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Fractionation and Enzymatic Hydrolysis of Soluble Protein Present in Waste Liquors from Soy Processing

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A waste effluent from a soymeal concentrates plant was centrifuged and ultrafiltered by successive processing in membranes of 10, 30, and 50 kDa, with further concentration of the resulting stream using a 5 kDa membrane. The separated fractions (5−10, 10−30, 30−50, and >50 kDa) were subjected to chemical, nutritional, and functional characterization. Resuspension of the retentates in salt-containing systems improved the protein solubility, the emulsifying capacity, and the gelation capacity, whereas the emulsion stability and the foam capacity and stability decreased with respect to the values obtained using distilled water, and the oil absorption capacity was not affected. The digestibility of the fraction >50 kDa was comparable to that of casein. The fractions of higher molecular mass (30−50 and >50 kDa) were subjected to enzymatic hydrolysis with a commercial protease, to give products with improved emulsifying activity and stability, particularly for hydrolysates with a degree of hydrolysis between 20 and 30%.

KEYWORDS: Ultrafiltration membranes; soy protein; proteolysis; functional properties

INTRODUCTION

Processes for producing soy protein concentrates by acid extraction lead to waste liquid effluents containing mono- and oligosaccharides, salts, and soluble protein. The separation and valorization of individual components from this effluent are alternatives to the conventional wastewater treatment. Valorization of waste streams could be based in the nutritional quality and functionality of acid soluble vegetal protein and the biological activity of soy oligosaccharides, which are gaining commercial importance for their prebiotic activity. Both the soluble vegetal protein and the soy oligosaccharides can be used in the formulation of foods, cosmetics, and pharmaceuticals.

Membrane technologies are used in food and pharmaceutical industries as environmentally friendly methods for concentrating and/or fractionating waste liquid effluents. Protein recovery by membrane operation is a well-known technology, and applications in soy industry have been extensively reported for producing isolates either from full fat soybeans (*1*, *2*) or from defatted soy flour (*3*), as well as for the fractionation of soy protein isolates (*4*) and hydrolysates (*5*, *6*). Membrane-fractionated proteins have better functional properties than those produced by isoelectric precipitation (*4*, *7*). In addition, oligosaccharides (*6*, 8) and phytate (*2*) can be efficiently separated from protein by membrane processing.

Enzymatic hydrolysis improves the functional properties of soy proteins (*6*, *9*, *10*), leading to products widely used for special nutrition. Proteolysis gradually unfolds the protein chains (developing hydrophobicity), reduces the incidence of allergenic factors, and can result in the formation of small peptides with biological (antioxidant, antihypertensive, antithrombotic) activities. On the other hand, peptides below 1000 Da are much less bitter than fractions with higher molecular mass (*11*).

This work deals with the experimental assessment of separation, recovery, and enzymatic modification of the protein present in the effluent from a soy concentrates plant (denoted ESCP). The global objective is to utilize the valuable compounds present in this waste stream (protein, sugars) by selectively separating and concentrating them in membranes. Sequential processing in ultrafiltration membranes with increasing cutoff was used for separating different protein fractions, which were characterized. Further enzyme hydrolysis was carried out with fractions having high molecular masses and/or inhibitory and allergenic activities. The nutritional value and functionality of resulting hydrolysates were evaluated.

MATERIALS AND METHODS

Materials. The liquid effluent (denoted ESCP) from a pilot plant of soy concentrates (manufactured by extraction with acetic acid−acetate buffer at room temperature) was kindly provided by MOYRESA (A Coruña, Spain). The liquors contained 12.50 g protein/L, 15.00 g suspended solids/L, 2.20 g ash/L, 8.07 g monosaccharides/L, 18.69 g disaccharides/L, 23.01 g higher oligosaccharides/L, and 44.53 g/L of other nonvolatile components.

Ultrafiltration. Fractionation of the soluble protein from ESCP was accomplished by ultrafiltration (optionally with a previous centrifugation step) through a Filtron unit (Pall Corporation, Madrid, Spain) fitted with 5, 10, 30, or 50 kDa molecular mass cutoff Omega membranes (*12*). The permeates obtained from the membranes of 10, 30, and 50

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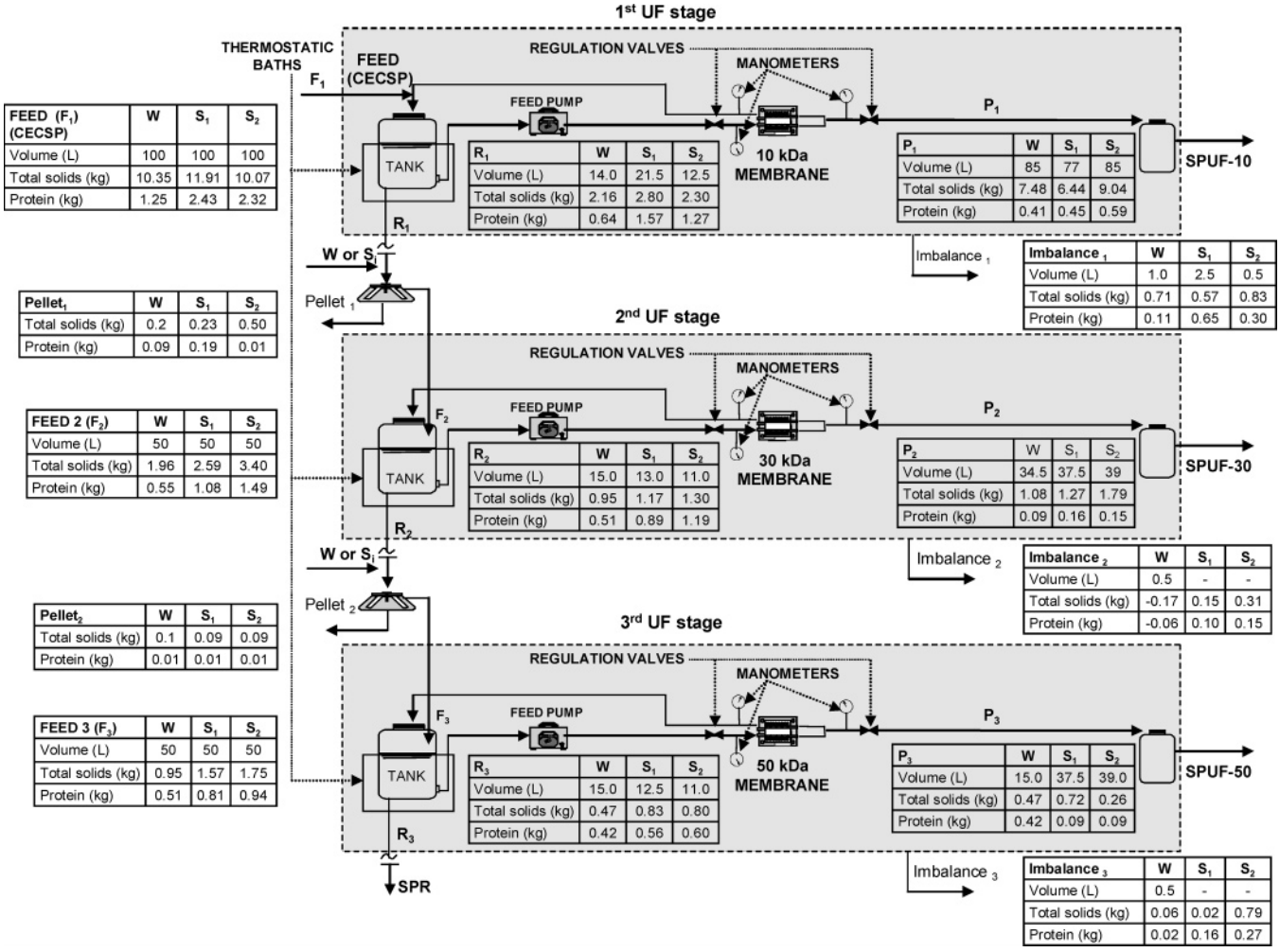

Figure 1. Flow diagram and proximate composition in solids and protein in the streams involved in the successive UF process using distilled water (W), ammonium acetate (S_1), or diammonium phosphate (S_2) as agents for dilution during processing of the centrifuged effluent from a plant of soy protein concentrates (CESCP). F, feed to the UF membrane; P, R, permeate and retentate from the UF membrane; and SPUF, soy protein ultrafiltered through the corresponding UF membrane and further retained in the 5 kDa membrane.

kDa were concentrated through 5 kDa membranes and dehydrated by freeze drying. The retentate from the 50 kDa membrane was dehydrated as described above. The centrifuged ESCP (denoted CESCP) was processed in multiple steps according to the flow diagram shown in **Figure 1** using a sequence of membranes with increasing cutoffs, as previously used by Deeslie and Cheryan (*5*). CESCP was first passed through the 10 kDa membrane (first UF stage), and the retentate was diluted with either distilled water or salt solutions to achieve a volumetric protein concentration similar to the one of CESCP. This solution was further submitted to ultrafiltration through the 30 kDa cutoff membrane (second UF stage), and the retentate was diluted again before filtration through the 50 kDa membrane (third UF stage). The permeates from the different membranes were further concentrated using the 5 kDa membrane and freeze-dried to yield the following fractions: soy protein ultrafiltered through the 10 kDa membrane (denoted SPUF-10), soy protein ultrafiltered through the 30 kDa membrane (denoted SPUF-30), soy protein ultrafiltered through the 50 kDa membrane (denoted SPUF-50), and soy protein retained by the 50 kDa membrane (denoted SPR). When required, the protein concentrates were diluted in salt-containing solutions to avoid protein precipitation of the retentate streams.

Protein Hydrolysis. Hydrolyses of SPUF-50 and SPR fractions were carried out using the commercial protease Flavourzyme 500L (kindly provided by Novo Nordisk, Spain), with 500 LAPU (leucin amino peptidase units)/g enzyme. The enzyme/substrate ratios used were in the range of 10−25000 LAPU/g protein. Hydrolyses were carried out for 0.5, 1, 2, or 4 h in stirred vessels at 50 °C. The enzyme was deactivated by heating the sample for 10 min at 70 °C. The degree of hydrolysis (DH) was measured using TNBS (trinitrobenzene sulfonic acid) as proposed by Adler-Nissen (*13*). The sample (250 μL) was mixed with phosphate buffer, pH 8.2 (2 mL), and 1% TNBS (Sigma Chem. Co.) (2 mL) in capped tubes, which were incubated at 50 °C for 1 h. Then, 4 mL of 0.1 N HCl was added to each tube and the absorbance was measured at 340 nm after standing for 30 min at room temperature and quantified using a calibration line made with D,L-leucine (Sigma Chem. Co.). The total number of amino groups was determined in a sample completely hydrolyzed with 6 N HCl at 120 °C for 24 h.

Analytical Methods. *Moisture and ash* were determined according to standard methods (*14*). The protein content in the solids was analyzed for total nitrogen by Kjeldhal, using the factor 6.25. The protein concentration in the liquid phase was determined by the Lowry method with bovine serum albumin (Sigma Chem. Co.) as the standard.

Gel permeation chromatography (GPC) of the samples was conducted on BioGel P-100 and BioGel P-30 columns (BioRad). The degassed gel was equilibrated with 0.1 M phosphate buffer, pH 7.0, packed in a column (2.5 cm × 75 cm), and contacted for 48 h with phosphate buffer for equilibration. The void volume was determined using γ-globulin (145 kDa). One milliliter of sample containing 10 mg protein/mL was loaded onto the column and eluted with phosphate buffer. The column was calibrated with albumin (molecular mass of 66 kDa), carbonic anhydrase (29), myoglobulin (17), cytochrome C (12.4), and vitamin B_{12} (1.35). Fractions of 3.0 mL were collected using an automatic fraction collector (Biologic Biofrac Fraction Collector),

and the absorbance of the fractions was measured using a Biologic Quadtec UV−vis detector at 280 nm.

In vitro digestibility was measured by the apparent digestibility coefficient (ADC) assay using trypsin, chymotrypsin, and peptidase (*15*). Available lysine was determined by the TNBS method, based on the reaction with the ϵ-amino group, characteristic of the lysine available to the organisms, using D,L-lysine (Merck, Darmstadt, Germany) as a standard (*16*).

Functional Properties. *Emulsifying Properties*. The emulsifying activity index is abbreviated EAI, and the emulsifying stability index is abbreviated ESI. Twenty milliliters of a 0.1% (w/v) protein solution (pH 7.0) were homogenized with 6.6 mL of soy oil during 1 min in an Ultraturrax T-50. Immediately after homogenization, aliquots of 50 μL were diluted to 5 mL with 0.1% sodium dodecyl sulfate (SDS), and the absorbance (500 nm) was determined using an Agilent 8453 spectrophotometer. The parameters ESI and EAI were calculated according to the following equations (*17*):

$$\text{ESI (min)} = T_0 \cdot t/(T_0 - T_{10})$$

$$\text{EAI (m}^2\text{/g)} = 2 \cdot [2.303 \cdot A \cdot D/(\phi \cdot C \cdot L \cdot 10)]$$

where T_0 and T_{10} are the turbidities measured at the beginning and after 10 min, t is the time (10 min), A is the observed absorbance, D is the dilution factor, ϕ is the volume fraction of the dispersed phase (oil), C is the weight of protein per unit volume (g/L) of aqueous phase before the emulsion is formed, and L is the path length of the cuvette (cm).

Whippability and Foam Stability. A 0.25 g amount of each sample was mixed with 25 mL of distilled water in an Ultraturrax for 2 min. After blending, each sample was transferred into a 100 mL graduated cylinder. The blender jar was rinsed with 5 mL of distilled water, which was added to the graduated cylinder. The volume was recorded before and after whipping and measured as the percent of volume increase due to whipping. Foam volume changes in the graduated cylinder were recorded at intervals of 1, 10, 30, 60, 90, and 120 min.

Gelation. Sample dispersions of 2, 4, 6, 8, 10, 12, 14, and 16% (w/v) were prepared in 50 mL of distilled water according to Coffmann and García (*18*). Each dispersion was adjusted to pH 7.0 with 0.1 N NaOH or 0.1 N HCl and mixed for 2 min. Five milliliter aliquots of the dispersions were poured into test tubes, heated to 100 °C in a water bath for 1 h, and cooled to 4 °C for 2 h. The least gelation concentration (LGC) was determined after heating in boiling water for 1 h followed by cooling for 2 h at 4 °C.

Oil Absorption. A 0.5 g amount of each sample was mixed with 50 mL of oil in an Ultraturrax, allowed to stand at room temperature for 30 min, and centrifuged at 5000*g* for 30 min, and the liquid retained by the solids was measured. The oil absorption capacity was expressed as g of oil bound per g of dry sample (*19*).

RESULTS AND DISCUSSION

Preliminary SDS−polyacrylamide gel electrophoresis (PAGE) assays of the protein in ESCP revealed the presence of 59.6, 38.9, 32.9, 28.9, 24.4, and 19.7 kDa fractions. The protein fractions with molecular masses in the range of 38.9−19.7 kDa corresponded to the ones reported for trypsin inhibitors (*20*) and allergenic factors (*21*). Separation of these fractions by membrane technology and further enzyme hydrolysis to (i) destroy undesirable factors or (ii) improve functionality was addressed in the present work. Enzyme modification of the highest molecular weight protein fraction recovered was also proposed with the aim of improving nutritional and functional properties for their application as additives or foods.

Effect of the Presence of Suspended Solids in the ESCP. The effluent used in this work contained (as an average) 1.50 wt % of suspended solids, which can affect the ultrafiltration process in two opposite ways: causing direct fouling by deposition and promoting turbulence in the liquid feed, which would lead to decreased polarization concentration and thus to increased membrane fluxes (*22*). In this field, Noordman et al. (*23*), dealing with the ultrafiltration of soy flour extracts, reported that rinsing membranes with 1% suspended milled beans (25 μm) resulted in higher fluxes and avoided reversible fouling. To test if the plant effluents as received (containing suspended solids) could be suitable for membrane processing and the effects caused by the removal of suspended solids, permeate fluxes were measured for streams ESCP and CESCP in experiments with 10, 30, or 50 kDa membranes. No significant differences in the normalized stationary permeate fluxes (NPF_{st}) were observed between ESCP and CESCP in assays with the 30 and 50 kDa membranes, whereas the NPF_{st} determined for the 10 kDa membrane was twice as high for CESCP than for ESCP (**Figure 2a−c**). **Figure 2d−f** shows the protein rejection for the 10, 30, and 50 kDa membranes (measured by the slope of the lines). The higher rejection was observed in the experiment with ESCP using the 10 kDa membrane (**Figure 2d**). CESCP showed lower rejections than ESCP in the assays with 10 and 30 kDa membranes. As a general trend, the protein rejection in experiments with CESCP decreased when the cutoff of membranes increased. The following experiments were performed using CESCP, owing to its better performance with the 10 kDa membrane.

Successive Ultrafiltration. Figure 1 shows data on the streams involved in the successive ultrafiltration of CESCP through membranes of increasing cutoffs, when the retentates were mixed with distilled water (column W). With this experimental set up, more than 90% of the solids and 80% of the protein initially present in the streams fed to the membrane units were recovered in the corresponding permeates and retentates. The imbalances were ascribed to retention of solutes into membranes and tubes by experimental error caused by the extensive manipulation of samples. However, the amounts and compositions of the several streams provided a semiquantitative estimate of the behavior of components along the three ultrafiltration steps. The main solutes permeating the 10 kDa membrane were buffer salts, proteins, and mono- and disaccharides. The interstage reposition of water (stream W in **Figure 1**) decreased the ionic strength of the medium, leading to solute precipitation.

Freeze-dried aliquots of streams P1, P2, and P3 in **Figure 1** were assayed for total solids and protein. Half of the protein initially present in CESCP was retained in this membrane, most of it having a molecular mass higher than 50 kDa. The SPR and SPUF-10 fractions accounted for 33.6 and 32.8% of the protein contained in CESP, whereas SPUF-30 was present in low proportions. The mass fractions of proteins in freeze-dried solids obtained from streams P1, P2, P3, and R3 increased with the molecular masses of the fractions to reach values of 0.055, 0.083, 0.17, and 0.89 g protein/g freeze-dried solids, respectively.

The GPC elution profiles of the ESCP liquor and the higher molecular mass fractions ultrafiltered from the CESCP are shown in **Figure 3**. The chromatogram of the ESCP (**Figure 3a**) showed peaks at 22.4, 6.4, and 1.9 kDa. The peaks appearing at elution volumes higher than 280 mL corresponded to small peptides (with molecular masses below 690−300 Da), which were not retained by membranes. The peak eluting at 92 mL corresponded to the elution volume of the column (Vo). The peaks with molecular masses in the range of 32−39 kDa, which were previously separated by SDS−PAGE, could not be resolved by GPC. The GPC chromatogram of the suspended solids separated by centrifugation (**Figure 3b**) showed a peak

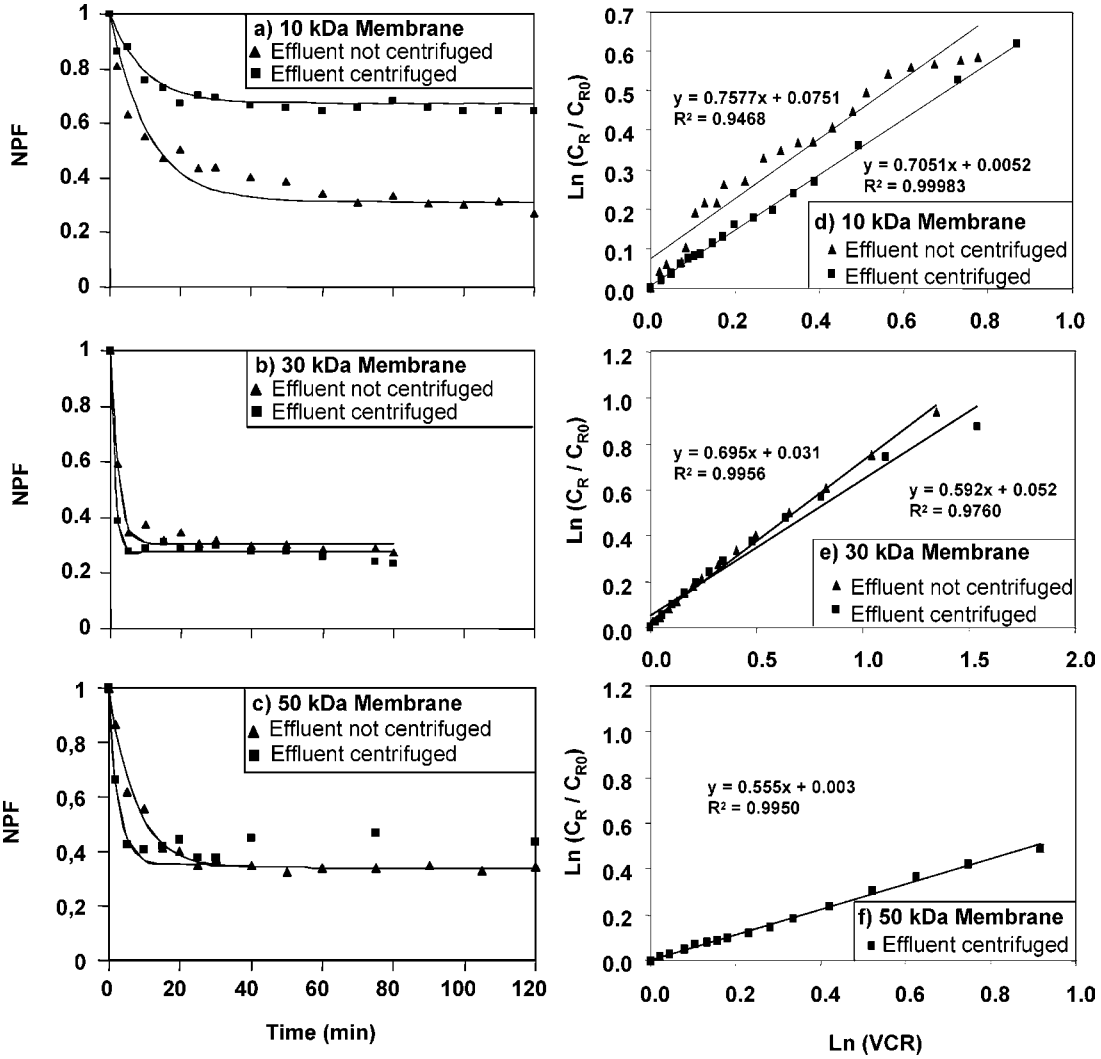

Figure 2. Effect of the processing time on the normalized permeate flux (NPF) for the effluents not centrifuged (ESCP) and centrifuged (CESCP) and interrelationship between the degree of concentration (measured by the concentration ratio C_R/C_{R0}) and the volume concentration ratio (VCR) for the same effluents.

at the elution volume of the column, a minor peak eluting at 152 mL (corresponding to 17 kDa units), and peptides with molecular masses below 1000 Da. The GPC profiles of the fractions SPUF-50, freeze-dried SPUF-50, SPR, and freeze-dried SPR are shown in **Figure 3c−f**, respectively. Both ultrafiltration and lyophilization could cause significant changes in the protein secondary structure (*24*) by inducing changes in the α- and *β*-helixes and probably favoring aggregation by intermolecular disulfhydryl binding. The SPUF-50 fraction showed two major peaks with molecular masses in the range of 400−700 Da and minor ones at 36, 22, 9, 5, and 2 kDa, these three latter disappearing in the lyophilized sample. The major peaks of the SPR freeze-dried fraction have molecular masses lower than 1000 Da, and those of 52 and 10 kDa are not present in the freeze-dried sample.

Dilution of the Protein Concentrates by Salt-Containing Solutions. Salt systems were used to dilute the retentate streams to overcome the problems of protein precipitation observed in the experiments using water as a dilution agent. The effect of salts on protein solubility during protein extraction has been studied using different natural sources, including tomato seeds (*25*), pigeonpea (*26*), *Rosa rubiginosa* (*27*), and hazelnut (*7*). Potassium and ammonium salts were preferred in this work because they provoke slight modifications of pH, which is an influential variable on protein solubility. The salts were compared at 0.5 M, except potassium phosphate at 1 M, and evaluated with regard to the protein solubility by measuring the percentage of protein solubilized and that which remains soluble after centrifugation. Ammonium acetate was selected since it provided the highest initial solubility (96.8%), followed by ammonium chloride (80.9%), potassium chloride (78.6%), diammonium phosphate (78.5%), potassium phosphate (73.1%), ammonium sulfate (75.9%), magnesium sulfate (73.6%), potassium phosphate (73.1%), 1% Tween 80 (73.6%), and distilled water (65.5%). Although SDS provided 98.6% initial solubility, after centrifugation, only 77.3% remained soluble. Diammonium phosphate was also selected due to the lowest reduction in solubility (3%) and, thus, the lower amount of solids separated after centrifugation.

Figure 1 also shows the results achieved in the successive membrane processing when ammonium acetate (S_1) or diammonium phosphate (S_2) solutions were used for dilution. In comparison with the experiments in which dilution was carried out with distilled water, the presence of salts increased the steady state permeate fluxes by three times, and membrane fouling was reduced allowing a higher number of operational cycles. The major fractions were SPUF-10 and SPR, which accounted for 19.3 and 25.9% of the initial protein, respectively, when

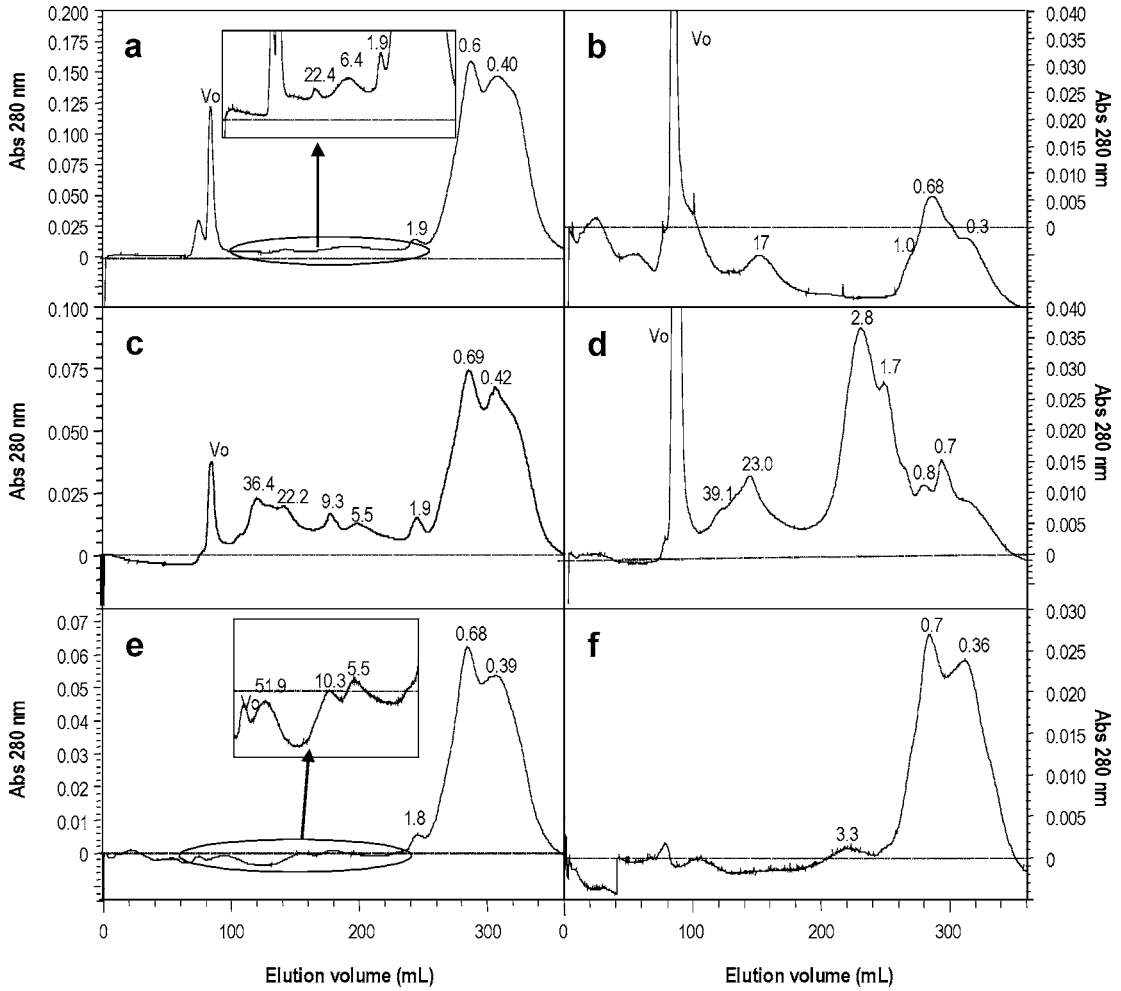

Figure 3. GPC elution profiles for (**a**) ESCP, (**b**) the precipitate obtained by ESCP centrifugation, (**c**) protein in exhausted liquor (stream R_3 in **Figure 1**), (**d**) freeze-dried sample of exhausted liquor (stream R_3 in **Figure 1**), (**e**) protein in permeate liquor (stream P_3 in **Figure 1**), and (**f**) freeze-dried sample of liquor permeate (stream P_3 in **Figure 1**).

diammonium phosphate was used, and for 24.3 and 23.0%, respectively, when ammonium acetate was used. The mass fractions of proteins present in freeze-dried solids were similar, no matter if the dilution was made with water or salt solutions. When using ammonium acetate solutions for dilution, P1, P2, P3, and R3 contained 0.038, 0.053, 0.076, and 0.33 g protein/g, respectively, whereas in experiments with diammonium phosphate the corresponding mass fractions were 0.046, 0.093, 0.099, and 0.46 g protein/g, respectively.

Table 1 summarizes the nutritional and functional properties of the fractions obtained by the successive ultrafiltration of CESCP. SPR showed the highest ADC, near the result determined for the standard casein. Membrane processing of high molecular mass proteins may result in denaturation (*24*), which can improve the digestibility. The in vitro digestibility of the low molecular mass fractions was below the values reported for protein isolates from rice protein (87%) (*28*), *R. rubiginosa* (70%), (*27*) and hazelnut (88%) (*7*). The digestibility of SPUF-50 was comparable to that of sesame seed isolates produced by isoelectric precipitation (*29*), whereas the digestibility of SPUF-30 and SPUF-10 is similar to the results reported for chickpea isolates (*30*). The ADC of the fractions prepared by resuspension of the retentate in ammonium acetate was in the range of 66−69%, comparable to those of sesame seed isolates (*29*) and higher than values reported for pea albumins and globulins (*31*).

As a general trend, the lower molecular weight of a given protein fraction resulted in higher emulsifying capacity, possibly due to both the presence of sugars and the improved surface properties of small peptides (*32*), which facilitate the diffusion of peptides in oil−water interfaces and enhance the interaction between proteins and lipids. The emulsifying capacity of the protein fractions does not present a clear correlation with regard to the salt system. The presence of salts doubled the EAI of the SPR fraction in the absence of salts, whereas the processing with ammonium acetate or diammonium phosphate solutions increased by 13−15 times the EAI of SPUF-50 with respect to the values obtained for processing with distilled water. SPR presented an EAI comparable to those reported by King et al. (*33*) for soy isolates produced by isoelectric precipitation and similar to that of CESCP. The protein fractions giving more stable emulsions were those retained in low cutoff membranes. Emulsions made with isolates from experiments with mineral salts were, in general, less stable than those obtained with distilled water. The fractions with higher protein content showed EAI and ESI in the range reported for other soy concentrates and isolates. SPUF-50 and SPR showed an EAI similar to those reported for other soy protein ultrafiltration isolates (*34*), whereas Chove et al. (*35*) reported values three times higher for soy protein fractions obtained by precipitation. The high molecular mass fractions showed ESI values similar or lower than those of SPUF-30 and SPUF-10, which presented values

Table 1. Nutritional and Functional Properties of the Protein Fractions Separated by Ultrafiltration[a]

	distilled water (W)				0.5 M ammonium acetate (S_1)				0.5 M diammonium phosphate (S_2)			
	SPR	SPUF-50	SPUF-30	SPUF-10	SPR	SPUF-50	SPUF-30	SPUF-10	SPR	SPUF-50	SPUF-30	SPUF-10
ADC (%)	82.84 ± 2.0	75.19 ± 1.8	68.87 ± 3.0	65.67 ± 2.0	68.5 ± 0.4	66.9 ± 0.0	67.0 ± 0.3	67.2 ± 0.5	69.22 ± 0.7	66.77 ± 0.4	66.81 ± 0.9	67.45 ± 0.5
μg lysine/16 g N	1075 ± 13.3	5892 ± 32.2	9516 ± 10.6	4957 ± 83.2	943.3 ± 141	5020 ± 663	3838 ± 110	1700 ± 62	1051	4531 ± 282	4162 ± 381	1325 ± 289
EAI (m^2/g)	106.7 ± 41.7	97.34 ± 24.3	825.7 ± 291	1565 ± 903	226.3 ± 17	1479 ± 196	1617 ± 62.98	1363 ± 67	234.9 ± 20	1319 ± 150	619.9 ± 80	1636 ± 102
ESI (min)	27.60 ± 1.1	23.35 ± 0.58	32.49 ± 2.9	34.61 ± 4.8	17.04 ± 0.5	21.79 ± 7.51	26.57 ± 5.36	20.57 ± 1.7	23.40 ± 0.58	30.56 ± 8.9	32.88 ± 5.12	20.44 ± 1.0
FF (%)	71.7	58.3	16.7	0	33	-	0	0	36.7	44.7	16.7	0
FS (mL)	51.5–31	47.5–31	35–30	no	40–33	-	no	no	41–30	40–30	no	no
LGC (%)	16	no	no	no	14	no	no	no	14	no	no	no
OA (g/100 g)	10049 ± 240	9628 ± 773	8867 ± 451	8552 ± 848	7765 ± 285	8846 ± 612	8049 ± 344	7067 ± 596	8444 ± 1261	8875 ± 270	9281 ± 1186	9594 ± 393

[a] FF, foam formation; FS, foam stability (0–120 min); and OA, oil absorption.

comparable to those reported for isoelectrically precipitated soy isolates (*34*). Chove et al. (*35*) reported ESI values slightly higher than those of SPR.

As expected, the foaming capacity of the fractions decreased with the molecular weight (*5*, *9*, *10*). Processing with salt-containing solutions did not improve the foaming capacity of the protein fractions in comparison with the ones obtained with water. SPR showed the highest ability for stabilizing air/water interfaces, a finding that could be in relation with some denaturation during processing resulting in an increased hydrophobic character (*32*, *36*). The foaming ability of SPUF-50 obtained in the processing with diammonium phosphate was comparable to that of SPR obtained under similar conditions.

Good foaming proteins should adsorb at the air−water interface and undergo rapid conformational changes, reducing the surface tension and forming cohesive, viscoelastic films. Foam stability is also favored by a slight heat denaturation (*32*). None of the low molecular weight fractions formed foams, a behavior similar to the one of SPUF-30 obtained with ammonium acetate. The most stable foams were formed by SPR coming from the experiment with distilled water, with results comparable to those reported for sesame protein isolates (*37*). No significant differences were observed between the foaming capacities and the stabilities of SPUF-50 and SPR. Poor foaming properties have been reported for 5 and 10 kDa soy peptides (*5*) and soy hydrolysates (*38*).

An inverse variation pattern is expected for emulsifying and foaming properties of proteins, owing to the opposite influence of the structure and surface properties in both variables (*32*). The foaming capacities of SPR and SPUF-50 obtained with distilled water were comparable to those reported by Liadakis et al. (*25*) for tomato seed isolates. Okezie and Bello (*20*) reported foaming capacity of 84% for commercial soy isolates, whereas Marcone and Kakuda (*39*) reported values of 15.6% for soy isolates produced in phosphate buffer, a similar result to that for SPUF-30.

SPR was the only fraction with the ability for gel formation, particularly when obtained in the presence of salts, whereas the oil absorption capacity was excellent for all of the fractions.

Enzyme Hydrolysis of High Molecular Weight Proteins. High molecular weight proteins were subjected to enzymatic hydrolysis to avoid the protease inhibition ability and the allergenic effects, as well as for improving their functional properties. A commercial protease was selected on the basis of its pH compatibility with the original pH of the feedstock, which allowed direct operation without acidification or neutralization, thus avoiding the development of bitter or unpleasant flavors (*40*).

In preliminary assays, operational conditions defined by long times and high enzyme-to-protein ratios (8 h and 25.000 LAPU/g protein) and shorter times and lower enzyme loadings (4 h and 10000 LAPU/g protein) were assessed for comparative purposes. As no significant differences on the DH were observed between these experiments, new conditions (8 h and 500 LAPU/g protein; 4 h and 100 LAPU/g protein) were considered. The results achieved for SPUF-50 showed that time was an influential variable on DH and that the DH achieved after 8 h using 500 LAPU/g was comparable to those achieved in the preliminary experiments. On the basis of this finding, 500 LAPU/g protein was selected as an upper limit for the enzyme loading. Similarly, SPR hydrolysis with 100 LAPU/g protein led to DH similar to the ones obtained at higher enzyme loading. Data of time course of DH in the range of 0−4 h for the various enzyme loadings assayed are shown in **Table 2**. The variation

Table 2. Nutritional Properties of the Protein Hydrolysates from the Two Fractions Produced by Ultrafiltration Technology

	SPUF-50											
	degree of hydrolysis (%)				available lysine (μg lysine/16 g N)				ADC (%)			
time (h)	10	50	100	500	10	50	100	500	10	50	100	500
0.5	19.4	29.4	28.8	55.1	3056	2767	2854	4462	67.1	66.9	67.1	66.9
1	23.1	32.3	33.3	56.1	2583	2342	2354	3514	67.8	66.5	66.9	66.4
2	24.4	35.6	37.3	59.1	2797	2610	3368	3730	67.8	66.7	67.1	66.9
4	28.1	37.6	40.3	63.4	2628	2572	3119	3342	67.1	66.5	66.7	66.4

	SPR								
	degree of hydrolysis (%)			available lysine (μg lysine/16 g N)			ADC (%)		
time (h)	10	50	100	10	50	100	10	50	100
0.5	10.5	24.9	31.9	509	556	673	75.8	75.1	73.9
1	14.4	27.3	36.6	597	733	756	76.0	75.1	72.5
2	18.8	29.9	38.3	585	889	714	75.8	74.5	69.6
4	20.6	37.8	42.1	582	453		72.9	74.7	72.2

Table 3. Functional Properties of the Hydrolysates from the SPUF-50 and the SPR Fractions

	SPUF-50			SPR		
	10 LAPU 0.5 h	100 LAPU 2 h	500 LAPU 0.5 h	10 LAPU 1 h	50 LAPU 2 h	50 LAPU 4 h
DH (%)	20	35	55	14	30	40
EAI (m^2/g)	1559	1260	1428	361.7 ± 73	565 ± 59	562 ± 8
ESI (min)	45.00	47.88	35.81	69.0 ± 21	32.0 ± 1	29.5 ± 2
FF (%)	no	no	no	no	no	no
LGC (%)	no	no	no	no	no	no
OA (g/100 g)	9048	8379	8581	7111 ± 582	8815 ± 204	8501 ± 960

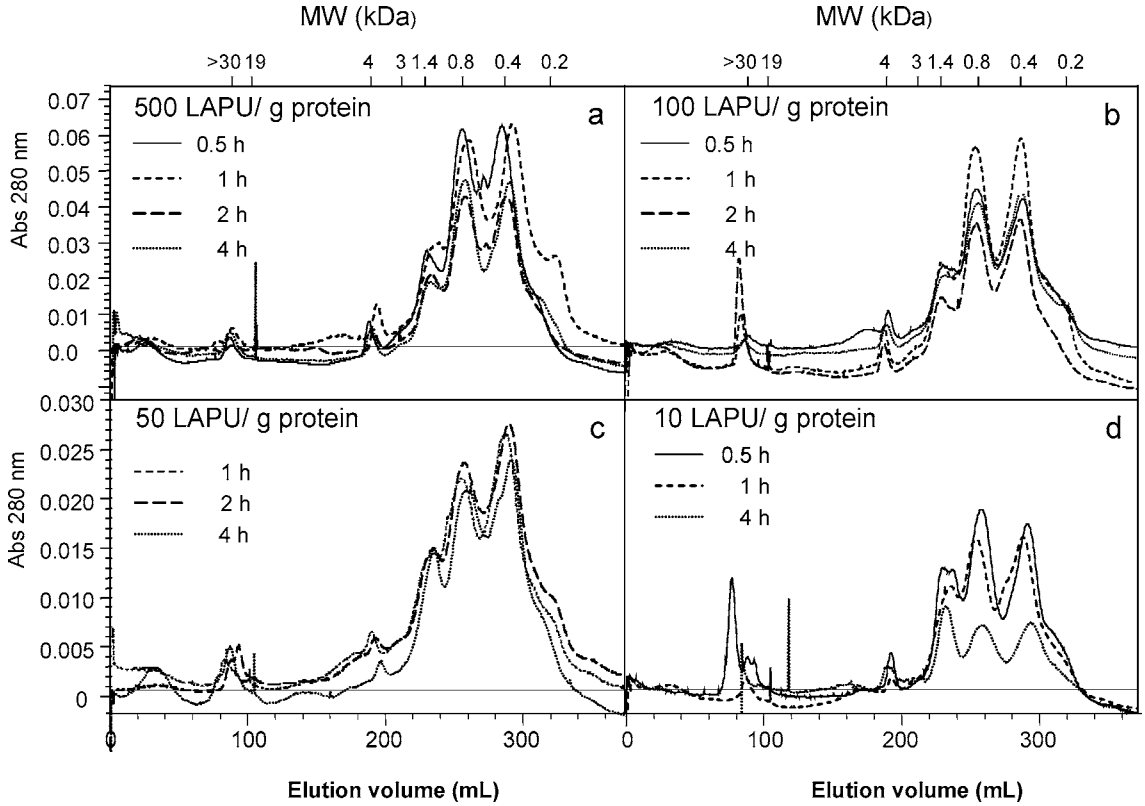

Figure 4. GPC elution profiles for hydrolysates from the SPUF-30 fraction produced using (**a**) 500, (**b**) 100, (**c**) 50, and (**d**) 10 LAPU/g protein.

pattern was similar under all of the conditions assayed. In experiments with SPUF-50, an increase of the enzyme loading from 100 to 500 improved DH by 58% after 2 h, but increasing the reaction time from 0.5 to 2 and 4 h caused comparatively little effect on DH (7 and 14% increase, respectively). In experiments with SPR, an increase in the enzyme loading from 10 to 50 LAPU/g protein doubled the DH after 4 h, and the maximum DH achieved was 40%. The DH obtained under the

severest conditions compared well with data reported for other protein isolates: Clemente et al. (*31*) reported a DH higher that 50% for chickpea protein using a mixture of alcalase 2.4L (0.4 Anson units/g protein) and Flavourzyme 500L (100 LAPU/g protein), whereas Bejosano and Corke (*41*) reported 11.8% DH for buckwheat protein using 91 units of pepsin A/mg of substrate, and Yim and Lee (*10*) reported 8% DH for soy isolates treated with *Mucor circinelloides* proteases.

Table 2 also summarizes the effects of the hydrolysis time and enzyme-to-substrate ratio on the nutritional properties of the hydrolyzed fractions. The in vitro digestibility of the hydrolysates from the SPR and the SPUF-50 fractions (82.8 and 75.2%, respectively) was similar to the results achieved for the nonhydrolyzed fractions. As a general trend, the hydrolysates from the two fractions showed a reduction in ADC and available lysine with respect to the substrates. Except for hydrolysates produced with 500 LAPU/g protein, the available lysine decreased after 1 h of hydrolysis. For the SPR fraction, minimum ADC values were determined for prolonged hydrolysis times, whereas the available lysine showed similar values along the hydrolytic process.

Table 3 shows the functional properties of hydrolysates obtained under selected conditions leading to different DHs but keeping satisfactory in vitro digestibility and lysine availability. Enzyme hydrolysis facilitates protein unfolding, exposing charged end groups and increasing the protein hydrophobicity. SPUF-50 and SPR hydrolysates showed EAI 10 and five times higher than the respective substrates, enhancement degrees higher than those caused by hydrolysis of sesame protein (*37*). ESI also increased upon hydrolysis, the increment being lower for SPUF-50 hydrolysates with 50% DH and for SPR hydrolysates with 30% DH. The oil absorption capacity of the hydrolysates was not affected by hydrolysis. The maximum functionality was achieved with 15−30% of hydrolysis. With the highest enzyme loading, the main peptide fractions have molecular masses under 1000 Da. As expected, hydrolysates did not show foaming or gelation properties (*5*, *9*).

Figure 4 shows the GPC profiles of SPUF-50 hydrolysates obtained with a BIOGEL P-30 column. **Figure 4a−d** shows the molecular mass profiles of SPUF-50 hydrolysates. The molecular mass profiles of hydrolysates with 10 LAPU/g showed little modification after 0.5 h, with a slight decrease in the area of peaks corresponding to >1 kDa and a slight increase in those <1 kDa. Hydrolysates produced with 50 LAPU/g showed a cyclic behavior, with formation of peptides of 1−19 kDa from higher molecular mass ones and degradation of those under 1 kDa. Hydrolysates with 100 LAPU/g formed lower molecular mass peptides progressively. Hydrolysates with 500 LAPU/g showed almost constant molecular mass distribution for hydrolysis times longer than 0.5 h. The fraction under 500 Da, composed of free amino acids, was minoritary. Too small peptides are undesirable, since they develop adverse flavor characteristics such as taste, color, and bitterness (*40*). Hydrolysates with intermediate and low DH usually provided the optimal functionality.

The chromatogram of the hydrolysates from the SPR fraction (data not show) showed lower resolution due to characteristics of the gel, particularly useful for separating higher molecular mass proteins but resolving the peptides with lower molecular masses at the end of chromatograms. Small peptides from vegetal proteins have biological and therapeutic applications, including antioxidant, antihypertensive, and anti-HIV activities. The radical scavenging capacity and antioxidant activity in emulsion of the protein fractions and hydrolysates isolated in the present work have been confirmed.

In conclusion, membrane technology is suitable for fractionating the acid soluble protein present in the industrial effluent from a soy concentrate plant. The present process was not intended as an alternative to the production of proteic products but to utilize the protein in a waste stream, with the double objective of (i) avoiding generation of contaminant streams and losses of commercially interesting compounds and (ii) recovery, concentration, and characterization of valuable nutrients. The protein products obtained by membrane processing showed good nutritional and functional properties, suggesting their possible utilization as food ingredients. Further enzymatic hydrolysis improved their functionality, particularly the emulsifying properties of the hydrolyzed protein. Studies to optimize the concentration through nanofiltration membranes of the soy oligosaccharides contained in permeates generated in the present process are ongoing.

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