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Removal of Polyphenols and Recovery of Proteins from Alfalfa White Protein Concentrate by Ultrafiltration and Adsorbent Resin Separations

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ABSTRACT

The peptides remaining after phenol removal from a co-product of the alfalfa industry have various applications: in the food industry (as an additive), in therapeutic nutrition (high-value protein, peptides with opioid or immunostimulant activities, etc.), and in cosmetics. Hydrolysis of alfalfa white protein concentrate (AWPC) by an industrial enzyme (Delvolase) improved its solubility and nutritional properties. But, colored peptidic hydrolysate was observed. This color change was due to phenolic oxidation at alkaline pH. A combined ultrafiltration process and sorption technique was investigated. A ZrO_2 Carbosep membrane (nominal molecular weight cutoff of 10,000 Da) allowed a 51% color reduction and a 96% peptide transmission. The colored peptide fraction in the permeate had a known and reproducible molecular-weight distribution. Permeate polyphenol removal was performed by a sorption technique. A polystyrene resin, Amberlite IRA900Cl, packed in a column, allowed a 92–95% phenol extraction while preserving peptide fractions.

Key Words. Alfalfa; Cross-flow ultrafiltration; Peptide separation; Polyphenol adsorption; Polystyrene resin, Amberlite

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INTRODUCTION

Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco: EC 4.1.1.39) is the world's most abundant leaf protein (1) and represents up to 70% of the leaf alfalfa proteins (2). Its aminogram is comparable to that of reference proteins such as β-casein or egg proteins, and is superior to that of other plant proteins (3). In spite of the high hydrophobicity of Rubisco, this protein presents an excellent solubility. The functional and nutritional properties of Rubisco represent a potential source of high-quality protein in animal and human feeds, and could be used in the food industry, therapeutic nutrition, or cosmetics. For several years, France-Luzerne, the leading European supplier of dehydrated alfalfa for animal feed, has been producing an alfalfa white protein concentrate (AWPC), mainly composed of white proteins (Rubisco). However, the marketing of AWPC in the human food industry was affected by the presence of phenolic constituents. Some of these are known to have anti-nutritional effects and to react with proteins and peptides. They also have a significant chemical reactivity with their hydroxyl groups, aromatic rings, and some polysaccharids (4, 5). Their oxidation in alkaline media leads to a brown-colored product. These unfavorable changes lead to a loss of product quality. To preserve the functional and nutritional properties of the final product, the ultrafiltration (UF) process seemed to be an attractive separation technique.

For the last ten years, UF has been a well-known technique in food industries for separating and concentrating macromolecule solutions (6–8) and in juice industries for clarification of fruit juices or wine (9–11). Moreover, UF is a "soft" technique because when products are ultrafiltered they are not subjected to chemical modifications. However, UF membranes in wide industrial use do not allow significant reduction in polyphenol concentration or the color of the product. Borneman et al. (9), Giovanelli and Ravasini (12) reported a removal of 40% and 12% polyphenols from apple juice, respectively. Since the resulting product retained enough color to affect marketing, other researchers combined the UF process with affinity sorptive separation.

This technique allowed adsorbtion of specific biomolecules. Two main methods were reported in the literature. In the first method, polyphenol compounds were completely removed by organic solvents. Rambourg and Monties (13) extracted alfalfa phenols by using polar solvents: 1-pentane with 2% methanol, 2-ethyl ether, 3-methanol, and 4-water. Even though this was a successful extraction method, it was not acceptable on an industrial scale because of the cost of solvent recycling. With the second technique, phenolic constituent removal was achieved by the inclusion of adsorbents in a batch reactor. The most widely used adsorbent was insoluble polyvinyl-N-pyrrolidone (PVP) (14–18). The phenolic OH groups can combine with strong-hydrogen bonds with the binding sites of PVP. Several authors (15, 19–22) showed that

polystyrene resins such as Amberlite XAD were effective for polyphenol removal. However, Lam and Shaw (23), and Loomis et al. (20) reported the more effective capacities of the anion exchangers, such as Dowex 1 and 2, to remove the plant phenols. Maier and al. (24) used adsorbent resins to obtain 25–65% color reduction from apple juice.

The aim of this study was to obtain a polyphenol–free constituent alfalfa peptide isolate, combining the advantages of a UF membrane and adsorbent. In this article the results of a study on the ability of inorganic UF membranes and adsorbent resins to remove phenolic compounds and recover peptides from AWPC is reported. The influence of several parameters on phenol removal and peptide recovery were studied: pH, temperature, amount of resin, contact time, and resin saturation. The extraction conditions were then tested and studied in a packed-bed adsorption column, before leading to a pilot scale study.

MATERIALS AND METHODS

Material and Reagents

Substrate

The alfalfa white protein concentrate (AWPC) at 8% (w/w) protein was supplied by France Luzerne (Châlons-en-Champagne, France). The protein solution was adjusted at pH 9.5 by 2M NaOH solution and diluted with distilled water to a final concentration of 3% (w/w).

Ultrafiltration Membrane Material

The UF module was composed of seven tubular ZrO_2 membranes (1.2 m long, 6 mM inner diameter, 0.16 m^2 filtering area) with a nominal molecular weight cutoff of 10 kDa (Carbosep M5). UF was performed with a tangential velocity of 4.49 $m.s^{-1}$ at 40°C.

Adsorbent Resins

High molecular weight (40 kDa) and cross-linked insoluble polyvinyl-N-pyrrolidone (PVP) was purchased from Sigma Chemical Co. (St. Louis MO, USA). Polystyrene resins, uncharged Amberlite XAD16, and cation and anion exchangers (Amberlite IRC75 and Amberlite IRA900Cl, Amberlite IRA93, Duolite A568) were supplied by Rohm & Haas S.A. (Chauny, France). Lewatit MP500 anion exchanger resin was purchased from Bayer. Magnesia and vegetable-activated charcoal were supplied by Prolabo (France).

The amount of resin was expressed as a percentage, i.e, grams of dry resin/100 mL of product (w/v).

Hydrolysis and Ultrafiltration

The hydrolysis of alfalfa white protein concentrate by Delvolase, a serine protease (Gist Brocades, Seclin, France) was investigated at pH 9.5 and at 40°C in a 8 L reaction vessel. pH, temperature, and reaction volume were continuously regulated by a pH-stat, a thermostat, and a level automatic system (Setric Hydrolysate was continuously ultrafiltered through a ZrO_2 tubular membrane (M5, Carbosep) with a permeate flow rate of 16.6 $1.h^{-1}\cdot m^{-2}$. The UF process was completed in a cross-flow mode to minimize the macromolecule accumulation onto the membrane surface.

The UF membrane was regenerated with several cleaning cycles: (1) NaOH (40 $g\cdot L^{-1}$; 60°C; 30 min), (2) water (60°C; 30 min), (3) HNO_3 (5 $g\cdot L^{-1}$; 60°C; 30 min), until the initial water permeate flux was achieved.

Adsorption Operation

Batch operation: Both alfalfa peptide permeate and dry adsorbent were stirred in a thermostat reaction vessel. When the adsorption reaction was achieved, adsorbent was removed by settling. *Laboratory scale column:* H = 18.5 cm, $\varnothing i$ = 2 cm, V = 58.199 cm^3. *Pilot scale column:* H = 58 cm, $\varnothing i$ = 10 cm, V = 4555 cm^3. Flow rate = 6.6 $mL\cdot min^{-1}$ and linear velocity of 2.11 $cm\cdot min^{-1}$.

Analytical Methods

Determination of Protein

The quantification of total protein ([pr]) was obtained with the Kjeldahl method and was expressed as total N × 6.25.

Determination of the Total Polyphenols

The concentration of total polyphenols ([Tpp]) was calculated by a modification of the standard method applied to beer. Tpp reacts with ferrous cations (Fe^{2+}), and the soluble complex exhibits an absorbance at 600 nm. The concentration of Tpp was determined by the relation $[Tpp] = A_{600nm}\cdot 820\cdot D$, where D is the dilution rate. The alfalfa peptide permeate was characterized by its absorption spectrum, between 200–500 nm. Absorbances were measured with a UV-VIS scanning spectrophotometer (Perkin-Elmer, Lambda 5).

Peptide Transmission

The transmission of peptide was calculated according to the equation $Tr\ (\%) = \frac{Np}{Nr}\cdot 100$, where Np and Nr were permeate and retentate protein concentrations, respectively.

TABLE 1
Equations for Several Hydraulic Resistances

Hydraulic resistances	Symbol	Equation
Membrane	R_m	$\frac{\Delta P}{\mu_w \cdot J_w}$
Overall fouling	R_f	$\frac{\Delta P}{\mu_p \cdot J_p} - R_m$
Irreversible fouling	R_{if}	$\frac{\Delta P'}{\mu_w \cdot J'_w} - R_m$

ΔP = transmembrane pressure (Pa); μ_w = water dynamic viscosity (Pa·s); μ_p = permeate dynamic viscosity (Pa·s); J_w and J'_w= water permeate fluxes before and after ultrafiltration operations ($m^3 \cdot s^{-1} \cdot m^{-2}$); J_p = permeate flux of protein solution ($m^3 \cdot s^{-1} \cdot m^{-2}$).

Hydraulic Resistances

After each UF run, the membrance fouling level was estimated by hydraulic resistances (Table 1), which were expressed in (m^{-1}).

Molecular Weight Distribution by Fast Pressure Liquid Chromatography

The molecular weight (MW) distribution of permeate was determined by fast pressure liquid chromatography (FPLC) on a Superdex peptide HR10/30 (Pharmacia, Uppsala, Sweden). Samples (50 μl) were eluted in 30% acetonitril–0.1% TFA buffer with a flow rate of 0.5 $mL \cdot min^{-1}$.

RESULTS AND DISCUSSION

Ultrafiltration Performances

Transmembrane Pressure

The evolution of transmembrane pressure, ΔP, versus time during the UF of AWPC hydrolysate is shown in Fig. 1. Since permeate flux was constant, ΔP increased slowly during the first 90 minutes before reaching a constant value: 1 bar. This initial increase described the membrane fouling as a function of operating time. The irreversible fouling of a membrane (R_{fi}), measured at the end of each run, was of the same order of magnitude as clean membrane hydraulic resistance (R_m):R_{fi}:R_m was 1.38. This ratio described the membrane fouling level, and it was similar to ratios determinated with other fluids: for instance, Nau et al. (6) and Taddéi et al. (25) have noted a R_{fi}:R_m ratio of 1.2–1.8

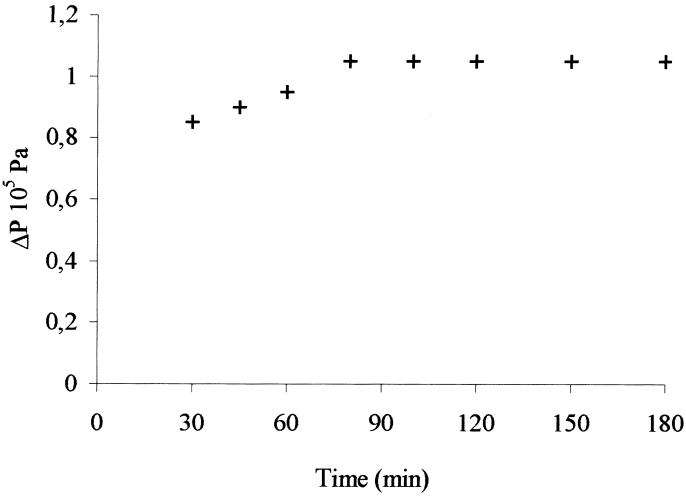

FIG. 1 Evolution of the transmembrane pressure according to time (pH 9.5 at 40°C, ZrO_2 tubular membrane with 10 kDa cutoff and 0.16 m^2).

and 2, respectively, when ultrafiltering β-casein and whey through M5 Carbosep membrane.

Quality of Permeate

The quality of permeate obtained with a pilot-scale ZrO_2 Carbosep membrane reactor was evaluated by several parameters: protein and phenolic concentrations according to UF time operation, molecular-weight peptide distributions, and absorption spectrum. As shown in Fig. 2, during the first 90 minutes of proteolytic reaction, soluble protein concentration in permeate increased rapidly until it reached a constant value of 21.7 $mg \cdot mL^{-1}$. A soluble protein equilibrium on both sides of the UF membrane (result not shown) was also observed, which indicated there was no significant rejection of hydrolysate by the membrane and no apparent product diffusion problems in the system (26). Thus, solubilized substrate in the reaction vessel went freely through the UF membrane to make up the permeate. This mass transfer was confirmed by the peptide transmission value, which was estimated at 70%.

Phenolic permeate concentration was constant (405 $mg \cdot L^{-1}$) during all UF processes (Fig. 2). With this separation technique, 51% reduction in phenolic concentration of APC hydrolysate was obtained (Fig. 3). These results were comparable to those obtained by Borneman et al. (9) for UF of apple juice

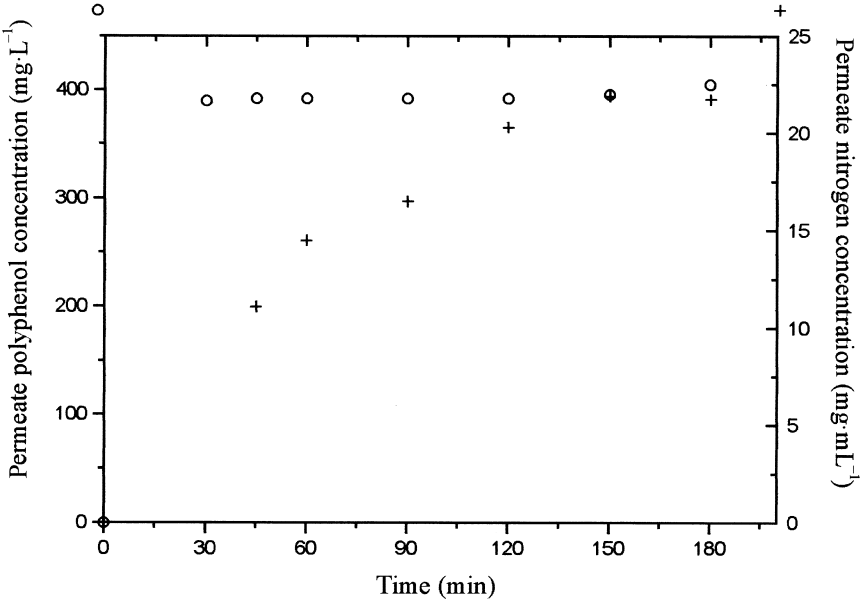

FIG. 2 Protein (+) and phenolic (o) concentrations of permeate during ultrafiltration of APC in a membrane reactor.

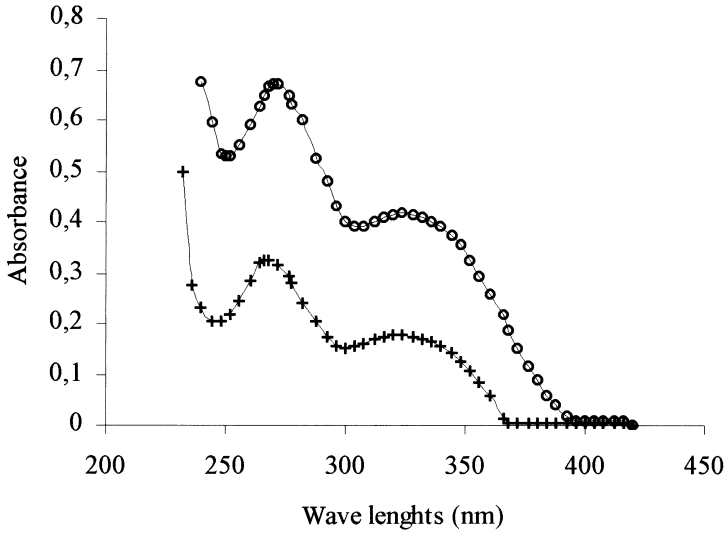

FIG. 3 Absorption spectra of hydrolysate (o) and permeate (+) AWPC (dilution 1/200).

through a 10-kDa membrane. The residual color in the permeate suggested that the remaining phenolic fraction of permeate was composed of small molecules (inferior to 10 kDa) that easily crossed through UF membranes. The alfalfa peptidic permeate (APP) was also characterized by its molecular weight distribution of peptidic fractions from 2360 Da to 90 Da (Fig. 4). In spite of color reduction of peptidic permeate by the UF process, this product must be treated again to produce an uncolored peptidic permeate.

Affinity Sorption Performances

Effect of Adsorbents

Ten adsorbents were tested for their effectiveness in removing a mineral adsorbent (magnesia), an organic adsorbent (vegetable-activated charcoal), insoluble polyvinyl-N-pyrrolidone (PVP), and seven polystyrene resins: an uncharged hydrophobic resin (Amberlite XAD 16), a cation exchanger (Amberlite IRC 75), and five anion exchangers (Amberlites IRA900CI and IRA93, Lewatit MP500CI, Duolite A568 and A103S). These experiments were carried out at pH 5.0, because this pH value reduces the initial permeate color and preserves the soluble peptidic solution (Table 2).

The various adsorbents tested bound polyphenols in different ways. Magnesia, PVP, and Amberlite IRC75 recovered more than 95% of initial protein but showed poor capacity to adsorb the phenolic compounds. Magnesia had also been tested at pH 9.5 since at this pH, magnesia decolorized bovine

TABLE 2
Capacity of 10 Adsorbents to Extract Polyphenols and to Recover Protein of APP

Nature of adsorbents	% Recovered protein	% Adsorbed polyphenols	mg of Adsorbed polyphenols/g dry resin (w/w)
Magnesia	98.5	1.4	0.12
Vegetable-activated charcoal	54.8	87.9	7.12
PVP	95.5	0	0
Amberlite XAD16	74.7	37.5	3.04
Amberlite IRC75	96.4	0	0
Amberlite IRA900Cl	82.8	65.9	5.34
Lewatit MP500Cl	84.0	50.1	4.06
Amberlite IRA93	92.1	58.2	4.72
Duolite A568	84.0	49.8	4.04
A103S	77.4	39.5	3.2

[pr] = 21 mg·mL^{-1}; [Tpp] = 405 mg·L^{-1}. Extraction with 5% of adsorbent (w/v) at pH 5.0 and at room temperature for 2 hours under agitation.

haemoglobin (29). Although 60% phenol adsorption was observed, protein remaining in solution was very low. At pH 5.0, insoluble PVP did not allow phenol removal of the APP. However, it has been widely used to extract polyphenols in solution (16, 20). This adsorbent is a strongly basic exchanger of anions. Its structure, made up of CO–N groups, is a stronger acceptor of protons and makes it possible to form a stable complex with OH groups of phenols, thanks to hydrogen interactions. Moreover, the PVP binds preferentially to polyphenols of a molecular weight higher than that of the chlorogenic acid (354 Da) (15). But the main AWPC phenols (coumestrol, apigenin 4,7 dihydroxyflavone, and coumaric acid) have a molecular weight below 270 Da. The results obtained with Amberlite IRC75 were corroborated by those of Fukushima et al. (18): cation exchangers had very weak affinities for plant polyphenols. At pH 5.0, polyphenols were not ionized and thus no interaction can be established between polymers and phenols.

Amberlite XAD16 and Amberlite A103S had similar performances, although their nature was different. These two resins presented a low capacity to remove phenols (38%) but retained 75% of the initial protein. Amberlite XAD16 is hydrophobic cross-linked polymer that derives its adsorptive properties from its potential macroreticular structure, its high surface area (800 $m^2 \cdot g^{-1}$), and the aromatic nature of its surface. Gray (16) suggested that the adsorption of polyphenols by polystyrene resins was predominantly due to hydrophobic interactions with the aromatic rings of phenol compounds. The chromatographic profile carried out by RP-HPLC (results not shown) confirmed the mechanism of hydrophobic peptide adsorption of the resin: hydrophobic peptides disappeared after contact of APP with Amberlite XAD16. This resin had a low capacity to remove polyphenol constituents (37.5%), as shown by results from Fukushima et al. (18), but disputed by those of Loomis et al. (20) and Maggi et al. (27). Indeed, the latter showed that Amberlite XAD16 extracted polyphenols in aqueous solution. This removal was all the more effective as the pH was acid. The adsorption of the aromatic acids and polyphenols on the Amberlite XAD16 was favored when the pH was close to the pKa. Loomis et al. (20) reported that phenols were strongly adsorbed by uncharged polystyrene resins such as Amberlite XAD, but the latter had little affinity for condensed tannins. Phenolic removal had also been tested in alkaline pH. At pH 9.5, the level of protein remaining improved (81.5%) but phenol removal decreased (24.6%). Amberlite A103S was an anion-exchanger polystyrene resin, but its phenol-removal capacity was inferior to that of anion exchangers tested in this work.

The best extraction rates were obtained with activated vegetable charcoal (87.9%) and Amberlite IRA93 (58.2%) but also with the other anion-exchanger polystyrene resins: Amberlite IRA900Cl (65.9%), MP 500Cl (50.1%), and Duolite A568 (49.8%). Giovanelli and Ravasini (12) had also re-

ported an important color reduction (22 to 47%) of apple juice, when they performed phenolic extraction with a different activated-charcoal amount. But when the phenol removal increased, the protein remaining in solution decreased: 45.2% and 7.9%, respectively, to activated vegetable charcoal and Amberlite IRA93. The three other anion exchangers presented an excellent protein yield (84%). These results confirmed those obtained by Loomis et al. (20), who used anion-exchanger resins containing quaternary ammonium groups on a styrene–divinylbenzene matrix [Dowex 1 Φ-$CH_2N^+(CH_3)_3$; Dowex 2 Φ-$CH_2N^+(CH_3)_2(C_2H_4OH)$]. The plant phenols bound with adsorbent by ionic and hydrophobic interactions. Amberlite IRA900C1 and Lewotit MP 500Cl behaviors were similar because they contained quaternary ammonium groups on a styrene divinylbenzene matrix.

These results suggested that Amberlite IRA900Cl was an excellent compromise between the capacity to extract polyphenols from APP (5.34 mg TPP/g dry resin or 65.9% of removal) and the protein remaining in solution (82.8%). All the following tests were carried out with the Amberlite IRA 900Cl.

Effect of Temperature

The effect of temperature on phenolic removing and peptidic recovering by Amberlite IRA900Cl was studied between 25°C and 60°C. No significant improvement was observed according to the temperature. Thus, for cost effectiveness, extraction of polyphenols was performed at room temperature (25°C).

Effect of pH

The influence of pH on the adsorption of polyphenols and the recovery of proteins by Amberlite IRA900Cl (strongly basic anion-exchanger resin) was studied at 5.0–9.5 pH, since peptide solubility decreased strongly for more acid pH. The peptide solution (pH 9.5) was adjusted with 0.5M–10M HCl to obtain the studied pH. Then it was put in contact with 5% (w/v) of resin and mixed for 2 hours at room temperature. Figure 4 showed the influence of pH on the recovery of proteins and on the removal of polyphenol molecules of APP. The optimal output of recovery of the protein compounds was obtained with pH 5.0 (82.8%), whereas polyphenol removal was reached at alkaline pH (pH 9.5): 81.2%. In the pH range from 5.0 to 9.5, the carboxyl groups of peptides were fully dissociated (COO^-). The amine groups were entirely protonated at pH 5.0, more or less protonated at pH 6.0–9.5, and fully unprotonated above pH 9.5 (28).

The ionization level of the carboxyl and amine groups at pH 5.0 could explain the results observed in Fig. 5. Whereas the positive binding site of Am-

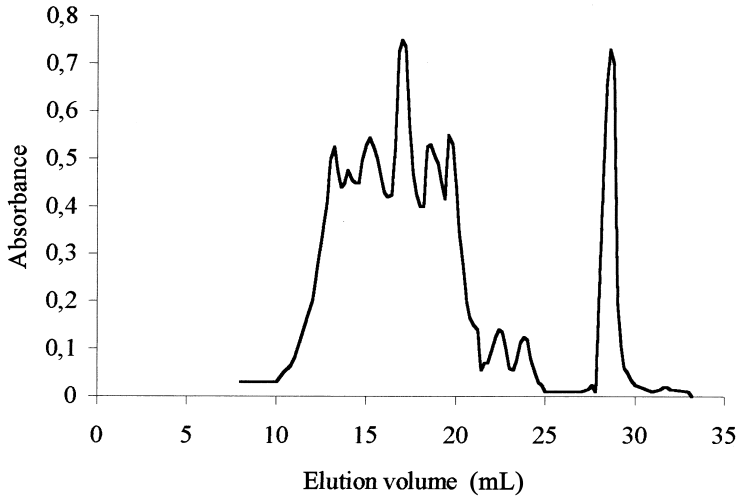

FIG. 4 Molecular weight distributions of the soluble peptidic fractions of the permeate in a membrane reactor in steady state.

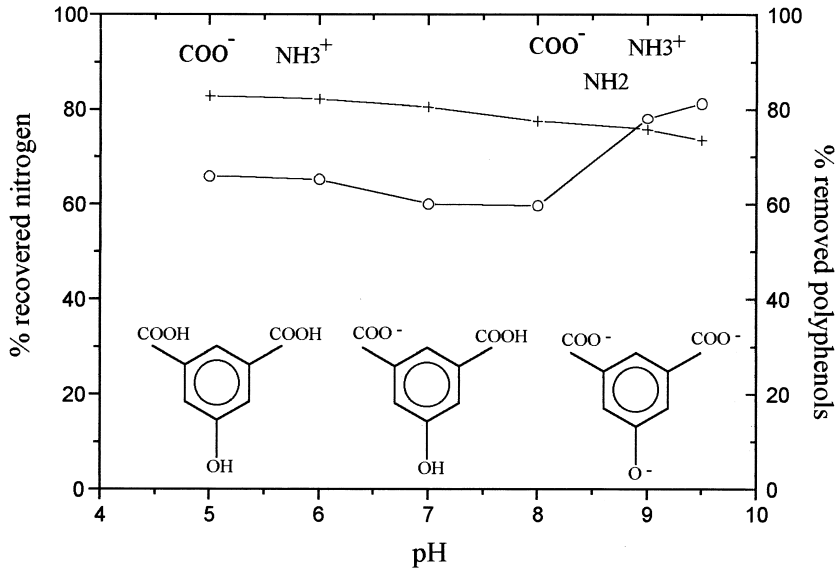

FIG. 5 Influence of pH on the removal of phenols (o) and the recovery of the protein (+) compounds from a solution of APP. $[pr] = 21\ mg \cdot mL^{-1}$ and $[Tpp] = 405\ mg \cdot L^{-1}$. Contact with 5% Amberlite IRA900Cl under agitation for 2 hours at room temperature.

berlite IRA900Cl resin (quaternary ammonium) pushed back the amine group (NH_3^+) peptides, they attracted the more or less ionized polyphenol hydroxyl groups as well as the peptide carboxyl groups. Hydrophobic interactions between the structure of polymers and polyphenols were also established. For a neutral pH, the hydroxyl groups of the aromatic ring of phenols were not charged (OH). That was why a resin capacity decrease in ability to bind polyphenols can be observed. For raised pH, the phenolic compounds were strongly ionized. Moreover, the strong alkalinity of the functional groups of the resin [$-N^+(CH_3)_3$] allowed the combination of all anions, including those slightly dissociated. At this pH, a sudden increase of the amount of removed polyphenols by the resin (81.2%) was noted. These results confirmed the reports of Lam and Shaw (23) and Gray (16) of the binding capacity of Dowex 1-X8 (anion-exchanger resin) with plant phenols, and those of Loomis et al. (20), who suggested that the high pH used by Lam and Shaw (23) and Gray (16) was not necessary. Moreover alkaline pH increased the ionization of phenolic hydroxyl groups and thus promoted oxydation of phenols, with subsequent protein modification. High pH also increased the proportion of protein amino groups in the reactive-NH_2 form, making them more susceptible to additional reactions (20).

Effect of the Amount of Resin

The influence of the amount of resin on the removal of phenols of APP and the recovery of protein compounds was studied in a range of 1–20% (w/v) (Fig. 6). Small amounts of resin (1%) preserved 97% of initial protein, but only 13% of polyphenols were adsorbed. In this case, the polystyrene resin was quickly saturated. The amount of polyphenols (405 $mg \cdot L^{-1}$) was largely in excess with respect to the number of resin-binding sites. For increasing amounts of resin (5–20%), protein concentrations decreased progressively (82.8–62.3%), whereas removed phenol concentrations tended toward a constant value (86%) as soon as the amount of resin was equal to or higher than 10%. In these conditions, the number of binding sites of resin relative to polyphenol was in excess. Ionized (COO^-) groups of peptides can bridge with free binding sites of resin, leading to a decrease of recovered proteins. Moreover, in spite of a large excess of resin relative to polyphenols, 14% of phenols did not react with adsorbent. These results could be explained by the structure and the molecular size of the phenolic compounds, which inhibited interactions by binding the sites of the resin; thus, selectivity of the resin with respect to some phenol constituents was noted. Since the objective was to preserve the greater peptide concentration, the next experiments were carried out with 5% (w/v) of resin. In these conditions, phenol (65.9%) and protein (82.8%) outputs were acceptable.

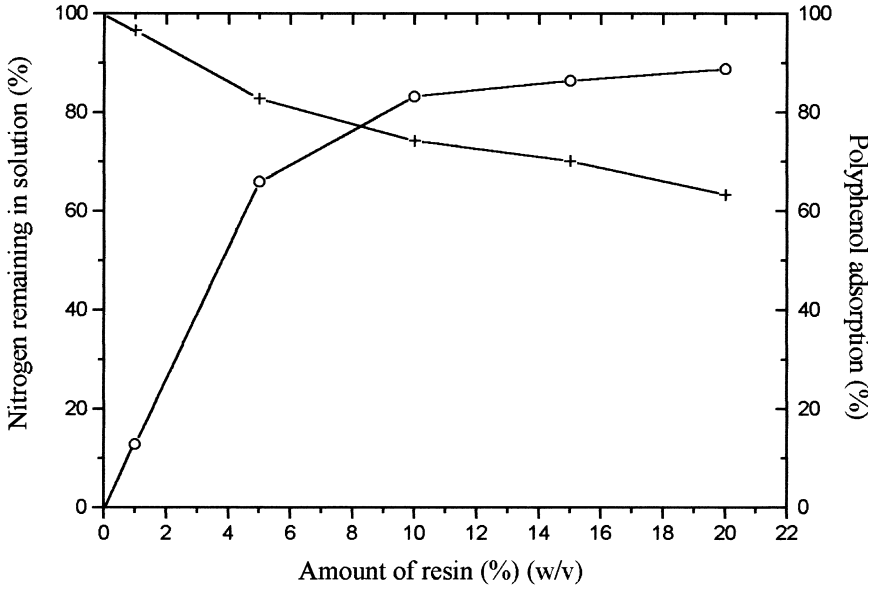

FIG. 6 Influence of the amount of resin on the polyphenolic removal (o) and the protein recovery (+) compounds from APP. $[pr] = 21\ mg \cdot mL^{-1}$ and $[Tpp] = 405\ mg \cdot L^{-1}$. Contact between APP (pH 5.0) and Amberlite IRA900Cl under agitation for 2 hours at room temperature.

Effect of Residence Time

Polyphenol adsorption and protein recovery at different times of contact with Amberlite IRA900CI were investigated between 30 minutes and 24 hours (Fig. 7).

Lower contacts (30 min) between resin and APP led to a low adsorption of phenolic compounds (30%), but Amberlite IRA900Cl required a minimal time of contact with the product to be operational (30 min). Between 30 minutes and 2 hours, the amount of removed polyphenols increased suddenly, thus increasing from 30 to 66% before being constant. Longer contact times did not significantly improve extraction. However, an additional 2.9% polyphenol removal was obtained between 2 and 24 hours. This behavior suggested a steric hindrance of the polyphenol molecules with respect to resin binding sites. It is assumed that polyphenolic modification favored a polymerization reaction between phenolic molecules (buildup of a phenol–phenol complex). Thus, 2.9% polyphenols remaining in solution reacted with the free sites of other phenolic molecules. These results suggested that a residence time of 2 hours re-

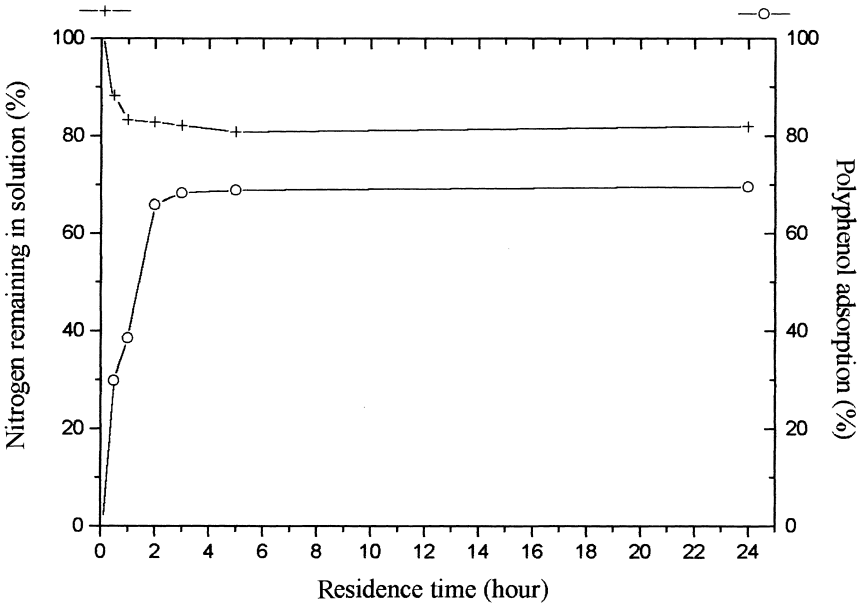

FIG. 7 Influence of residence time on the polyphenolic removal (o) and the protein recovery (+) from a solution of APP. $[pr] = 21\ mg \cdot mL^{-1}$ and $[Tpp] = 405\ mg \cdot L^{-1}$. Contact with 5% Amberlite IRA900Cl at pH 5.0 under agitation at room temperature.

moved 65.9% of polyphenols and recovered 82.8% of the protein compounds from APP.

Substitution of the Saturated Resin by New Resin

The best extraction performance of the APP by Amberlite IRA900Cl (5%) is obtained with a contact time of 2 hours. However, the final product still retains 35% of the initial polyphenols. To remove this phenolic fraction, fresh resin replaced the saturated resin and this operation was repeated until a peptide solution free from polyphenols was obtained, i.e., a colorless solution. The results are summarized in Table 3.

By changing the resin three times (every two hours), 90% of initial polyphenols were extracted, but a 5% protein loss appeared at the time of the third passage (77% instead of 82%). More substitutions of resin did not improve the quality of APP. In spite of these successive extractions, 10% of the initial polyphenols remained in solution. It was possible, on the one hand, that this fraction was made up of polyphenols having a molecular structure that did not

TABLE 3
Influence of Fresh Resin Contribution on the Decolorization of the APP

Number of substitutions: Ri	% Recovered protein (±1.3%)	% Removed polyphenols (±1.9%)
R1	82.3	65.9
R2	81.9	85.9
R3	76.8	90.6
R4	76.8	89.8

[pr] = 21 mg·mL^{-1}; [Tpp] = 405 mg·L^{-1}. Contact with 5% of Amberlite IRA900Cl at pH 5.0 under agitation for 2 hours at room temperature.

bridge with resin, and on the other hand, these polyphenols were bound in a covalent way or not to peptides in solution. It seemed impossible to remove these 10% polyphenols under these working conditions. The results confirmed the assumption put forth previously: there was a steric hindrance of polyphenols on the resin surface.

The aim of this study was to produce a decolorized protein solution at a pilot scale and in a continuous mode, yet the pH of the APP solution did not evolve during extraction process. Thus, it is possible to work with resin in a column since, with this new configuration, APP would continuously come in contact with new resin during its elution through the column.

Extraction of Polyphenols from APP in a Column Process

The batch-extraction step allowed the selection of one adsorbent and of the operating conditions. Batch conditions were then transposed in an equivalent column process. To respect the product amount: dry resin amount ratio (i.e., 5%), APP (pH 5.0) was eluted at 6.66 mL·min^{-1} (or 7 BV·h^{-1}) through a column with a bed volume (BV) of 58.2 cm^3. As shown in Fig. 8, after 10 BV, removal of polyphenols in the column decreased because 43% of the initial phenols were present in the permeate output. At 10 BV, peptide concentration was optimum (18 mg·mL^{-1}). With a lower flow rate (1 mL·min^{-1}), column performances decreased after 20 BV. The column configuration allowed significantly increased phenol extraction: 96% of the initial peptides were recovered and 92% of the polyphenol compounds were removed. It would be impossible to reach 100% of phenolic extraction under these conditions, since these 8% remaining polyphenols (results similar to those found for the batch process) were probably bound covalently to proteins of the APP.

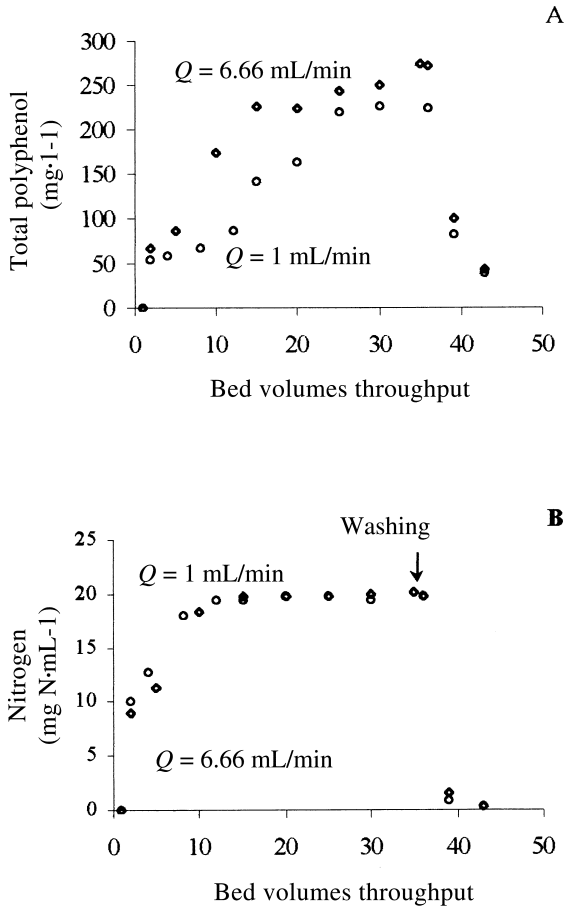

FIG. 8 (A) Phenolic removal (o) and (B) protein recovery (+) from a solution of APP. [*pr*] = 21 mg·mL^{-1} and [*Tpp*] = 405 mg·L^{-1}.

Influence of the Flow Rate

To increase the removed phenol amount, several flow rates were tested: 0.48–6.66 mL·min^{-1}. The amount of removed phenol constituents (92%) was constant, whatever the product flow rate (Fig. 9).

Recovered peptide concentration decreased when the flow rate was increased: a flow rate of 0.48 mL·min^{-1} allowed for maximal protein output (96%), whereas flow rates inferior to 2 mL·min^{-1} led to low protein concen-

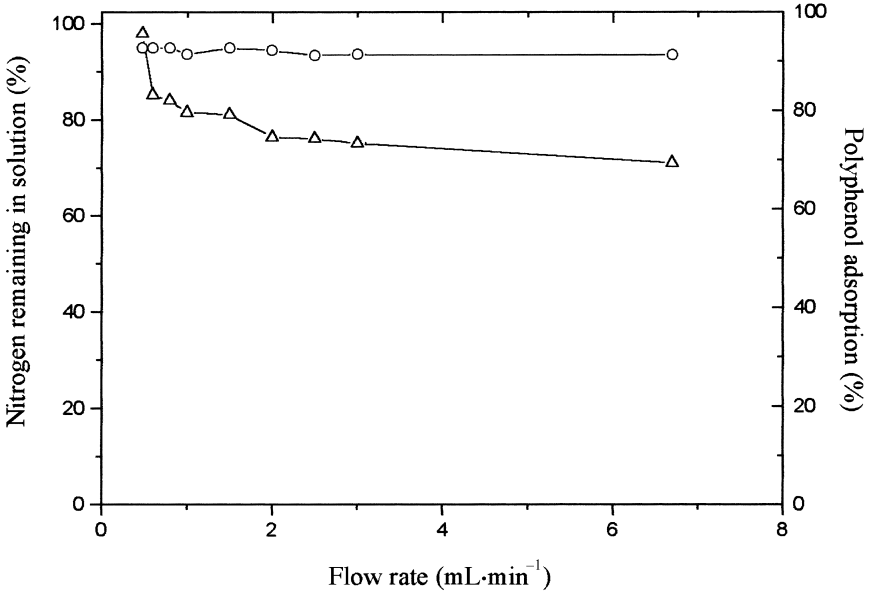

FIG. 9 Influence of the product flow rate on the phenolic removal (o) and the protein recovery (Δ) from a solution of APP. $[pr] = 21$ mg·mL^{-1} and $[Tpp] = 405$ mg·L^{-1}.

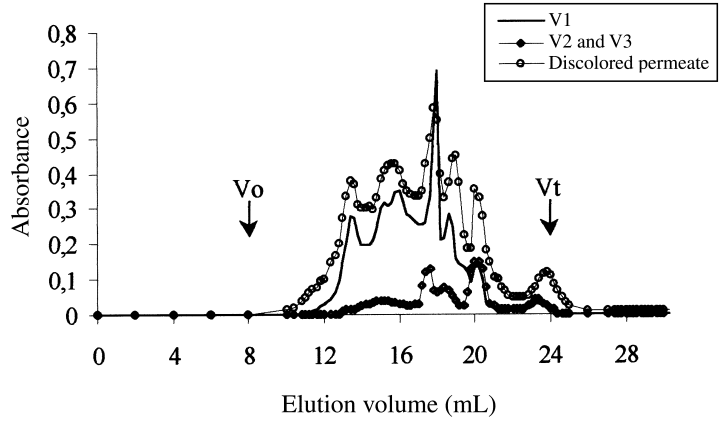

FIG. 10 Influence of product flow rate on the recovery of peptide fractions. $Q = 3$ mL·min^{-1}, pH 5.0 at room temperature.

TABLE 4
Pilot Scale Production of a Peptide Permeate Without Polyphenol from an Alfalfa Protein Concentrate

Process steps	Amount of protein (g)	Recovered protein yield (%)	Amount of polyphenol (g)	Phenolic extraction yield (%)
Hydrolysate	1224	100	33.4	0
Permeate	816	67	15.8	53
Discolored permeate	750	92	1.8	89
Total yield (%)		61		94

trations (up to 75%). Since APP was characterized by a 2,360–90 Da peptide fraction, it is possible that large peptides diffused quickly through the column, whereas others permeated more or less inside the resin pores and would be eluted later. Since only one bed volume runs with APP solution, it can be hypothesized that short peptides cannot be eluted. This is why the peptide concentration decreased. In this case, the resin-column process behaved like ion-exchanger and adsorption chromatographies. To confirm this assumption, a bed volume ran with APP, followed by several water bed volumes (Fig. 10). The first bed volume (V_1) recovered 74.3% of peptide constituents. The next two (V_2 and V_3) recovered short peptides. These results confirmed the feasibility of working with a high product flow rate to preserve performances of the packed-bed adsorption column.

Pilot Scale Discoloration

Forty liters of alfalfa peptide permeate were run through the pilot packed-bed adsorption column containing IRA900Cl. The performances obtained with this column were similar to those of laboratory-scale columns (Table 4). The removed phenol yield was 94%, whereas the total recovered protein yield was 61%.

CONCLUSION

This study has showed the feasability of combining the advantages of UF membrane and sorption techniques to develop and optimize the production of alfalfa-peptide discolored permeate on a pilot-plant scale. Thus, with a continuous ZrO_2 Carbosep membrane (10-kDa) reactor and a permeate flux of 16.6 $1 \cdot h^{-1} \cdot m^{-2}$, an alfalfa peptidic permeate yield of 340 g product$\cdot h^{-1} \cdot m^{-2}$ was reached. This UF process was followed by a phenolic removal of alfalfa peptidic permeate. This extraction was achieved in a packed-bed adsorption column with a polystyrene resin, Amberlite IRA900Cl. This step led to 94%

initial polyphenol decrease. The overall purification and separation process continually produced, a reproducible and characteristic peptidic decolorized permeate. To promote this peptidic decolorized permeate, an electrodialysis then an atomization of the product will be completed. The functional and nutritional properties of the peptidic uncolored powder, which represents a potential source of high-quality protein, have been studied in detail and will be published in a subsequent article.

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