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Selective Separation of Peptides Contained in a Rapeseed (*Brassica campestris* L.) Protein Hydrolysate Using UF/NF Membranes

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The ability of a charged UF membrane to fractionate the small peptides found in a rapeseed protein enzymatic hydrolysate, according to their charge characteristics, was investigated. The complexity of such a hydrolysate has required the setting up of technological alternatives to isolate the small peptides, to obtain a more efficient separation among the numerous peptide species. A preliminary step consisted of precipitation followed by filtration with a 3000 g/mol molecular weight cutoff (MWCO) membrane to obtain a solution concentrated in small peptides. The possibility of fractionating these small peptides by a charged 1000 g/mol MWCO membrane was investigated. The study enabled us to assess the contribution of electrostatic interactions during fractionation. The effect of pH and ionic strength on the peptide transmission was studied. The ionic strength contribution was considered by studying the effect on the selectivity of a desalting step by nanofiltration on a 500 g/mol MWCO membrane. Peptide transmission was lower at pH 9 than pH 4, and it was the lowest at pH 9 and low ionic strength. Ionic strength had a significant influence at pH 9 but showed no influence at pH 4. The amino acid analysis and capillary electrophoresis revealed that negatively charged (acid) peptides were found in lower proportions in the permeate. The opposite trend was observed for basic peptides, whereas neutral peptides were found in the same proportion in the retentate and the permeate. These results can be explained, according to the Donnan theory, by the existence of attractive and repulsive forces at the membrane−solution interface. Selectivity between basic and acid peptides was as high as 1.90 at pH 9 and low ionic strength. A rough sketch of a membrane-based process is proposed to fractionate rapeseed peptide mixtures. Results obtained were reproducible within 10%.

KEYWORDS: Rapeseed protein hydrolysate; *Brassica campestris* L.; ultrafiltration; nanofiltration; peptide; fractionation selectivity

INTRODUCTION

Peptides, and notably small peptides (<1000 g/mol), are molecules that present a wide range of functional, nutritional, and biological properties. They thus present a great interest for food, pharmaceutical, and cosmetic industries. Because of the increasing fear of consumers toward animal-based products, the interest in peptides from plant origins is rising. Enzymatic hydrolysis of plant proteins leads to a great variety of peptides, which gives an enhanced value to the agricultural byproducts. For instance, defatted rapeseed (*Brassica campestris* L.) meal is currently intended for animal nutrition because of its high protein content (from 30 to 45%) and its well-balanced amino acid composition. For the past decade, a few studies have dealt with the production of enzymatic hydrolysates from that plant byproduct, to increase its field of applications (*1*).

However, two bottlenecks put a brake on the industrial production of small peptides contained in such plant protein hydrolysates. First, small peptides stand in an extremely complex mixture together with amino acids, oligopeptides, and numerous other substances such as phenolic compounds and fibers. Second, biologically active peptides often present a particular physicochemical characteristic, such as the charge, which is essential to their activity (*2*). As a consequence, the development of plant peptides requires purification and fractionation. The separation of small peptides from larger compounds such as oligopeptides by membrane processes, especially ultrafiltration, is a well-known technique. On the other hand, the fractionation of small peptides is classically achieved by chromatographic methods. These techniques are very efficient to fractionate small peptides according to their charge, size, or hydrophobic properties (*3*). However, the scale-up issue of a chromatographic method generates some high costs because of the use of organic solvents and because of the low productivity of this technique.

For that reason, several studies have focused on the small peptide fractionation by nanofiltration membranes. These studies have dealt with the separation of peptides from synthetic solution

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(*4*) or well-identified peptides from β-casein hydrolysates (*5*−*7*) and also polypeptides from gliadins (*8*) or β-lactoglobulin hydrolysates (*9*−*11*). Few authors have studied the influence of ionic strength (*5*, *6*). A predominant contribution of electrostatic interactions in the peptide fractionation has been noticed in all of these works. Selectivity of the separation was based on the peptide and membrane charges (*4*, *6*, *12*). Actually, peptides with the same charge as the membrane (coions) were concentrated in retentate whereas peptides with the opposite charge (counterions) were preferentially transmitted in permeate. This phenomenon was explained by the Donnan principles (*13*), based on the development of attraction and repulsion forces between ionic solutes and membranes. In fact, the concentration of coions is lower in the membrane than in the solution, whereas, on the contrary, the concentration of counterions is higher. This difference in ion concentrations at the membrane−solution interface creates a potential difference. This is called the Donnan potential, which is responsible for the repulsion of coions and the attraction of counterions by the charged membrane. The extrapolation of the Donnan theory to the separation of peptide mixtures may be restricted because of coupling and competitive effects (*7*). The use of electromembrane filtration has also been proposed to fractionate charged peptides from a α_{s2}-casein hydrolysate (*14*). However, although more selective, the industrial development of this technology could be difficult, as compared to classical nanofiltration.

From observations made during fractionation of relatively simple peptide solutions, the aim of this work is to understand fractionation of complex hydrolysates to further develop a membrane-based process to fractionate rapeseed peptide mixtures. To stand in the best conditions to study the small peptides fractionation, we initially settled a process to obtain a small peptide-enriched solution from plant hydrolysate. This solution was used to confirm the potential of nanofiltration membranes to fractionate, at least partially, the numerous charged peptides standing in that mixture. The mineral composition of the hydrolysates was taken into account just as some variations in pH values. Thus, we determined the right operating conditions allowing the amplification of charge effects during the fractionation. Validation of process ability to product fractions of various charge properties was performed through the amino acid analysis and the original use of capillary electrophoresis. Indeed, the extensive number of various peptides (more than 100) contained in solutions, but each one in very low amounts, made the usually used reversed phase liquid chromatographic methods (*6*, *9*) unuseful here: Separation could not be resolutive enough. The separation selectivity is discussed as a function of pH and ionic strength because of the predominant role of ionic interactions during fractionation.

EXPERIMENTAL PROCEDURES

Preparation of the Hydrolysate. Industrial rapeseed meal, produced by Novance (Compiègne, France) and obtained by solvent extraction, was used as the protein source (nitrogen content, 35% w/w). To prepare a protein concentrate, rapeseed defatted meal (30 kg) was suspended in 300 L of 0.2 M NaOH. The proteins were extracted by stirring for 30 min at ambient temperature and recovered by centrifugation at 3500*g* with a centrifugal decanter (Alfa Laval, Sweden). The supernatant pH was adjusted to the protein isoelectric point (pH 4.0), and the precipitate formed was recovered by centrifugation at 3000*g* with a centrifugal plate separator (Veronesi Separatory, Italy). The precipitate was washed with distilled water and recovered by centrifugation as described above. This protein concentrate (nitrogen content, 70% w/w) was hydrolyzed in a 20 L reactor (Biolafitte, France) equipped with a stirrer, a pH electrode, and a temperature regulation system. Used were the following hydrolysis parameters: substrate concentration, 5% w/v; enzyme/substrate ratio, 1/10; enzyme, Alcalase 2.4 L (Novozymes, Bagsvaerd, Denmark); pH 9 (maintained by addition of 8 M NaOH); and temperature, 60 °C. The hydrolysis was stopped after 5 h by heating at 90 °C for 10 min at a degree of hydrolysis of 28%, as determined by the pH stat technique (*15*, *16*). The hydrolysate was aliquoted in 2.5 L parts and freezed for further use.

Filtration Experiments. Millipore (Bedford, MA) provided the three different membranes used in this work (PLAC, PLBC, and Nanomax 50). The PLAC and PLBC membranes had a flat sheet configuration and molecular weight cutoff (MWCO) values of, respectively, 1 and 3 kDa. They were composed of regenerated cellulose and exhibited anionic characteristics. The filtering area of both membranes was 0.1 m^2. The Nanomax 50 membrane was a 500 Da MWCO spiral-wound cartridge. Its filtering area was 0.4 m^2. The active layer was a polyamide/polysulfone thin film composite.

Membrane experiments were performed with a cross-flow ProScale system (Millipore) equipped with a temperature regulation system, two pressure gauges (at the inlet and outlet of the retentate), and a mass flow meter (Bopp & Reuther, Mannheim, Germany) to measure the permeate flow rate. Each filtration experiment was performed at 25 °C with an initial feed volume of 2 L. The feed pH value was adjusted either to 4 with HCl or to 9 with NaOH. These pH values were chosen to obtain a distribution of peptides with some negative and positive charges at both pH values. For each experiment, the following procedure was operated as follows: permeate recycling for 10 min to stabilize membrane charge, concentration until the volume concentration ratio equaled 2, and then diafiltration with several successive diavolumes (four with PLBC and Nanomax 50 membranes or two with PLAC membrane). A diavolume was completed when the recovered permeate volume corresponded to the retentate volume at the end of concentration step (1 L). Diafiltration was performed with distilled water, adjusted to the pH value of the feed with NaOH or HCl. Each experiment was performed at least in duplicate. The filtration unit was regenerated after each experiment by continuous washing with NaOH solution (0.1 M, 60 min, 25 °C) and distilled water until the permeate reached pH 7. Water permeability was checked to ensure effective cleaning. Membranes were stored at 4 °C in 0.05% (w/v) of NaN_3 to prevent any microbial growth. Before the filtration experiments, the hydrolysates were centrifuged in order to remove insoluble substrate fragments and then adjusted at pH 4 to precipitate the high molar mass substances, notably the aggregating peptides (*17*, *18*). The supernatant, with a peptide concentration of 3.0% (w/v), was the feed phase for the filtration on the PLBC membrane to purify small peptides from larger compounds. The feed flow rate was 3 L min^{-1}, and the transmembrane pressure was 5.0×10^5 Pa. Permeates obtained after concentration and diafiltration steps were freeze-dried and then pooled together for further operation.

Because the hydrolysate contained a great amount of salt (use of pH stat and acid precipitation), one part of the 3 kDa permeate could be desalted to assess the influence of ionic strength on further fractionation selectivity. Desalting was performed with a Nanomax 50 membrane at a transmembrane pressure of 10.0×10^5 Pa and a feed flow rate of 7 L min^{-1}. The feed phase (i.e., PLBC membrane permeate) was adjusted to a peptide concentration of 0.5% (w/v). Desalting was monitored during filtration through conductivity measurements using a WTW LF96 conductivity meter. The final retentate was freeze-dried for further fractionation. The PLAC membrane was used for this fractionation study with a feed flow rate of 3 L min^{-1} and a transmembrane pressure of 5.0×10^5 Pa. The feed peptide concentration was adjusted to 0.5% (w/v) with purified water, at a selected pH value. The permeate and the final retentate were freeze-dried for further analysis.

The small peptide recovery yield (R) was calculated according to the following equation:

$$R = \frac{m_p(t)}{m_i} \times 100 \quad (1)$$

R is based on the ratio between the small peptide mass content in

permeate, $m_p(t)$, and the small peptide mass of the feed, m_i. m_i and $m_p(t)$ were weighed, and they are given in grams.

Another ratio (ρ) in amino acid concentration between permeate (C_p) and retentate (C_r) was calculated by:

$$\rho = \frac{C_p}{C_r} \quad (2)$$

ρ_A, ρ_B, and ρ_N refer, respectively, to acidic, basic, and neutral amino acids ratios. Concentrations are given in mg per 100 g of dry matter.

The selectivity is defined by:

$$S_{B/A} = \rho_B/\rho_A \quad (3)$$

Analytical Methods. Size-exclusion chromatography analyses were carried out with a Superdex Peptide HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) and a solvent mixture of ACN/H_2O/TFA (40/60/0.1, v/v/v). An isocratic elution was performed at 0.6 mL min^{-1}, and wavelengths of detection were 214 nm for peptide determination and 310 nm for phenolic compounds determination. The phenolic compounds were measured in peptide solutions without any treatment and were expressed from a calibration curve made with sinapic acid (224 g/mol). The molar mass distribution was determined using a calibration curve made with papain (23000 g/mol), insulin (5750 g/mol), a custom-synthesized peptide of 28 amino acids (3254 g/mol), a custom-synthesized peptide of 22 amino acids (2592 g/mol), a custom-synthesized peptide of 12 amino acids (1364 g/mol), β-interleukine (1005 g/mol), kemptide (772 g/mol), Lys-Tyr-Lys (437 g/mol), Leu-Leu-Leu (357 g/mol), Ala-Met (220 g/mol), and L-His (155 g/mol). All standards were purchased from Sigma Chemical Co. (St. Louis, MO) or custom synthesized at the Laboratoire de Chimie Physique Macromoléculaire (Nancy, France).

The Kjeldahl method was employed for the total nitrogen content calculation with a conversion factor of 6.25 for peptide mass determination. Cations (Na^+, K^+, and Ca^{2+}) concentrations were determined by atomic absorption spectroscopy using a SpectrAA-20 from Varian (Palo Alto, CA). Detection of Na^+, K^+, and Ca^{2+} was performed at, respectively, 330.3 (range, 0−150 mg/L), 285.2 (0−0.8 mg/L), and 422.7 nm (0−6 mg/L). Anion (Cl^-, SO_4^{2-}, PO_4^{2-}, and NO_3^-) concentrations were determined by anion-exchange chromatography using columns AG9 (precolumn) and AS9 (column) on a HPLC apparatus (Dionex, Sunnyvale, CA). The elution rate was 1.3 mL min^{-1} with a buffer composed of sodium carbonate (12 mM) and sodium bicarbonate (10 mM).

The determination of total amino acid composition was obtained after acid hydrolysis of the peptide solutions. The samples, acidified with 6 N HCl, were sealed in tubes under nitrogen and incubated at 110 °C for 24 h. Amino acids were derivatized with 9-fluorenylmethyl chloroformate and *o*-phtahaldehyde (*19*) and analyzed by high-performance liquid chromatography (HPLC). The HPLC apparatus was a HP 1090 Liquid Chromatograph system (Hewlett-Packard, Palo Alto, CA). Separations were performed with a reversed-phase column (Hypersil 5μ C18, Interchim, Montluçon, France) using two solvents. These solvents were (A) 20 mM sodium acetate containing 0.024% triethylamine and 0.5% tetrahydrofurane (pH 7.2) and (B) acetonitrile/methanol/100 mM sodium acetate (40/40/20, v/v/v) (pH 7.2). The solvent was delivered to the column as follows: time 0.0−17.0 min, elution with 100% A; 17.0−18.0, elution with A/B (40/60); 18.0−25.0, elution with 100% B. The same procedure, but without acid hydrolysis, was used to determine the free amino acid content. The amino acid concentrations were calculated from a calibration curve made with an amino acids kit (Sigma Chemical Co.). Aspartic acid and glutamic acid represented the acid amino acids, whereas lysine, arginine, and histidine constituted basic amino acids. Acid hydrolysis resulted in the deamidation of the amide groups present on asparagine and glutamine to yield aspartic acid, glutamic acid, and ammonia. Acid hydrolysis also destroyed tryptophan, which was weakly represented in rapeseed proteins (*20*).

Capillary electrophoresis was used to obtain the peptide maps of permeate and retentate, to characterize the variations in the composition of charged peptides. Capillary electrophoresis is probably one of the most efficient instrumental setups for the study of peptide fractionation in reason of the high ability of this technique to separate charged analytes (*21*). The capillary electrophoretic separations were performed on a PACE 5000 system (Beckman, Fullerton, CA) equipped with an UV detector, an automatic injector, a fluid-cooled column cartridge, and a System Gold data station. Fused silica capillary dimensions were 57 cm in length (50 cm at the detection window) × 50 μm i.d. The buffer was made up of 50 mM borate buffer at pH 9. The running voltage was 20 kV during separation. All runs were performed at 25 °C with detection at 214 nm. Samples were prepared at a concentration of 1 g/L in low conductance HPLC grade water. The anode electrode was coincident with the capillary injection terminal. Migration was run under normal polarity (anode to cathode).

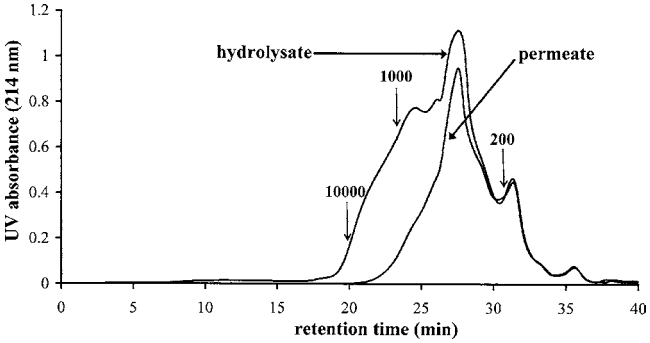

Figure 1. Molar mass distributions of the rapeseed protein enzymatic hydrolysate and permeate obtained by a pretreatment step. LC was carried out on Superdex Peptide HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) with a constant flow rate of 0.6 mL/min of ACN/H_2O/TFA (40/60/0.1, v/v/v).

RESULTS AND DISCUSSION

Characterization of the Hydrolysate. To efficiently assess the influence of charge on fractionation selectivity, the first objective was to decrease the contribution of size criterion. Therefore, the first step was to purify small peptides to obtain peptide solutions with a molar mass distribution as narrow as possible and almost exclusively composed of peptides. A pretreatment step by acid precipitation to remove high molar mass substances (particularly the aggregating peptides), followed by an ultrafiltration of the supernatant with a low MWCO membrane (3 kDa), allowed the concentration of small peptides (**Figure 1**).

Table 1 reports the composition of the initial hydrolysate and of the permeate resulting from this pretreatment step and illustrates the high purity in small peptides (<1000 g/mol). The total nitrogen content in permeate was 87 vs 76% in the hydrolysate. Ninety percent of this nitrogen matter presented a molar mass lower than 1000 g/mol, i.e., composed of less than 10 amino acids, whereas this part was only 67% in the protein hydrolysate. The part of free amino acids in total nitrogen matter was only 8%. The recovery yield of small peptides was quite high: 75% of the small peptides contained in the original hydrolysate were recovered after the pretreatment step. The mineral content of hydrolysate was strongly dominated by sodium and chloride ions because of the use of NaOH to regulate pH during enzymatic hydrolysis and HCl to acidify the hydrolysate during the acid precipitation step to remove high molar mass substances. The concentration in phenolic compounds has been studied since these molecules are involved in the dark color and astringency of rapeseed protein-based products. Rapeseed meal contains 15−18 g kg^{-1} of phenolic acids (*22*), which is five times higher than the soybean meal content. In the 3 kDa permeate, this content is dropped to 8 g

Table 1. Composition of the Hydrolysate and of the Permeate Obtained by Ultrafiltration with a PLBC Membrane

	hydrolysate	permeate
total nitrogen content (%, w/w)	76	87
molar mass distribution (%)		
>3000 g/mol	20	1
3000–1000 g/mol	13	9
<1000 g/mol	67	90
free amino acids (%)	4	8
recovery yield of small peptides (%)		75
minerals (mg kg^{-1})		
chloride (Cl^-)	30	424
sulfate (SO_4^{2-})	7	8
phosphate (PO_4^-)	2	3
nitrate (NO_3^-)	2	2
sodium (Na^+)	369	428
potassium (K^+)	14	16
calcium (Ca^{2+})	1	1
total ions	425	882
phenolic compounds (g kg^{-1})	10	8

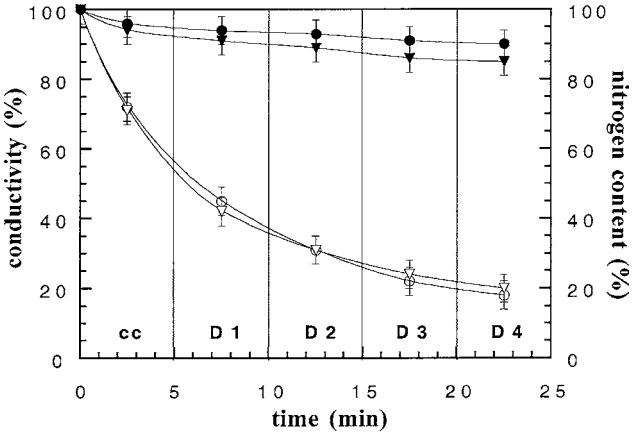

Figure 2. Evolution of conductivity (pH 4, ○; pH 9, ▽) and nitrogen content (pH 4, ●; pH 9, ▼) of retentate during a desalting step with a Nanomax 50 membrane. Values are normalized by feed values. Cc refers to concentration step; D1, D2, D3, and D4 refer to the diavolume numbers. Each value is the mean of triplicates ± standard error.

kg^{-1}, which shows the ability of that process to isolate small peptides toward unwanted components of high molar mass such as polyphenols (MM > 3000 g/mol).

Desalting. The efficiency of a desalting process of hydrolysates can thus be evaluated by two parameters: the desalting level and the loss in peptide material (*23*, *24*). Therefore, we have assessed the quality of this desalting by monitoring the global conductivity of the retentate and the loss in nitrogen content, due to transmission into permeate (**Figure 2**). These data have been normalized by the feed (3 kDa permeate) values: Conductivity was measured and expressed as a residual percentage of the initial conductivity, and in the same way, the nitrogen content determined by the Kjeldahl method was expressed as a percentage of initial nitrogen content. The influence of feed solution pH value on these two parameters has also been investigated. At the end of the last diafiltration step, the separation led to an 80−83% conductivity reduction and a 10−16% nitrogen content reduction in the retentate. Therefore, conductivity reduction was large whereas nitrogen content loss was low, whatever the pH value.

To better understand the desalting step, we have determined the ionic composition of desalted and nondesalted hydrolysates. Thus, we have measured the total concentration of four anions (Cl^-, SO_4^-, PO_4^-, and NO_3^-) and three cations (Na^+, K^+, and Ca^{2+}) mainly found in rapeseed meal. The results are consistent with those obtained with conductivity measurements. At pH 4 and pH 9, this demineralization step has led to an 80% decrease in salt concentration. The transmission of ions was not significantly affected neither by the nature of these ions (cations or anions) nor by the change in pH value. It means that the electrostatic interactions between ionic solutes and membrane have a low extent whatever the pH of the solution, maybe because of a low membrane charge density (*25*). It can be also noticed that chloride and sodium concentrations represented, respectively, about 95% of anions and cations, as expected. The higher concentration in cations at pH 9 was only due to the use of NaOH to increase the hydrolysate pH from 4 to 9.

Table 2. Amino Acid Composition of Permeates after Desalting of the Hydrolysate on a Nanomax 50 Membrane and of Hydrolysate before Fractionation; Analysis Made by RP-HPLC (C18 Column)[a]

	mg/100 g					
	permeate of desalting pH 4		permeate of desalting pH 9		hydrolysate before fractionation	
	free	total	free	total	free	total
Ala	50	76	20	58	42	2795
Arg	197	294	92	227	301	7913
Asx[b]	6	68	8	99	14	8705
Cys	4	14	1	29	0*[c]	0*
Glx[d]	34	178	53	358	353	13034
Gly	38	95	33	109	56	4066
His	10	57	8	11	100	2396
Ile	6	29	1	58	201	3157
Leu	92	114	31	78	626	5628
Lys	26	72	6	25	212	3818
Met	4	22	29	39	0*	2015
Phe	19	43	53	79	420	3307
Pro	61	221	182	285	1070	4027
Ser	50	67	41	61	38	2611
Thr	31	45	16	57	140	4826
Trp	ND[e]	ND	ND	ND	ND	ND
Tyr	17	31	16	38	102	2404
Val	17	51	25	65	234	4630
total	662	1477	615	1676	3909	75334

[a] Total quantities of each amino acid were determined after acid hydrolysis whereas quantities of each free amino acid were determined without acid hydrolysis. [b] Asx, aspartic acid and asparagine. [c] 0*, concentration too low to be determined. [d] Glx, glutamic acid and glutamine. [e] ND, no determination was performed.

The separation mechanism of nanofiltration membranes is based upon the "sieve effect" and the "charge effect" (*26*). We have determined the nature of the low quantity of nitrogen transmitted to have a better understanding of the influence of the solute size in the separation process (**Table 2**). Free amino acids (≈100−150 g/mol) represented 45% of the total nitrogen matter in permeate at pH 4 (662 vs 1477 mg/100 g) and 37% at pH 9 (615 vs 1676 mg/100 g), whereas this part was only 8% in the initial solution (**Table 1**). This observation shows the better transmission of the smallest molecules (i.e., free amino acids) as compared to the largest (i.e., peptides). Both this result and the important transmission of anions and cations as compared to the nitrogen matter show that the molecule size is the predominant parameter in this separation. The variations between the two pH values in the charged amino acids concentrations (i.e., Arg, Lys, His, Asx, and Glx) indicate a slight contribution of charge effects in the separation of nitrogen matter, but the sieve effect remains the most important (**Table 2**). To sum up, this desalting process eliminated the majority of the smallest molecules (mainly ions and free amino acids) while the retentate remained with a similar composition in peptides. These results show the efficiency of this polyamide

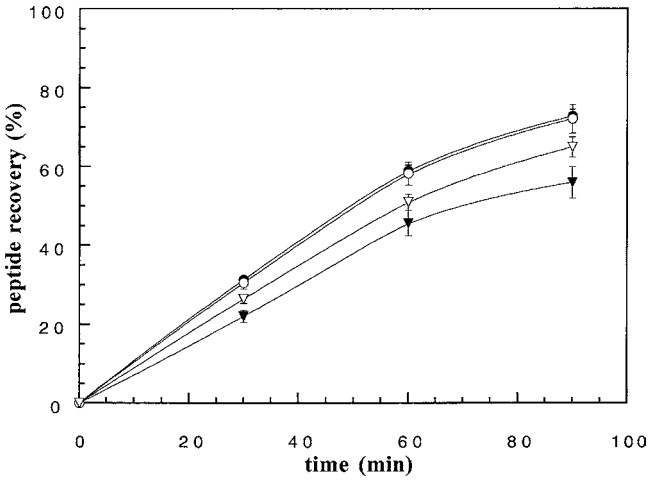

Figure 3. Peptides recovery yield (R) in permeate vs time for various pH values and ionic strengths with PLAC membrane (not desalted pH 4, ○; desalted pH 4, ●; not desalted pH 9, ▽; and desalted pH 9, ▼). Each value is the mean of triplicates ± standard error.

membrane to desalt hydrolysates without significant variation in peptide composition because of a low transmission of peptides. Although losing free amino acids could appear to be a drawback of this process from an economical standpoint, it can also be considered as an advantage in the case of particular applications. Indeed, from a nutritive point of view, amino acids are less assimilated than small peptides (di-, tripeptides) that they constitute (*27*). Then, when fractions are intended to substitute or complement diets, high purity in small peptides is more interesting than mixtures of amino acids and small peptides.

Fractionation of Peptides. A study of the peptide sieving, an amino acid analysis, and capillary electrophoresis was employed to validate the process ability to product fractions with different charge properties. This fractionation study was performed with a 1 kDa MWCO membrane and various hydrolysates to assess pH (pH 4/pH 9) and ionic strength (desalted/not desalted) influences on peptides fractionation. Because of the carboxylic groups ($-COO^-$) standing on this cellulose acetate membrane, it is negatively charged at both pH 4 and pH 9. The charge is, however, more strongly negative at pH 9 because of a complete dissociation of carboxylic groups at alkaline pH values (*28*). In the same way, acid peptides are negatively charged and basic peptides are positively charged at both pH 4 and pH 9, and this charge is stronger at pH 9 for acid ones and at pH 4 for basic ones.

Peptide Sieving. We first investigated the influence of pH and ionic strength on peptide sieving. **Figure 3** shows the evolution of peptide recovery in permeates during the fractionation. Recovery was always higher at pH 4 than at pH 9 because of a weaker negative charge of the membrane, leading to less rejection of acid peptides. Concomitantly, the proportion of positively charged peptides increases when the pH decreases; a higher sieving of these peptides occurs at pH 4. These two complementary phenomena finally lead to a higher transmission of peptides.

Ionic strength revealed influent in the case of pH 9 but not at pH 4. Indeed, as seen above, electrostatic interactions are great at pH 9 when the membrane charge is the most important, whereas they are not so significant at pH 4. However, these interactions can be weakened by a screening of the charges (*9*): At high ionic strength, the high diffusion coefficient of small ions induces a screening of both membrane and peptide charges. These small ions (mainly Na^+ and Cl^-) are found in great quantities in the nondesalted pH 9 hydrolysate (1040 mg/kg, among which 60% cations) as compared to the desalted hydrosylate (177 mg/kg). It is thus obvious that the membrane charge is significantly screened by these numerous cations, thus decreasing the repulsive forces between the membrane and the negatively charged peptides.

Table 3. Influence of pH Value and Ionic Strength on Ratios of Acid (ρ_A), Basic (ρ_B), and Neutral (ρ_N) Amino Acids and on Basic/Acid Selectivity ($S_{B/A}$) after Fractionation of Various Hydrolysates with a PLAC Membrane[a]

	pH 4		pH 9	
	not desalted	desalted	not desalted	desalted
ρ_A	0.88 (±0.03)	0.77 (±0.07)	0.79 (±0.07)	0.70 (±0.02)
ρ_B	1.18 (±0.05)	1.32 (±0.09)	1.46 (±0.01)	1.33 (±0.03)
ρ_N	1.02 (±0.01)	1.06 (±0.02)	1.01 (±0.04)	1.10 (±0.05)
$S_{B/A}$	1.34	1.71	1.85	1.90

[a] The standard deviations from the mean value in ratios are given between parentheses.

As a result, the sieving was more important at pH 9 without desalting (final recovery = 65%) as compared to pH 9 with desalting (final recovery = 56%), whereas it was not affected by ionic strength at pH 4 (final recovery = 72% in both cases). This first study on peptide fractionation shows that the pH and ionic strength influence the peptide separation by their contribution to the electrostatic interactions, especially at pH 9.

Amino Acid Analysis. The amino acid content of permeate and retentate solutions was analyzed to confirm the implication of these charge effects during the fractionation of charged peptides. In fact, after an acid hydrolysis of peptides, the composition in free amino acids is representative of the composition of peptides in amino acids. The selectivity of this membrane process toward charged peptides was assessed by studying the proportions of acidic, basic, and neutral amino acids that constituted peptides of permeate and retentate, for the various pH and ionic strength hydrolysates. **Table 3** shows that there were some differences in partitioning of amino acids according to their charge characteristics. Whatever the ionic strength or the pH value, the ratio in acid amino acids concentration between permeate and retentate was always lower than 1. So, acid amino acids (and, as a consequence, acid peptides) were in lower proportion in the permeate than in the retentate. This means that acid peptides were preferentially repelled by the membrane in all cases.

The opposite trend was observed for basic peptides, which are attracted by the membrane and transmitted, whereas neutral peptides occurred in the same proportion in the retentate and the permeate. This fractionation according to the charge was more acute when the pH increased: At high ionic strength, acid peptide rejection increased by 10% (ρ_A decreased from 0.88 to 0.79), whereas basic peptide transmission increased by 25% (ρ_B increased from 1.18 to 1.46). As a result, global selectivity increased by nearly 50%. The fractionation selectivity between basic and acid peptides ($S_{B/A}$) was lower at pH 4 (1.71 with desalted hydrolysate) because of a decrease in electrostatic interactions due to a lower negative charge of the membrane.

The influence of ionic strength on charge fractionation was dependent upon pH. Indeed, at pH 4, an increase in ionic strength resulted in a higher transmission of acid peptides and a lower transmission of basic peptides, leading then to a lower selectivity (1.34 vs 1.71). These results are consistent with those previously reported (*29*). At pH 9, although the global selectivity

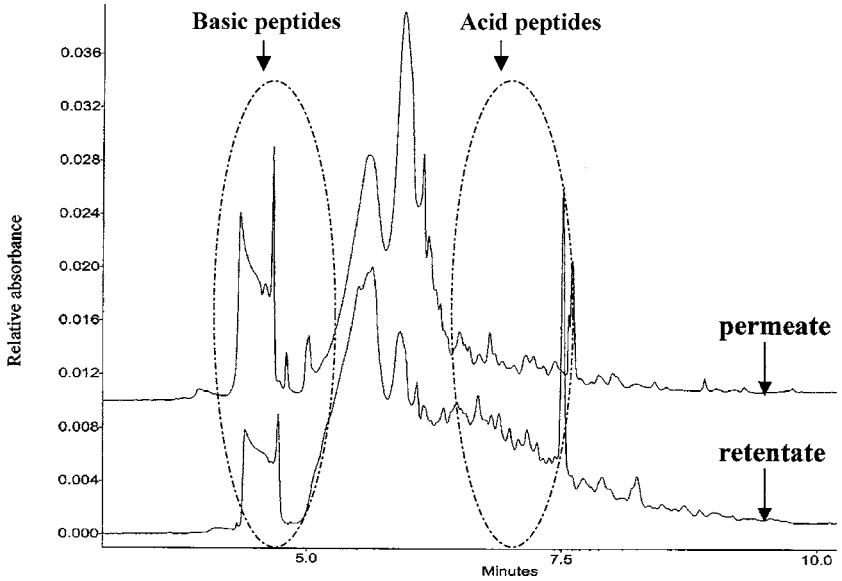

Figure 4. Electrophoregrams of permeate and retentate obtained after nanofiltration experiment on a PLAC membrane of a desalted hydrolysate at pH 9. Conditions: voltage, 20 kV; buffer, 50 mM borate, pH 9; capillary length, 57 cm (50 cm to detector).

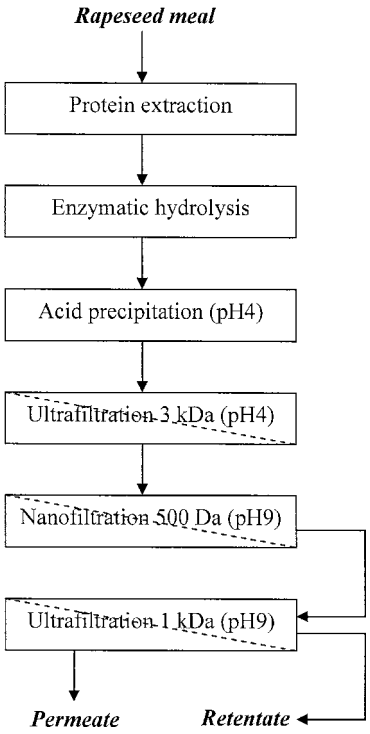

Figure 5. Schematic flow sheet of the membrane-based rough sketch process proposed for the production of peptide fractions with distinct charge properties from rapeseed meal.

decreased slightly with increasing ionic strength (1.85 vs 1.90), the lowest rejection of acid peptides at high ionic strength was gone together with a higher attraction of the basic ones. The explanation of the behavior of basic peptides at high ionic strength at pH 9 is based on competition phenomena between counterions (*30*). A rise in the higher mobility counterions (Na^+) concentration can lead to an increase in transmission of less mobile counterions (basic peptides) (*6*, *31*). Two comments can explain why this was observable at pH 9 and not at pH 4. First, the concentration in cations was more important at pH 9 (626 vs 445 mg/kg). Then, the amount of peptides with basic properties was lower at pH 9 as compared to pH 4. Thus, the ratio cation concentration/basic peptides concentration was much higher at pH 9, which could explain the observation of the higher transmission of basic peptides only at this pH value.

Capillary Electrophoresis. The electrophoregrams of permeate and retentate, obtained by the nanofiltration experiment with the desalted hydrolysate at pH 9, are given in **Figure 4**. With their positive charges, basic peptides were strongly attracted by the cathode. They migrated faster than acid peptides (with negative charges), which were attracted by anode and migrated to the detector only because of solvent flow (electroosmotic flow). The comparison of migration profiles shows a more important concentration of peptides with a high electrophoretic mobility (i.e., positively charged peptides) in permeate (between 3 and 5 min). It means that the concentration in basic peptides was higher in permeate than in retentate. On the contrary, peptides with the lowest electrophoretic mobility (after about 6.5 min) were found in larger concentrations in retentate, which illustrates the predominance of acid peptides in this fraction. As a consequence, these data are in good agreement with those obtained by amino acid analysis.

Finally, these results have underlined that changes in the pH value and/or the ionic strength modified the nature and the intensity of electrostatic interactions between coions and/or counterions and membrane. The highest selectivity was observed at pH 9 with low ionic strength. This result confirms those obtained with less complex peptides mixtures (*6*, *8*), which have also pointed out the best selectivity at alkaline pH without added NaCl, using various nanofiltration membranes. Therefore, because the charge effects largely influence the filtration selectivity, the control of pH and ionic strength would be a major concern in further works aimed at designing a selective process. By amino acid analysis and capillary electrophoresis, it was possible to ascertain that nanofiltration membranes were able to fractionate, at least partially, some small peptides stemming from a complex plant hydrolysate, according to their charge.

From a statistical point of view, it is noticeable that all of the results obtained were reproducible within 10%: Standard errors represented in **Figure 2** and standard deviations from mean values given in **Table 3** were lower than 10%; amino acid analyses performed on various fractions obtained by this process at different times showed variations in composition below 10%. Moreover, the total amino acid content of hydrolysate before fractionation determined in **Table 2** (75334 mg/

100 g dry matter) is consistent with the total nitrogen content given in **Table 1** (76% w/w).

Figure 5 sums up the rough sketch of process proposed in this work to be further investigated to achieve such a result. However, this fractionation was not complete. In this purpose, the use of chemically modified membranes with a higher negative charge density would be interesting since it should increase selectivity through an increase in electrostatic interactions. Moreover, the study of the influence of other parameters on the membrane separation, like peptide hydrophobic character, would lead to a better comprehension of the mechanism of peptides fractionation.

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