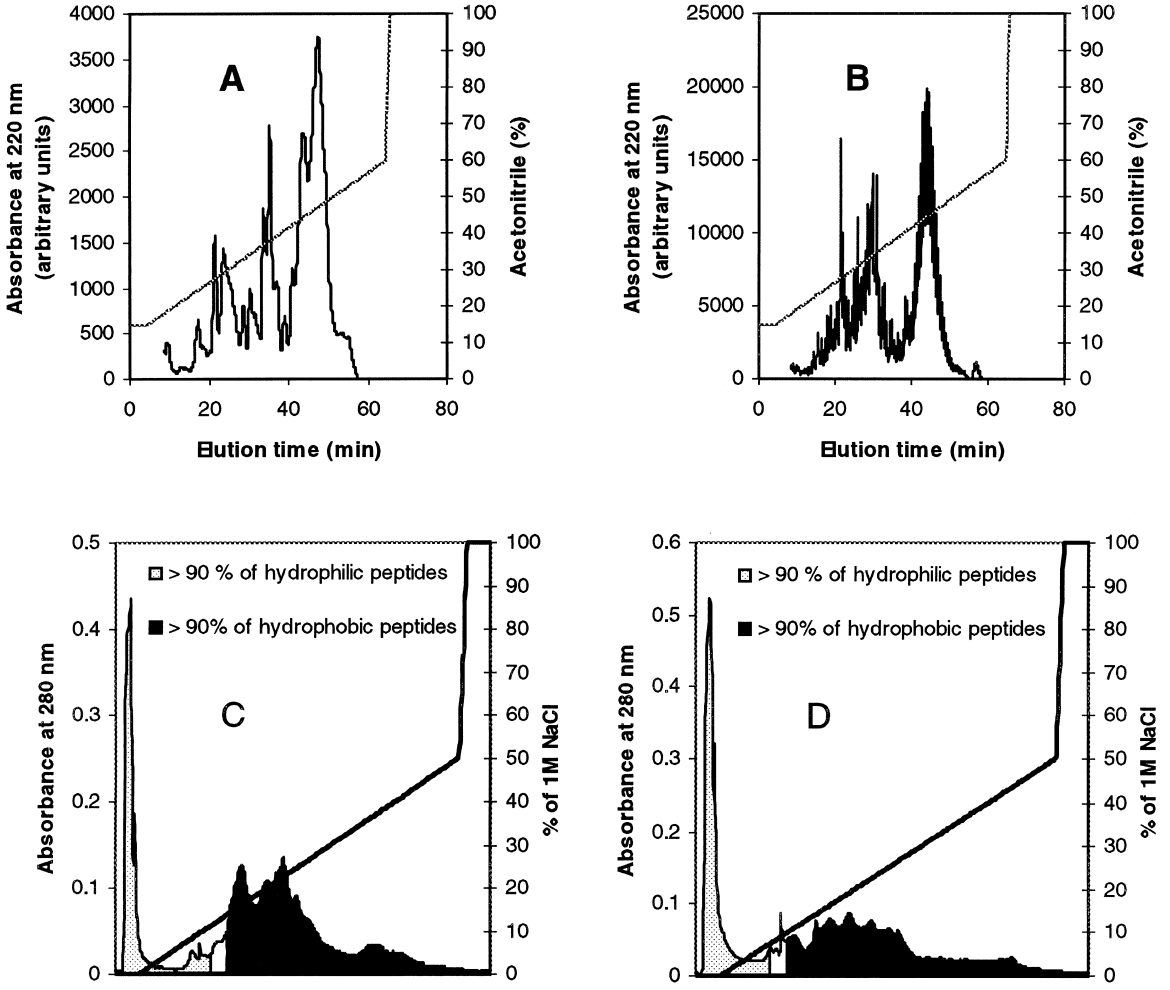

Fig. 1. RPC (A and B) and CEC (C and D) patterns of wheat gliadin (A and C) and gluten (B and D) hydrolysates.

much enriched in hydrophilic polypeptides (>90% of the polypeptides content) whereas the second one was enriched in more hydrophobic polypeptides (>90%).

3.2. Fractionation of hydrolysates by ultrafiltration

SEC patterns of permeate and retentate showed that they were composed of polypeptides with approximately the same apparent molecular size (≈17 000), even if the smallest polypeptides were recovered in permeate (not shown). Thus polypeptide size was not the major characteristics involved in the separation.

Transmissions of polypeptides were determined from RPC patterns of hydrolysates, permeates and retentates. Fig. 2 A shows the patterns of overall permeate and final retentate from RPC. They depended greatly on the characteristics of the polypeptides (Table 1): irrespective of the membrane (MWCO of 50 or 150 kg/mol), hydrophilic polypeptides were better transmitted (28 to 49%) than the more hydrophobic ones (less than 5%). High *Sf* values resulted from this. Gliadin hydrolysates exhibited higher transmissions of hydrophilic polypeptides than did gluten hydrolysates.

Finally, continuous diafiltration resulted in fractions with contrasted compositions: ‘‘hydrophilic’’

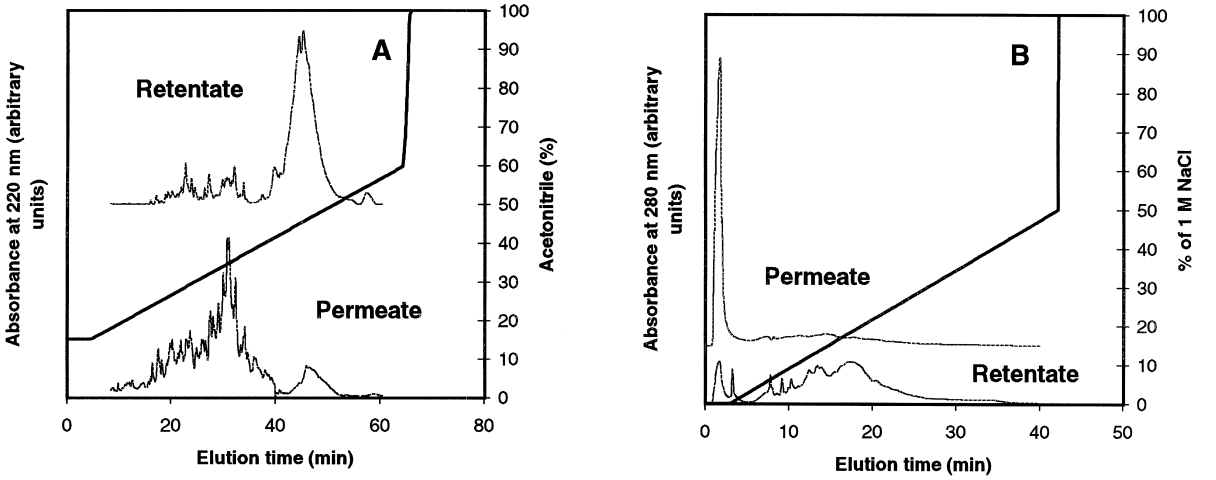

Fig. 2. RPC (A) and CEC (B) patterns of permeate and retentate (M4 membrane) obtained from gluten hydrolysates.

polypeptides accounted for 84 to 90% of the total polypeptide content in permeate; conversely, ''hydrophobic'' polypeptides accounted for 75 to 88% of the polypeptides in retentate. The recovery of polypeptides was good since the yield of hydrophilic polypeptides in the permeates and the yield of hydrophobic polypeptides in the retentates ranged from 67 to 92%.

In addition, assuming a 100% transmission of NaCl, the use of continuous diafiltration with a $V^*=4$ involved a decrease of NaCl content of the retentate by 54 from 0.065 *M* to 1 m*M*.

The use of M1 membrane instead of M4 membrane resulted in a slight increase of the transmission of the two kinds of polypeptides. Consequently, *Sf* value and purity of permeate (concentration of hydrophilic polypeptides) decreased.

CEC pattern of gliadin and gluten permeates show that they are composed almost entirely of polypeptides not retained by the column or eluted at NaCl concentration below 0.15 *M* (Fig. 2B).

On the other hand, retentates contained polypeptides with varied charge properties, but only 18% of those polypeptides were eluted at a NaCl concentration lower than 0.15 *M*. In both cases, RPC of the fractions resulting from CEC showed that the less positively charged polypeptides at pH 3.6 were the most hydrophilic ones.

Table 1
Fractionation of gliadin and gluten hydrolysates by continuous diafiltration with inorganic membranes[a]

Raw material hydrolysed	Gliadins		Gluten	
Membranes	M4	M1	M4	M1
Tr Φ_i	30	49	28	34
Tr Φ_o	2	3	2	4
Sf	18	16	14	9
[Φ_i] in permeate	89	84	90	84
[Φ_o] in retentate	83	88	75	77
Y Φ_i in permeate	67	79	79	76
Y Φ_o in retentate	80	70	92	85

[a] Tr: transmission of polypeptides, Φ_i: hydrophilic polypeptides, Φ_o: hydrophobic polypeptides, *Sf*: selectivity factor, [*i*]: concentration of *i* polypeptides in % of total polypeptides, Y_i: Yield of *i* polypeptides in % of total *i* polypeptides of the raw material.

3.3. Functional properties of the fractions

Foams were produced with solutions of polypeptide fractions obtained by ultrafiltration on M4 membranes of reduced gluten hydrolysates. They differed by the volume of liquid incorporated (Fig. 3A). Foams of hydrolysate and retentate had the highest density (0.133 and 0.167 respectively) and that of permeate the lowest (0.058). The properties of the polypeptide influenced even more the stability of the foams than their production. Foams of permeate and hydrolysate were not stable, characterized by a rapid drainage, coalescence and breaking of bubbles (Fig. 3B). On the other hand, foams of retentate showed an excellent stability: drainage was

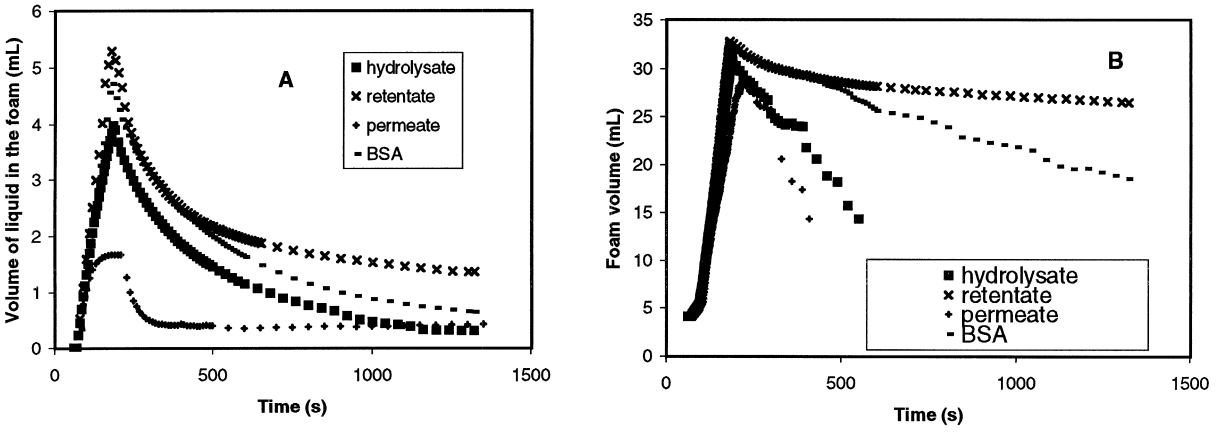

Fig. 3. Foaming properties of gluten polypeptide fractions compared to BSA at pH 6.5 (1 mg/ml). Kinetics of liquid intake and drainage (A) and volume stability (B).

slow and foam volume changed very little. The efficiency of retentate polypeptides to stabilize air/water interface was better than BSA.

4. Discussion

Limited enzymatic hydrolysis of gliadins and reduced gluten resulted in polypeptides differing both by their hydrophobicity and their charge: the more hydrophilic ones behaved during CEC as the less charged at pH 3.6. Conversely, the more hydrophobic polypeptides are positively charged at pH 3.6. Examination of sequence data indicated that hydrophobic, charged polypeptides must arise preferentially from the non-repetitive domains of prolamins, whereas the hydrophilic, near-neutral polypeptides arise from the repetitive domains.

Ultrafiltration separated in a similar way gliadin and reduced gluten hydrolysates into two polypeptide fractions differing mainly in their apparent hydrophobicity (retention on C_{18} grafted silica) and their charge. In accordance with Lucas et al. [15] who show that an uncharged protein is far better transmitted (size exclusion mechanism) than the same protein when highly charged, one can assume that hydrophobic polypeptides are less transmitted because they are positively-charged.

In overloading membrane process conditions, the first adsorption step is not determining for the steady-state equilibrium and it was not the scope of this study to look at the initial permeate fraction composition. However, zirconia is positively charged at pH 3.6 and charged polypeptides fouled membrane mainly by the dominant convection due to the applied pressure.

A higher purity of peptide fractions could be achieved by CEC, but this process is less adapted to cheap large scale production and it would require a dialysis or diafiltration step to desalt hydrophobic polypeptides eluted between 0.15 and 0.4 *M* of NaCl.

Furthermore, functional properties of UF fractions were clearly different [14]. Particularly, ''hydrophilic'' polypeptides of the permeate did not stabilize foams, whereas ''hydrophobic'' polypeptides of the retentate displayed excellent foaming properties, in relation to the amphiphilicity of the latter [2].

5. Conclusions

Analyses of gliadin hydrolysates by reversed-phase and ion-exchange chromatographies has confirmed that chymotryptic hydrolysates contained two types of polypeptides: the one (repetitive polypeptides) is hydrophilic and near neutral at acidic pH, whereas the other one (non repetitive) is more hydrophobic and positively charged at acidic pH.

The hydrolysates of gluten reduced by cysteine have characteristics similar to those of gliadins in terms of charge, hydrophobicity and molecular mass.

Both hydrolysates from gliadins or gluten can be

advantageously processed by inorganic ultrafiltration membranes as charged and hydrophobic polypeptides are weakly transmitted through the membranes. A continuous diafiltration process produced with high yields two fractions with contrasted compositions and functional properties. This diafiltration process, reducing salt concentration of retentate, seems to be well adapted to valorize these fractions in food industry.

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