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# Preparation of Whey Protein Hydrolysates Using a Single- and Two-Stage Enzymatic Membrane Reactor and Their Immunological and Antioxidant Properties: Characterization by Multivariate Data Analysis

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An initial 5% (w/v), followed thereafter with replacement aliquots of 3% (w/v), whey protein isolate (WPI) (ca. 86.98% Kjeldahl N × 6.38), was hydrolyzed using Protease N Amano G (IUB 3.4.24.28, *Bacillus subtilis*) in an enzymatic membrane reactor (EMR) fitted with either a 10 or 3 kDa nominal molecular weight cutoff (NMWCO) tangential flow filter (TFF) membrane. The hydrolysates were desalted by adsorption onto a styrene-based macroporous adsorption resin (MAR) and washed with deionized water to remove the alkali, and the peptides were desorbed with 25, 50, and 95% (v/v) ethyl alcohol. The desalted hydrolysates were analyzed for antibody binding, free radical scavenging, and molecular mass analysis as well as total and free amino acids (FAA). For the first time a quantity called $IC_{50}$, the concentration of peptides causing 50% inhibition of the available antibody, is introduced to quantify inhibition enzyme-linked immunosorbent assay (ELISA) properties. Principal component analysis (PCA) was used for data reduction. The hydrolysate molecular mass provided the most prominent influence (PC1 = 57.35%), followed by inhibition ELISA (PC2 = 18.90%) and the antioxidant properties (PC3 = 10.43%). Ash was significantly reduced in the desalted fractions; the protein adsorption recoveries were high, whereas desorption with alcohol was prominently influenced by the hydrophobic/ hydrophilic amino acid balance. After hydrolysis, some hydrolysates showed increased ELISA reactivity compared with the native WPI.

**KEYWORDS: Whey protein isolate; enzymatic membrane reactor; desalt; multivariate data analysis; inhibition ELISA; DPPH scavenging property; $IC_{50}$**

## INTRODUCTION

Enzymatic hydrolysis of proteins confers attractive properties on the hydrolysates such as markedly improved solubility at pH values in which the native protein has very diminished solubility (*1*). In addition, emulsifying, organoleptic, and bioactive properties are improved dramatically (*2*−*4*). Whey proteins, ideally waste products from cheese manufacture, are appreciated as a high-value group of proteins (*5*, *6*) and are currently manufactured using state-of-the-art membrane technology (*7*, *8*) to obtain high-purity products with promising applications in the functional foods as well as pharmaceuticals industries (*9*). Enzymatic whey protein hydrolysates (WPHs) may be used in food formulations such as hypoallergenic infant formulas (*10*−*13*) and sports foods (*14*). In addition, WPHs find acceptability in clinical nutrition as well as being a source of bioactive peptides (*15*−*17*).

Use of ultrafiltration (UF) membranes during hydrolysis for the production of enzymatic hydrolysates has continued to gain prominence in the food industry (*13*, *18*, *19*). In operation, the UF membranes are coupled to hydrolysis tanks (hence, enzymatic membrane reactor, EMR) for simultaneous in situ permeation of small hydrolysates (and retention of the enzyme and large polypeptides) during the hydrolysis process, which provides attractive advantages over conventional batch reactors (*13*, *19*). These advantages include immense savings due to reuse of the enzyme, removal of the enzyme-inhibiting hydrolysates, and production of hydrolysates with uniform molecular mass characteristics achievable with the use of the right membrane nominal molecular weight cutoff (NMWCO) size. Furthermore, inactivation of the enzyme, which is necessary at the end of hydrolysis in the conventional batch reactors, is obviated, which reduces production of side-reaction foulants such as lysinoalanine (*16*).

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Meanwhile, recent reviews compare EMR reactors with traditional size exclusion chromatography, with claims that membrane chromatography stands to provide better separation throughput because the process is free from diffusional restrictions (*20*, *21*). The EMR process has also attracted attention due to the need to produce a product that is immunologically safe for the consumer (*13*, *22*).

Removal of the alkali used to control the pH during protein hydrolysis is a technological and academic endeavor that requires investigations into cheaper and high-recovery methods to obtain "cleaner" products acceptable to consumers. In a recent review, use of a macroporous adsorption resin (MAR) to simultaneously desalt and debitter WPHs with high hydrolysate recovery was reported (*23*). Meanwhile, multivariate data analysis is currently applied in the food experiments to design, optimize, and analyze food process trends (*18*, *24*, *25*). However, there are no reported cases on the use of multivariate data analysis to reveal the influence of the hydrolysis in an EMR and posthydrolysis processing such as desalting on hydrolysate bioactive, immunological, and functional properties.

This paper reports a study on the effects of various membrane nominal molecular weight cutoff (NMWCO), hydrolysis in either a single stage or or two-stage scheme, desalting of WPI hydrolysates produced in an EMR, and use of various alcohol concentrations to desorb. Principal component analysis (PCA) was used for data reduction to enable multivariate data analysis.

## MATERIALS AND METHODS

Whey protein isolate (WPI; ca. 86.98% Kjeldahl N × 6.38) was bought from Fonterra Co-operative Group Ltd. (Auckland, New Zealand), whereas Protease N Amano G (IUB 3.4.24.28, *Bacillus subtilis*) was bought from Amano Enzymes Co. (Nagoya, Japan). A styrene-based MAR, branded DA201-C, was sourced from Jiang-Su Su-Qing Water Treatment Engineering Group (Jiang-Ying, Jiang-Su Province, China). The ultrafiltration modules consisted of two Millipore Corp. (Bedford, MA) tangential flow filtration (TFF) Pellicon 2 Mini filters: (a) Biomax 10 (polyethersulfone) cassette (size = 0.1 $m^2$) with a 10 kDa NMWCO and (b) PL series (regenerated cellulose) 3 kDa NMWCO cassette TFF membrane (size = 0.1 $m^2$). During use, a TFF cassette was mounted within a Millipore Pellicon 2 Mini Holder while a Millipore pump and the silicon tubes (MasterFlex 96410−17) were used to link the membrane and the hydrolysis tank.

**ELISA Reagents.** For the ELISA, anti-bovine whey protein antibodies (delipidized whole serum) developed in rabbit were bought from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG−horseradish peroxidase polymer was bought from Sino-American Biotechnology Co. (Shanghai, China). The microtiter high-binding ELISA plates (8 and 12 wells), the ELISA plate 96-well frames, and the well sealing film were all products of Bio Basic Inc. (Markham, ON, Canada). The substrate for peroxidise, 3,3′,5,5′-tetramethylbenzidine dihydrochloride monohydrate (TMB-2HCl·$H_2O$) was from Amresco Inc. (Solon, OH).

The substrate stock solution was made by dissolving ca. 30 mg/mL TMB-2HCl·$H_2O$ in dimethyl sulfoxide (DMSO) and stored under refrigeration (4 °C) until required for use. The coating buffer was 0.05 M sodium carbonate buffer, pH 9.6, whereas the washing buffer was 0.05 M sodium phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-T). The blocking buffer was made up of PBS-T with 5% (w/v) bovine serum albumin [BSA (PBS-BT)], whereas the assay buffer was 0.05 M sodium citrate, pH 4.8.

**Whey Protein Hydrolysis in an EMR**. WPI (ca. 5%, w/v, initially and thereafter the permeate replaced with 3%, w/v, aliquots) was hydrolyzed using Protease N (initially 3 g per 1000 mL of reaction volume for the single-stage hydrolysis regime) in a setup similar to the one given in **Figure 1**, as described earlier (*18*). The hydrolysis tank was maintained at 55 °C and the pH kept at 7.0 using 0.5 M or 1 M NaOH (*26*). The temperature was lowered upon the reactants leaving the reaction tank (to 50 and 40 °C for the Biomax and the PLBC regenerated cellulose TFF cassettes, respectively) using tap water with the help of a heat exchanger (C in **Figure 1**). The retentate temperature was raised upon leaving the membrane unit using a heat exchanger (H in **Figure 1**) close to 55 °C.

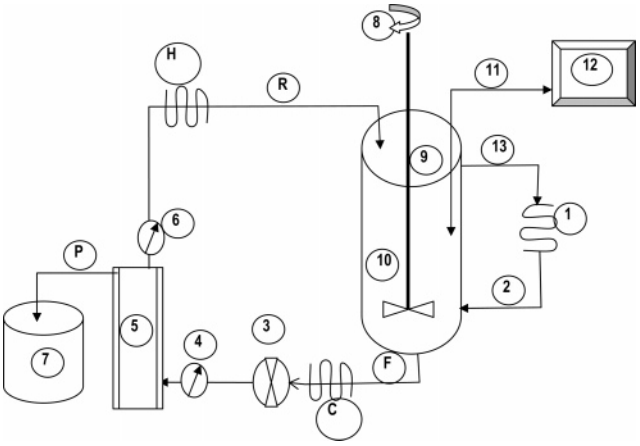


**Figure 1.** EMR in operation in the study in which the reactants were pumped to the membrane through which species small enough permeated as the product: 1, thermostatic water heater; 2, heating water inlet; 3, peristaltic pump; 4, feed pressure gauge; 5, Pellicon 2 cassette assembly; 6, retentate pressure gauge; 7, permeate collector; 8, stirrer; 9, stirring rod; 10, hydrolysis tank reactor; 11, pH-meter electrode; 12, pH-meter; 13, cool water from reactor; C, cooling of feed; F, feed from reactor; H, heating for the retentate; P, permeate; R, retentate.

The process was designated single-stage EMR when either the 10 or 3 kDa membrane was used without further hydrolysis of the issuing permeates. On the other hand, the two-stage hydrolysis was done by first fitting the EMR with the 10 kDa TFF membrane, hydrolyzing for 2 h, and its permeate being further hydrolyzed in the EMR fitted with the 3 kDa TFF membrane. The enzyme for the two-stage system was 1.5 g in each stage per 1000 mL of reaction volume. To aid in limiting the deposition of the WPI on the membrane surface as the gel polarization layer (GPL), a higher recirculation velocity (24−28.8 L/h) was applied to offer membrane surface deposit erosion (*13*, *18*, *27*), whereas higher permeate fluxes led to high enzyme leakages (*28*). The 10 kDa membrane permeate outlet was controlled using a screw clip to reduce the initial permeate flow rate to ca. 15 mL/min., which was found to be optimum in a previous paper (*18*). When the 3 kDa membrane was used, it was necessary to apply a back-pressure on the retentate outlet to achieve the initial permeate flow rate that was used (ca. 15 mL/min).

In operation, the small species in the feed (F in **Figure 1**) that was pumped to the membrane was sieved through and collected as the permeate (P in **Figure 1**) while the enzyme, native WPI, and large polypeptides were retained and recirculated as the retentate (R in **Figure 1**) back to the hydrolysis tank.

**Degree of Hydrolysis (DH).** The DH was evaluated by relating the increase in the concentration of the liberated α amino groups (α-$NH_2$) by use of 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) (*29*) according to the method of Adler-Nissen (*30*) with modifications. At the end of the EMR process, the retentate was completely drained into a beaker, and after thorough mixing, 0.5 mL each of the collected permeate (total volume already noted) and the retentate were drawn and separately mixed with 9.5 mL of hot (75 °C) 1% sodium dodecyl sulfate (SDS). The mixture was treated under sonication at 75 °C for 10 min to solubilize the hydrolysates and inactivate the enzyme (*30*). The total concentration of α-$NH_2$ in the permeate and retentate was then determined by mixing 10 or 20 μL of the sample with 240 or 230 μL of SDS, respectively, and a further 2.0 mL of 0.2125 M sodium phosphate buffer with vortex mixing. After equilibration at 50 °C, 1 mL of 0.1% TNBS was added with vortex mixing, and the reaction was allowed to proceed in a hooded thermostatically controlled water bath regulated at 50 °C with total exclusion of light. After 1 h, the reaction was quenched by using 4 mL of 0.1 N HCl, cooled further under tap water and the absorbance of the blank and the samples were

read at 340 nm. A standard calibration curve was prepared using fresh L-leucine. A blank was made of 1% SDS instead of the samples, whereas the original concentration of the α-$NH_2$ groups in the WPI was determined by drawing 0.5 mL of the WPI just before addition of the enzyme. The DH for the EMR WPH was calculated using relationships in the equations

$$DH_{permeate}(\%) = \frac{([NH_2]_{permeate} \times V_{permeate})}{h_{tot} \times N_{permeate}} \times 100 \quad (1)$$

$$DH_{retentate}(\%) = \frac{([NH_2]_{retentate} \times V_{retentate})}{h_{tot} \times (N_{WPI} - N_{permeate})} \times 100 \quad (2)$$

$$DH_{EMR}(\%) = \frac{([NH_2]_{permeate} \times V_{permeate}) + ([NH_2]_{retentate} \times V_{retentate})}{h_{tot} \times N_{WPI}} \times 100 \quad (3)$$

where $[NH_2]_{permeate}$ = concentration of $NH_2$ in permeate (μmol/mL), $[NH_2]_{retentate}$ = concentration of $NH_2$ in retentate (μmol/mL), $N_{WPI}$ = nitrogen content in WPI substrate (86.98% of WPI substrate), $N_{permeate}$ = nitrogen content in the permeate, $h_{tot}$ = concentration of $NH_2$ per gram of WPI nitrogen (123.3 mg/g or 939.9 μmol/g of WPI nitrogen was used in this study), $V_{retentate}$ = volume of retentate (mL), and $DH_{permeate}$, $DH_{retentate}$, and $DH_{EMR}$ are the DH values for the permeate, retentate, and the total EMR hydrolysis process, respectively.

**Reactor Robustness and Product Conversion.** The reactor robustness was monitored by determining the residual enzyme activity ($A_{residual}$), the leaked enzyme activity ($A_{leakage}$), and the lost enzyme activity ($A_{loss}$). Additionally, the average permeate flux ($J_{average}$) and the amount of the product converted and recovered in the permeate were calculated as apparent sieving ($S_{apparent}$), using procedures explained earlier (*18*, *27*, *28*).

**Desalting.** Desalting of the WPH was done according to the method of Cheison et al. (*23*). Following hydrolysis, the collected permeate and/or retentate was immediately pumped through a glass column packed with macroporous adsorption resins (MAR) without further treatment. After all or any of the volume that just allowed WPH nitrogen to be detected in the eluate (monitored by a UV detector clocked at 220 nm), deionized water was pumped to rinse off NaOH and any unbound peptides.

Desorption with alcohol was achieved by first stirring an excess of 25% (v/v) of food grade ethyl alcohol mixed with the resin slurry over a magnetic stirrer until the solution was basically not golden yellow (color of dissolved peptides) or until 5 bed volumes (5 bv) of alcohol was used. The alcohol concentration was increased to 50% (v/v) and the desorption process repeated, and finally the alcohol concentration was raised to 95% (v/v). The collected alcohol fractions were concentrated under reduced pressure at 45 °C and the solid substance frozen (−18 °C) followed by freeze-drying to obtain the lyophilisate, which was used in subsequent studies. Otherwise, immediately preceding hydrolysis, the permeate (and/or retentate) was concentrated under vacuum and lyophilized and the powder desalted subsequently.

**Molecular Mass Determination**. The lyophilisates were analyzed for molecular mass distribution as previously reported (*26*) using a Waters 650E Advanced Protein Purification System (Waters Corp., Milford, MA).

**Total and Free Amino Acids (FAA) Determination.** Analysis of 17 amino acids was done following hydrolysis of the samples using 6 N HCl at 110 °C for 24 h. The content of FAA in the un-desalted WPH was determined after deproteination of the samples using 3.5% of 5-sulfosalicylic acid (SSA). The amino acids were analyzed after on-line derivitization with *O*-phthalaldehyde (OPA) and 9-fluorenylmethoxycarbonyl (F-MOC) for proline using reversed phase high-performance liquid chromatography (RP-HPLC) under conditions described previously (*26*).

**Inhibition ELISA.** Inhibition ELISA was done following a modification of the competitive inhibition ELISA protocol described by Svenning et al. (*31*). All of the wells except the outermost channels and wells (to avoid drying caused by the edge effect) were coated with 150 μL of WPI (ca. 50 mg/mL in the coating buffer). The plates were sealed and kept at room temperature (25 °C) for 2 h followed by further storage in a humidified box at 4 °C. After 18 h of incubation, the wells were emptied by inverting them over a waste beaker and dried further over dustless paper towels. After three washings with 250 μL of PBS-T followed by three washings with double-distilled water with 5 min of incubation (at 25 °C) between washings, the wells were blocked with 250 μL of the blocking buffer (PBS-BT), sealed, and incubated in a humidified box at 37 °C for 2 h followed by washing as above. Meanwhile, both the WPI and the desalted and/or un-desalted WPH samples were prepared in serial dilutions by first drawing 10 μL (for the WPI) or 20 μL (for the WPH) of the samples into a small centrifugation cuvette (total volume = 100 μL). After mixing over a vortex mixer, another 10 μL of WPI or WPH was drawn from the first cuvette to the next and the process repeated until the fifth cuvette with addition of the coating buffer to make up the volume. Into the cuvettes was added 20 μL of anti-bovine whey whole antiserum (1:5000 in coating buffer), and the contents were vortex-mixed and preincubated at 25 °C for 2 h to enable conjugation between the antibody and the antigenic WPI or WPH. After the blocked wells had been washed as above, 100 μL of the preincubated samples and WPI with the anti-bovine whey antiserum were added to the wells, sealed, and kept at 37 °C for 2 h. The contents were emptied and the wells washed.

Because of the large number of samples handled and the time-consuming nature of the tests, overnight storage was necessary at some stage or another. When storage was required, the wells were filled with 150 μL of the coating buffer, sealed, and kept refrigerated (4 °C) until the next morning (16 h). After washing, 100 μL of goat anti-rabbit−peroxidase polymer (1:1000 in PBS-T) was carefully delivered to the well bottom, sealed, and incubated for 2 h in a humidified box at 37 °C. The wells were washed with PBS-T followed by double-distilled water, and 100 μL of the substrate (0.25 mL of the ca. 30 mg/mL TMB stock solution in DMSO added to 19.745 mL of the 0.05 M sodium citrate, pH 4.8, assay buffer and 5 μL of 33% $H_2O_2$) was added. The wells were sealed and incubated in a dark humidified box at 37 °C for between 5 and 10 min. The reaction was terminated with the addition of 50 μL of 2 M $H_2SO_4$, and the reactants were read at 450 nm using a SpectraMax Plus$^{384}$ microplate reader; the results were acquired and processed using SoftMax Pro 4.6 (ROM version 1.16), all from Molecular Devices Inc. (Sunnyvale, CA).

To cater for the nonspecific binding (NSB), several wells were coated with WPI and blocked followed by the addition of the goat anti-rabbit−peroxidase polymer (1:1000 in PBS-T). The WPI/WPH−anti-bovine whey antiserum conjugate was excluded in these wells. To prepare wells for the blind blank, the WPI/WPH conjugation stage was omitted, and instead 20 μL of anti-bovine whey antiserum was mixed with 80 μL of the coating buffer and added into wells in duplicate. A third control was made of TMB to account for autodeterioration of the substrate in which the WPI/WPH−anti-bovine whey antiserum conjugate and the goat anti-rabbit−peroxidase polymer were excluded. This was used to zero the plate reader.

In our modification, WPI was used to coat all of the wells to eliminate differences in NSB and chances of differences in avidities of plate−WPH binding (*32*). Both of the blanks and the sample WPH determinations were done together each time to ensure accuracy of the determinations. The amount of the colored product formed by the enzyme was interpreted to be proportional to the amount of the enzyme-linked antibody that binds. This, in turn, was directly related to the amount of the antibody that was not inhibited by the WPI or WPH and was therefore available to bind the antigenic WPI coated to the wells. The inhibition of the anti-bovine whey antiserum by the WPI/WPH was calculated using the relationship in eq 4.

$$\text{inhibition}(\%) = \frac{OD_{blank} - (OD_{sample} - OD_{NSB})}{(OD_{blank} - OD_{NSB})} \times 100 \quad (4)$$

where $OD_{blank}$ is the optical density (absorbance) at 450 nm of the wells without the WPI/WPH−anti-bovine whey antiserum conjugate but in which only the anti-bovine whey antiserum was added. $OD_{NSB}$ is the optical density for nonspecific binding representing readings for the wells in which neither the anti-bovine whey antiserum nor the WPI/

WPH−anti-bovine whey antiserum conjugate was used. $OD_{sample}$ is the optical density of the wells containing the WPI/WPH samples conjugated with the anti-bovine whey antiserum.

The results were plotted (percent inhibition on the *y*-axis and WPI/WPH concentration on the *x*-axis) to generate a curve for each sample. Curve transformation was performed to linearize it from which, for the first time in competitive inhibition ELISA, a new quantity called $IC_{50}$ was calculated to relate the sample concentration and inhibition. This value was defined as the concentration of the sample to inhibit antigen−anti-bovine whey antiserum binding by 50% and was understood to be low for highly antigenic samples and higher for less antigenic samples.

**Antioxidant Activity Determination.** Free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*33*) scavenging was used to quantify the antioxidant properties of the desalted WPH. Approximately 524 $\mu$mol/L DPPH was dissolved in methanol, and the antioxidant activity was determined by the addition of DPPH and the five serial dilutions of ca. 50 mg/mL WPI or ca. 100 mg/mL WPH dissolved in 0.05 M sodium carbonate/bicarbonate, pH 9.6. The reaction was allowed to go on for 30 min, and the absorbance of the residual DPPH was read at 514 nm. A blank consisted of buffer instead of the WPI/WPH. The DPPH scavenging activity was calculated using the relationship in eq 5.

$$\text{DPPH scavenging (\%)} = \left(\frac{OD_{blank} - OD_{sample}}{OD_{blank}}\right) \times 100 \quad (5)$$

where $OD_{blank}$ is the absorbance (514 nm) of the blank reaction mixture and $OD_{sample}$ is the absorbance (514 nm) of the reaction mixture with the WPI/WPH. The antioxidant scavenging capacity was determined using five serial dilutions, and suitable curves (or linearizations) were plotted from which the amount of WPI/WPH to reduce DPPH concentration by 50% ($IC_{50}$) was calculated.

**Analytical Procedures.** The protein concentrations in the hydrolysate permeates and the retentate were determined using the standard Kjeldahl method (*34*), whereas for the desorbed fractions and for ELISA as well as the free radical scavenging activity the soluble nitrogen concentrations were determined using the method of Lowry et al. (*35*) with bovine serum albumin (BSA) as the standard. The spectrophotometric determinations were done in duplicate using two blanks (to obtain four readings) and the means and standard deviation calculated for each.

**Statistical Analysis.** The protein determinations in the permeate, retentate, and the desorbed fractions were done in duplicate with two blanks (total four readings), and the means and standard deviations were calculated. The results from the determinations were fitted into SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL) for the principal component analysis (PCA) and multivariate data analysis.

## RESULTS AND DISCUSSION

Whey protein hydrolysis was carried out in a setup described in detail elsewhere (*18*, *28*) and similar to that depicted in **Figure 1**. The WPI was hydrolyzed either in a single stage with the EMR fitted with a 10 or 3 kDa TFF membrane filter or using a two-stage scheme whereby the EMR was fitted with the 10 kDa followed by further hydrolysis with the EMR fitted with the 3 kDa. The protein recoveries for the single- and two-stage EMR operation regimes are presented in **Figure 2**. Only the 10 and 3 kDa membranes were available for this study and were used for comparison because most bioactive peptides and those with reduced immunogenicity range below 3 kDa in molecular mass distribution (*12*, *36*). The membrane used in the EMR influenced the amount of product recovered in the permeate, with the lowest recovery being recorded from the two-stage process (overall 24.17%) as opposed to when the 10 kDa TFF was used in the single-stage (68.48%). The 3 kDa membrane was able to provide a recovery equivalent to only 45.24% when used in the single-stage. When used as a second stage, however, 52.86% of the first-stage permeate was recovered (**Figure 2**).

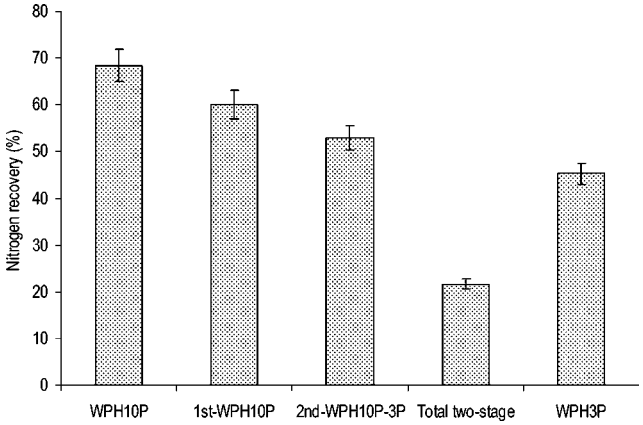


**Figure 2.** Nitrogen recovery in enzymatic membrane reactor (EMR) schemes during hydrolysis in the single- and two-stage EMR schemes.

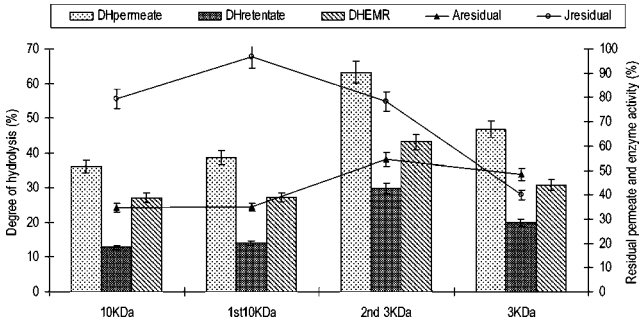


**Figure 3.** Degree of hydrolysis of the permeate ($DH_{permeate}$), retentate ($DH_{retentate}$), and the total reactor hydrolysates ($DH_{(EMR)}$) as well as the residual enzyme activity ($A_{residual}$) and the residual permeate flux ($J_{residual}$).

The most prominent influence of the 3 kDa TFF membrane whether used in the single- or two-stage scheme was savings on the enzyme. The highest $A_{residual}$ value (54.50%) was recorded when the EMR was run in the two-stage mode. In this way, the enzyme that leaked from the first stage was available for reuse in the subsequent stage, which may have implications on the cost of the enzyme (**Figure 3**). The single-stage EMR mode with the 3 kDa TFF membrane also conserved the enzyme ($A_{residual}$ value of 48.44%), whereas the 10 kDa TFF led to severe enzyme leakage ($A_{residual}$ value of 34.63%). However, the savings on the enzyme were reversed by decay in permeate flux ($J_{average}$ value of 40.00% for the single stage) in the 3 kDa EMR as opposed to a stable 79.41% for the single-stage 10 kDa scheme.

Earlier on it was determined that if the hydrolysis reaction mixture was recirculated before, during, or immediately after addition of the enzyme, there was a severe decline in the permeate flux. Thus, the first 5 min simulating a batch hydrolysis process without recirculation was chosen as reasonable to enable the WPI to be hydrolyzed to peptides that could be sieved as well as reducing the flux declines due to WPI deposits on the EMR as the GPL (*18*). During the first 5 min, a DH equivalent to ca. 15−20% was reached.

The greatest advantage of the second stage was the significantly higher permeate flux stability ($J_{average}$ value of 78.26%). This stability in the permeate flux may be explained by the reduced tendency of the extensively hydrolyzed WPH obtained from the first stage to deposit as the GPL as opposed to the native WPI. It was indeed reported earlier (*18*, *28*) that when the temperature of the membrane surface was high enough, the WPI solubility was equally high and the enzyme solubilized the WPI deposits on the membrane, thus reducing the formation of the GPL by providing a co-detergence property on the EMR.

**Table 1.** Proximate Analysis and Molecular Mass Distribution of Desalted and Un-desalted Whey Protein Hydrolysates (WPH)

| | proximate analysis (%) | | mol mass distribution (%) | | | |
|---|---|---|---|---|---|---|
| product | protein | ash | <600 Da | 601–1500 Da | 1501–3000 Da | >3000 Da |
| $WPH_{10P}$[a] | 78.87 | 6.77 | 77.56 | 16.95 | 5.21 | 0.28 |
| $WPH_{10P}$-25 | 83.20 | 4.30 | 52.97 | 29.47 | 14.19 | 3.37 |
| $WPH_{10P}$-50 | 98.50 | 2.75 | 63.12 | 25.39 | 9.28 | 2.21 |
| $WPH_{10P}$-95 | 97.94 | 2.82 | 70.23 | 24.31 | 4.78 | 0.68 |
| $WPH_{10R}$[b] | 73.97 | 7.32 | 58.24 | 22.00 | 10.41 | 9.36 |
| $WPH_{10P-3R}$[c] | 74.41 | 7.42 | 74.19 | 18.57 | 6.69 | 0.56 |
| $WPH_{10P-3R}$-25 | 95.08 | 0.82 | 54.30 | 33.29 | 11.18 | 1.24 |
| $WPH_{10P-3R}$-50 | 98.12 | 1.05 | 62.63 | 26.91 | 9.14 | 1.33 |
| $WPH_{10P-3R}$-95 | 97.68 | 0.92 | 77.33 | 18.99 | 3.20 | 0.48 |
| $WPH_{10P-3P}$[d] | 78.63 | 9.64 | 87.54 | 10.76 | 1.67 | 0.03 |
| $WPH_{10P-3P}$-25 | 85.53 | 2.09 | 79.28 | 15.78 | 4.61 | 0.33 |
| $WPH_{10P-3P}$-50 | 96.53 | 1.55 | 83.29 | 13.59 | 2.83 | 0.29 |
| $WPH_{10P-3P}$-95 | 93.06 | 1.60 | 77.40 | 20.72 | 1.81 | 0.08 |
| $WPH_{3P}$[e] | 80.01 | 8.56 | 79.03 | 18.26 | 2.61 | 0.10 |
| $WPH_{3P}$-25 | 85.03 | 5.05 | 73.36 | 19.50 | 6.54 | 0.60 |
| $WPH_{3P}$-50 | 92.29 | 3.33 | 80.37 | 14.70 | 3.93 | 0.99 |
| $WPH_{3P}$-95 | 94.85 | 1.54 | 78.53 | 18.73 | 2.28 | 0.46 |
| $WPH_{3R}$[f] | 91.77 | 7.49 | 64.02 | 24.02 | 9.74 | 2.21 |

[a] $WPH_{10P}$ = whey protein hydrolysates permeable through a 10 kDa membrane. The values 25, 50, and 95 denote the concentration of alcohol to desorb. [b] $WPH_{10R}$ = whey protein hydrolysates retained by a 10 kDa membrane. [c] $WPH_{10P-3R}$ = whey protein hydrolysates permeable through a 10 kDa membrane but retained in a 3 kDa membrane. [d] $WPH_{10P-3P}$ = whey protein hydrolysates permeable through a 10 kDa membrane and further permeable through a 3 kDa membrane. [e] $WPH_{3P}$ = whey protein hydrolysates permeable through a 3 kDa membrane. [f] $WPH_{3R}$ = whey protein hydrolysates retained by a 3 kDa membrane.

The regenerated cellulose 3 kDa TFF membrane filter has a maximum operating temperature limit of 40 °C, at which conditions of WPI solubility and viscosity are reduced (*18*, *27*) apart from Protease N activity being lower (*26*, *28*). The polyethersulfone-based 10 kDa TFF membrane, on the other hand, can withstand harsher conditions (temperature limit of 50 °C) that are suitable for good product hydrodynamics and enzyme activity (*18*, *26*); hence, the $J_{average}$ values were high even after 5 h (79.41%) and 2 h (96.88%) (**Figure 3**). Additionally, continuous removal of the hydrolysates in the permeate eliminated product inhibition on Protease N (*26*) with the result that higher DH values were attainable as opposed to when the batch reactor hydrolysis was carried out.

The DH of protein hydrolysates is an important parameter that relates well with the bioactive, functional, and immunological properties (*11*, *12*, *37*). Generally, the DH is determined using the pH-stat, the osmometric method, and the colorimetric methods (TNBS, ninhydrin, and OPA) (*38*). The DH can be used to estimate the average peptide length and is currently poorly characterized in EMR operations. Thus, a simple novel modification of the TNBS method (*30*) was made and used in this study with the advantage that the traditional concept of the DH in the batch reactor was retained. In this way the average peptide chain length (*38*) could be estimated and corroborated by the molecular mass of the hydrolysates (**Table 2**). The FAA content was also determined as a guide to their release in the EMR, and the results point to limited production of FAA, high DH values notwithstanding. Thus, the EMR provides a product with reduced FAA, which is desirably less hyperosmotic (*11*).

**Desalting.** Upon adsorption of the WPH onto MAR, the ash content was reduced during rinsing while the resins retained the peptides. Desorption was achieved by reversing solvent antagonism with the use of a detergent-like solvent, ethyl alcohol (*23*). The ash was high following hydrolysis (**Table 1**) in an EMR, although even at high DH values recorded in this study the ash content was significantly below the ca. 15% recorded previously from batch hydrolysis at about DH = 15% (*23*). The highest ash content was found in the second-stage hydrolysate owing to the cumulative permeation of the alkali through both the 10 and 3 kDa TFF membranes. Generally, the ash content in the retentates was lower than that for the corresponding permeates. After desalting, the ash reduced in all of the desorbed fractions to below 5% (**Table 1**).

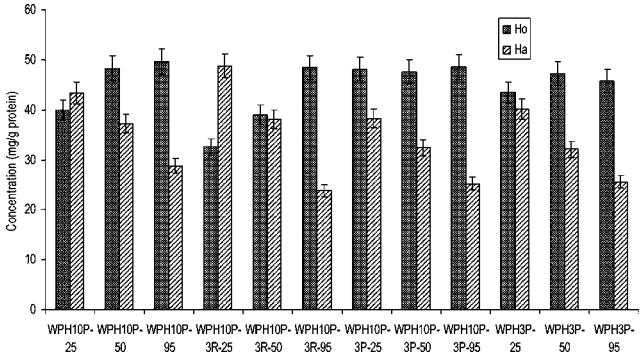


**Figure 4.** Concentration of hydrophobic ($H_oAA$)/hydrophilic (HAA) amino acids in the desalted whey protein hydrolysate (WPH) fractions.

**Table 2.** Recovery Efficiency of Whey Protein Hydrolysates (WPH) Produced in an Enzymatic Membrane Reactor (EMR) and Desalted Using Macroporous Adsorption Resins (MAR)

| | desorbed fractions | | |
|---|---|---|---|
| product | 25% | 50% | 95% |
| $WPH_{10P}$[a] | 63.90 | 31.95 | 4.14 |
| $WPH_{10P-3P}$[b] | 71.87 | 18.82 | 9.31 |
| $WPH_{10P-3R}$[c] | 63.60 | 31.96 | 4.44 |
| $WPH_{3P}$[d] | 72.91 | 22.47 | 4.62 |

[a–d] Refer to the footnotes of **Table 1** for definitions.

Desorption with MAR resulted in various migrations of peptides into the fractions recovered by 25% (between 63.90 and 72.91% of total product recoveries), 50% (between 18.82 and 31.95%), and 95% (between 4.41 and 9.31%) alcohol (**Table 2**). Generally, the recovery was higher for the fraction recovered with 25% alcohol than previously recorded (*23*). The highest content of peptides recovered in the 95% alcohol concentration was the product from the second-stage EMR, which also corresponded to the lowest recovery in the 25% alcohol fraction recovery (**Table 2**). MAR was shown in a previous study to exhibit typical hydrophobic interaction properties with the peptides favored by high temperature, low pH, and high ash content (*23*). Indeed, the highly hydrophobic fraction desorbed using >50% alcohol may be removed and processed further, which leads to a product with less salt and bitterness (*23*). In the present study, the same trend was largely reproduced with the fractions desorbed using 25% alcohol being composed of less hydrophobic amino acids ($H_oAA$) than the subsequent fractions (**Figure 4**). The total amino acid analysis revealed a remarkable relationship between the concentration of the $H_oAA$ and the alcohol concentration. Thus, with increasing concentration of ethyl alcohol used for desorption, the content of the hydrophilic amino acids (HAA) decreased (**Figure 4**) while the *Q* values and bitterness (see ref *38* and the literature cited therein for a review of the *Q* value) increased (results not shown). The results mean that the content of the hydrophobic amino acids (and possibly too the ratio to the HAA) provided the greatest driving force in peptide separation during desalting

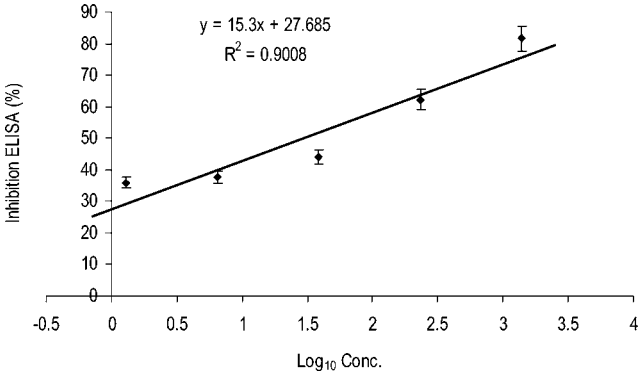


**Figure 5.** Transformed inhibition ELISA curve for desalted enzymatic membrane reactor (EMR) whey protein hydrolysates (WPH) desorbed with 25% alcohol ($WPH_{10P}$-25). From the regression equation, the $IC_{50}$ value was calculated.

and desorption. Unlike the previous results (*23*), however, the peptide chain length did not play a significant role in determining the desorption with alcohol, possibly because of the elevated DH of the EMR hydrolysates (**Figure 3**) as opposed to ca. 15% recorded in a batch reactor for the previous results (*23*). Elevated DH values resulted in short peptides and exposure of the amino acids in the peptide matrix, which excluded formation of secondary structures induced by the aqueous media in order for the polypeptides to attain a conformation that requires less energy (*23*).

Generally, when the 10 kDa membrane was used, the desorbed WPH were composed of higher amounts of peptides with molecular masses of less than <600 Da (**Table 1**) than previously reported, which seems to suggest that the separation was more significantly influenced by the amino acid composition than the peptide lengths. For the 3 kDa and the second-stage 3 kDa membrane, however, the proportion of peptides with molecular mass of <600 Da in the fractions desorbed with 50% alcohol were marginally more than in the fractions desorbed with 95% alcohol. In addition, the peptide recovery was high with no side reactions imparted on the desalted hydrolysates, whereas alcohol could be removed, recycled, and reused.

**Immunological Properties.** Two fundamental modifications were introduced in this study to help determine the ELISA reactivity of WPI/WPH. Use of WPI to coat all of the wells was used as opposed to previous studies in which the hydrolysates were used to coat the assay wells (*13*, *31*). The use of WPI throughout reduced the value of the NSB quantity while ensuring only single values for the NSB and the blind blank reading were used in each analysis in order to reduce errors. Furthermore, queries have been raised concerning differences in the avidities of binding between the primary allergen and the hydrolysates (*32*). This was likely reduced owing to the use of WPI only.

Additionally, a quantity known as an $IC_{50}$ value—being the concentration of WPI/WPH to cause 50% inhibition of the primary antibody (rabbit anti-bovine whey antiserum), thus making it unavailable to bind the well-coated WPI—was introduced and used for the first time in competitive inhibition ELISAs to aid in data interpretation (**Figure 5**). This quantity provided a better scaling quantity compared to that in other papers, where quantities such as percent reductions in immunogenicity were used (*13*). $IC_{50}$ values are common in inhibition assays [antioxidant and angiotensin-I converting enzyme (ACE) inhibition], and the benefits are well documented (*33*, *37*). Use of this quantity may provide a reference library for comparisons from various studies because the statistic is a weighted value, which may be reproducible and has more significance in comparative studies. It is hoped that its use will gain currency in inhibition ELISA, too.

**Table 3.** Immunological and Antioxidant Properties of Desalted and Un-desalted Whey Protein Hydrolysates

| product | ELISA $IC_{50}$ ($\mu$g) | effect | DPPH $IC_{50}$ (mg) |
|---|---|---|---|
| WPI | $8.8 \times 10^{-4}$ ($R^2 = 0.9242$) | | N/A[g] ($R^2 = 0.0197$) |
| $WPH_{10R}$[a] | $5.22 \times 10^{-9}$ ($R^2 = 0.9784$) | increased | N/A |
| $WPH_{10P}$[b] | $1.22 \times 10^{-7}$ ($R^2 = 0.7156$) | increased | N/A |
| $WPH_{10P}$-25 | 28.74 ($R^2 = 0.9008$) | decreased | 153.74 ($R^2 = 0.8625$) |
| $WPH_{10P}$-50 | 727.78 ($R^2 = 0.6224$) | decreased | 46.25 ($R^2 = 0.953$) |
| $WPH_{10P}$-95 | $4.27 \times 10^{-7}$ ($R^2 = 0.209$) | increased | 103.26 ($R^2 = 0.988$) |
| $WPH_{10P-3R}$[c] | $7.55 \times 10^{-7}$ ($R^2 = 0.8909$) | increased | N/A |
| $WPH_{10P-3R}$-25 | $5.5 \times 10^{-4}$ ($R^2 = 0.8776$) | decreased | 100.19 ($R^2 = 0.9266$) |
| $WPH_{10P-3R}$-50 | $2 \times 10^{-2}$ ($R^2 = 0.7352$) | decreased | 71.82 ($R^2 = 0.9549$) |
| $WPH_{10P-3R}$-95 | $4.8 \times 10^{-10}$ ($R^2 = 0.4153$) | increased | 18.79 ($R^2 = 0.9687$) |
| $WPH_{10P-3P}$[d] | $1.60 \times 10^{-5}$ ($R^2 = 0.8909$) | increased | N/A |
| $WPH_{10P-3P}$-25 | $1.84 \times 10^{3}$ ($R^2 = 0.3947$) | decreased | 92.84 ($R^2 = 0.9279$) |
| $WPH_{10P-3P}$-50 | $8.44 \times 10^{7}$ ($R^2 = 0.3069$) | decreased | 45.07 ($R^2 = 0.9806$) |
| $WPH_{10P-3P}$-95 | $2.87 \times 10^{-3}$ ($R^2 = 0.9916$) | decreased | 10.65 ($R^2 = 0.8592$) |
| $WPH_{3P}$[e] | N/A ($R^2 = 0.0109$) | | N/A |
| $WPH_{3P}$-25 | N/A ($R^2 = 0.0005$) | | 82.37 ($R^2 = 0.9414$) |
| $WPH_{3P}$-50 | $3.26 \times 10^{4}$ ($R^2 = 0.451$) | decreased | 52.00 ($R^2 = 0.992$) |
| $WPH_{3P}$-95 | $1.18 \times 10^{4}$ ($R^2 = 0.6491$) | decreased | 46.73 ($R^2 = 0.9761$) |
| $WPH_{3R}$[f] | $1.08 \times 10^{-6}$ ($R^2 = 0.8909$) | increased | N/A |

[a–f] Refer to the footnotes of **Table 1** for definitions. [g] N/A = values not useful for calculation or were not determined.

The antibody-binding properties of proteins and their hydrolysates is a widely applied characterization technique for ELISA reactivity and prediction of protein/hydrolysate allergenicity. In this study, the WPI–anti-bovine whey antibody binding was high. The quantity $IC_{50}$ was used to quantify the amount of WPI/WPH able to conjugate and bind with the anti-bovine whey antibody. This quantity was low for highly immunoactive WPH due to the high concentration of the present epitopes, which reduced the effective concentration of the hydrolysates required to bind the antibody by 50%, making it unavailable to bind the antigenic WPI coated to the wells. WPI had a calculated $IC_{50}$ value of $8.8 \times 10^{-4}$ $\mu$g with a high coefficient of correlation ($R^2$) equal to 0.9242. The high correlation between the concentration and the antibody binding ability of the WPI/WPH provided a reliable means of calculating the $IC_{50}$ values (**Figure 5**). $IC_{50}$ values higher than those for native WPI indicate reduced immunogenicity and, it is hoped, lower allergenicity. For all of the un-desalted hydrolysates except the permeate from the single-stage 3 kDa TFF membrane ($WPH_{3P}$) there was some protein dose-dependent relationship with ELISA inhibition values (**Table 3**).

The retentates from the hydrolysis schemes had remarkably lower $IC_{50}$ values (high immunological property) than the

permeates in all cases with the 10 kDa retentates recording the highest antibody binding capacity ($5.22 \times 10^{-9}$ μg, $R^2 = 0.9784$) followed by the products $WPH_{10P-3R}$ ($7.55 \times 10^{-7}$ μg, $R^2 = 0.8909$) and $WPH_{3R}$ ($1.08 \times 10^{-6}$, $R^2 = 0.8909$). Desalting on MAR led to differences in immunogenicity owing to differential migration of epitopic fragments with the concentration of alcohol probably influenced by the amino acid composition and to some extent by the molecular masses of the WPH (**Table 1**). Thus, the fraction $WPH_{3P}$-25, the permeate through the single-stage 3 kDa TFF desalted and desorbed using 25% alcohol, recorded some inhibition to the anti-bovine whey antibody, but the inhibition was not related to the concentration of the hydrolysates ($R^2 = 0.0005$), indicating an almost nonimmunological property by the fraction. Good correlations between the concentration of the peptides and inhibition to the anti-bovine whey antibody ($R^2 > 0.9$) were recorded for the desorbed fractions $WPH_{10P}$-25 ($R^2 = 0.9008$) and $WPH_{10P-3P}$-95 ($R^2 = 0.9916$). Following hydrolysis and desalting, some fractions showed more immunological properties than the un-desalted parent WPH and/or WPI (effect marked increased in **Table 3**). However, the fractions with lower $IC_{50}$ values than WPI showed relatively poor coefficients of correlations ($R^2 \leq 0.5$), which makes the result less reliable (**Table 3**). The coefficients of correlation for the un-desalted WPH were generally higher than those for most of the desalted fractions.

Hydrolysis and heat-denaturing of β-lactoglobulin was postulated to lead to the exposure of putatively immunoactive epitopew, which makes some hydrolysates more immunogenic than the native protein from which they are obtained (*39*). Thus, although enzymes are used to eliminate specific epitopic sites in native immunoactive proteins, enzyme specificities may hinder complete reduction of antibody-binding properties of immunogenic proteins. This may be reversed by employing bioengineering tools such as site-directed genetic engineering to produce enzymes targeting specific epitopic pockets in parent proteins to eliminate ELISA reactivity and hence protein/hydrolysate antigenicity.

Use of proteins and their hydrolysates is influenced to some extent by allergenicity (*31*) and bitterness (*23*, *40*). There are procedures to process protein hydrolysates to render them safe for populations that show allergenic responses (*41*). Thus, the reactive epitopes are cleaved by the enzyme, leading to reduced allergenic reactivity (*12*). Although heat was reported to denature the proteins, the process led to a rearrangement of the protein structure, which in turn exposed formerly hidden allergenic sites and made the peptides more immunogenic than the native proteins (*42*, *43*). This phenomenon may explain the increase in immunogenicity of some of the hydrolysates compared to the native WPI (**Table 3**).

Ultrafiltration membranes have been used to separate the immunoactive fragments of hydrolysis, which were found to be related to the molecular mass sizes (*44*), whereas both hydrolysis and use of ultrafiltration membranes were proposed as a means to deal with protein allergy (*45*). Although there seems to be no consensus (probably due to the differences in the assay proteins and protocols), hydrolysates with average molecular masses of >1400−3000 Da seem to show some allergenic responses, whereas those composed of fragments lower than 1400 Da showed no response in studies (*12*, *44*). Enzyme hydrolysis is one of the methods used to reduce allergenicity of whey proteins as is heat denaturing (*39*, *46*, *47*).

The safety of proteins and protein hydrolysates and food is traditionally defined in terms of chemical and microbiological safety, but allergenicity is expected to increasingly become an issue of concern with consumers in the future. Production of hypoallergenic hydrolysates is attractive for infant formulations such that selection of enzymes and the extent of hydrolysis (DH) and conditions that decimate the immunoactive epitopes will attract interdisciplinary research crossing the medical and food science and technology fields. The EMR produced peptides with reduced immunoreactivity, and its use in the production of hypoallergenic hydrolysates holds potential for the functional foods/pharmaceuticals industry. The $IC_{50}$ values are easy to compare and relate and point to the likelihood that when the immunogenicity of protein hydrolysates reduces severalfold over that of the native protein, it is easily underestimated. The $IC_{50}$ values are sensitive to residual immunogenicity, which becomes useful when in the evaluation of the effectiveness of enzymatic hydrolysis in reducing and/or eliminating the epitopes.

**Antioxidant Properties.** The free radical scavenging ability of native WPI was practically nil and did not show any dose dependence (**Table 3**). Likewise, the un-desalted WPH showed some unclear relationship with DPPH, a pointer to the inherent practicability of the DPPH assay with samples containing interfering salts (*33*, *48*). After hydrolysis and desalting, however, all hydrolysates showed markedly improved antioxidant properties. The highest DPPH-scavenging activities were recorded with the single- and second-stage 3 kDa TFF membrane hydrolysates (**Table 3**). Significantly, the highest antioxidant activities (lowest $IC_{50}$ values) were recorded for the fraction permeable through the 10 kDa and further permeable through the 3 kDa TFF membranes ($WPH_{10P-3P}$) desorbed with 95% alcohol ($IC_{50} = 10.65$ mg) and the retentate in the 3 kDa ($WPH_{10P-3R}$) also desorbed using 95% alcohol ($IC_{50} = 18.79$ mg). Generally, the fractions desorbed using 25% alcohol showed poor free radical scavenging.

Antioxidant activity of WPI was reported by Peña-Ramos et al. (*49*) in a lipid oxidation measured as thiobarbituric acid-reactive substances (TBARS). However, in the present study, the property was largely undetected. This disparity may be due to the detection methods, whereas the DPPH system has been shown as doubtful as an index of antioxidant activity (*48*). WPI did scavenge DPPH in our study, although the effect was not dose-dependent and hence the $IC_{50}$ value was not computable ($R^2 = 0.0197$). The best dose-dependent relationship was recorded for both the single- and two-stage hydrolysates permeable through the 3 kDa TFF membrane fractions desorbed with 50% alcohol ($WPH_{3P}$-50 and $WPH_{10P-3P}$-50), corresponding to 99.2 and 98.06% correlation. In addition, the hydrolysates desorbed with 95% alcohol from the permeate of the single-stage 10 kDa were explained by 98.8% (**Table 3**). There was an apparent relationship between the antioxidant properties of the whey protein peptides and the amino acid composition with the presence of the aromatic/imino amino acids being the most prominent determinants (*49*). The results of this study show that the desalted fractions with the high DPPH free radical scavenging were largely composed of low amounts of hydrophilic amino acids and generally ca. 45 mg of amino acids per 100 g of protein (**Figure 4**). EMR hydrolysates showed increased DPPH free radical scavenging activity, which was better than that for the native WPI.

**PCA.** Multivariate data analysis was performed by use ofPCA. The Kaiser−Meyer−Olkin (KMO) measure of sampling adequacy (a value that lies between 0 and 1, with high values being good) was calculated to be 0.579, which was considered to be adequate. The PCA communalities, which indicate the amount of variance shared with at least one of the remaining variables, were high (range of 0.726 for $H_oAA$ to 0.984 for both

**Table 4.** Component Matrix and Rotated Matrix for the Principal Components

| | component matrix[a] | | | rotated component matrix[a] | | |
|---|---|---|---|---|---|---|
| original variable | 1 | 2 | 3 | 1 | 2 | 3 |
| membrane NMWCO[b] | **–0.667**[g] | **0.522** | 0.341 | –0.389 | **0.789** | 0.244 |
| alcohol concentration[c] | **–0.695** | **–0.608** | –0.096 | **–0.863** | –0.319 | 0.122 |
| peptides with mol mass of <600 Da | **–0.970** | 0.205 | –0.032 | **–0.837** | 0.532 | –0.007 |
| peptides with mol mass of 601–1500 Da | **0.883** | –0.390 | 0.140 | **0.713** | **–0.644** | 0.166 |
| peptides with mol mass of 1501–3000 Da | **0.988** | 0.046 | –0.074 | **0.920** | –0.334 | –0.162 |
| peptides with mol mass of >3000 Da | **0.830** | 0.087 | –0.220 | **0.762** | –0.270 | –0.301 |
| $IC_{50}$ for ELISA[d] | –0.116 | **0.890** | 0.030 | 0.193 | **0.855** | –0.195 |
| $IC_{50}$ for antioxidant activities | –0.078 | –0.299 | **0.862** | –0.027 | –0.056 | **0.913** |
| $H_oAA$[e] | **–0.791** | –0.119 | –0.293 | **–0.823** | 0.116 | –0.188 |
| HAA[f] | **0.881** | 0.384 | 0.114 | **0.964** | 0.052 | –0.061 |

[a] Extraction method: principal component analysis (three components extracted). [b] Nominal molecular weight cutoff. [c] Concentration of ethyl alcohol used to desorb the desalted peptides from macroporous adsorption resins. [d] Concentration of peptides to cause inhibition of anti-bovine whey antibody by 50%. [e] Hydrophobic amino acid concentration. [f] Hydrophilic amino acid concentration. [g] Values in boldface show high correlation coefficients.

content of peptides with molecular mass of <600 and between 1501 and 3000 Da, respectively), which indicates that the variables fitted well with the factor solutions (*18*). The PCA extracted three PCs, whence the $IC_{50}$ values for antioxidant and ELISA were log-transformed to eliminate skewing in the scatter plots. The first PCA (PC1) accounted for 57.35% of the total variance in the analysis, whereas PC2 and PC3 contributed 18.90 and 10.43%, respectively (results not shown). The three PCs accounted for a total of 86.67%, which implies the analysis can be limited to these with the risk of only losing 13.33% of the information.

From **Table 4**, it is evident that PC1 is closely associated with the concentrations of peptides with molecular mass of <600 Da (0.97) and between 1501 and 3000 Da (0.99). The fact that the coefficients for the $IC_{50}$ values for ELISA (−0.12) and antioxidant activities (−0.08) are negative and nearly negligible means that PC1 is probably a molecular mass separation characteristic of the hydrolysates, which is driven by the membrane NMWCO and hydrolysis scheme (−0.67) as well as the alcohol to desorb (−0.70). Indeed, the negative coefficients of these parameters means that lower NMWCO and alcohol concentration provided better driving forces on MW separation (*18*, *28*). Interestingly, the amino acid concentration provides evidence that PC1 was favored by lower values for $H_oAA$ (−0.79) and higher values for HAA (0.88). The implications are that the higher content of HAA (which was recorded when lower alcohol was used, **Figure 4**) played a significant role in the hydrolysate migration. It is no wonder, then, that the highest amount of hydrolysate nitrogen was consistently recovered in the 25% alcohol desorbed fraction. On the other hand, PC2 is depicted in these results to be associated prominently with ELISA $IC_{50}$ values (0.89), which is possibly driven by the alcohol to desorb (−0.61) and the NMWCO (0.52). It is therefore an immunogenicity property of the hydrolysates and is favored by higher membrane NMWCO and lower alcohol concentration. PC3 is obviously an antioxidant property of the hydrolysates (0.86), which is apparently favored by high membrane NMWCO (0.34).

From an analysis of the PCA, the hydrolysates' properties may be ranked (*18*, *28*). Thus, the influence is significantly (57.35%) due to the molecular mass (caused by $H_oAA$/ HAA amino acid composition and membrane NMWCO), followed by the ELISA reactivity (18.90%) and antioxidant properties (10.43%). Additionally, the hydrolysates' properties were influenced most by the membrane hydrolysis system and the concentration of the alcohol used to desorb. The membrane system and NMWCO provided a means to produce short peptides with elevated DH values, with most peptides lying in the range of di- to pentapeptides (<600 Da). Meanwhile, alcohol provided crude separation based on the amino acid composition. The fact that all of the coefficients for the alcohol concentration used to desorb had a negative coefficient implies that lower alcohol concentrations (higher amounts of HAA and longer peptides) were favorable for separation and the immunological/ antioxidant properties. The EMR provided a useful technological process to produce peptides with reduced immunogenicity and increased antioxidant activity.

## LITERATURE CITED


(1) Adler-Nissen, J. Enzymatic hydrolysis of soy protein for nutritional fortification of low pH food. *Ann. Nutr. Aliment.* **1978**, *32*, 205−216.

(2) Chobert, J.-M.; Bertrand-Harb, C.; Nicolas, M.-G. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *J. Agric. Food Chem.* **1988**, *36*, 883−892.

(3) Spellman, D.; O'Cuinn, G.; FitzGerald, R. J. Physicochemical and sensory characteristics of whey protein hydrolysates generated at different total solids levels. *J. Dairy Res.* **2005**, *72*, 138−143.

(4) Pena-Ramos, E. A.; Xiong, Y. L. Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. *Meat Sci.* **2003**, *64*, 259−263.

(5) de Wit, J. N. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* **1998**, *81*, 597−608.

(6) Ha, E.; Zemel, M. B. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying benefits for health. *J. Nutr. Biochem.* **2003**, *14*, 251−258.

(7) Brans, G.; Schroen, C. G. P. H.; van der Sman, R. G. M.; Boom, R. M. Membrane fractionation of milk: state of the art and challenges. *J. Membr. Sci.* **2004**, *243*, 263−272.

(8) Zydney, A. L. Protein separations using membrane filtration: new opportunities for whey fractionation. *Int. Dairy J.* **1998**, *8*, 243−250.

(9) Chatterton, D. E. W.; Smithers, G.; Roupas, P.; Brodkorb, A. Bioactivity of $\beta$-lactoglobulin and $\alpha$-lactalbumin—technological implications for processing. *Int. Dairy J.* **2006**, *16*, 1229−1240.

(10) Pahud, J. J.; Monti, J. C.; Jost, R. Allergenicity of whey protein: its modification by tryptic in vitro hydrolysis of the protein. *J. Pediatr. Gastroenterol. Nutr.* **1985**, *4*, 408−413.

(11) Mahmoud, M. I.; Malone, W. T.; Cordle, C. T. Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. *J. Food Sci.* **1992**, *57*, 1223−1229.

(12) Alting, A. C.; Meijer, R. J.; van Beresteijn, E. C. Selective hydrolysis of milk proteins to facilitate the elimination of the ABBOS epitope of bovine serum albumin and other immunoreactive epitopes. *J. Food Prot.* **1998**, *61*, 1007−1012.

(13) Guadix, A.; Camacho, F.; Guadix, E. M. Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. *J. Food Eng.* **2006**, *72*, 398−405.

(14) Manninen, A. H. Protein hydrolysates in sports and exercise: a brief review. *J. Sports Sci. Med.* **2004**, *3*, 60−63.

(15) Cheison, S. C.; Wang, Z. Bioactive milk peptides: redefining the food−drug interphase−review. *Afr. J. Food Nutr. Sci. Agric. Dev.* **2003**, *3*, 29−38.

(16) Clemente, A. Enzymatic protein hydrolysates in human nutrition. *Trends Food Sci. Technol.* **2000**, *11*, 254−262.

(17) Meisel, H. Biochemical properties of peptides encrypted in bovine milk proteins. *Curr. Med. Chem.* **2005**, *12*, 1905−1919.

(18) Cheison, S. C.; Wang, Z.; Xu, S.-Y. Hydrolysis of whey protein isolate in a tangential flow filter membrane reactor: I. Characterisation of permeate flux and product recovery by multivariate data analysis. *J. Membr. Sci.* **2006**, *283*, 45−56.

(19) Deeslie, W. D.; Cheryan, M. Continuous enzymatic modification of proteins in an ultrafiltration reactor. *J. Food Sci.* **1981**, *46*, 1035−1042.

(20) Zeng, X.; Ruckenstein, E. Membrane chromatography: preparation and applications to protein separation. *Biotechnol. Prog.* **1999**, *15*, 1003−1019.

(21) van Reis, R.; Zydney, A. Membrane separations in biotechnology. *Curr. Opin. Biotechnol.* **2001**, *12*, 208−211.

(22) Prieto, C. A.; Guadix, A.; Gonzalez-Tello, P.; Guadix, E. M. A cyclic batch membrane reactor for the hydrolysis of whey protein. *J. Food Eng.* **2007**, *78*, 257−265.

(23) Cheison, S. C.; Wang, Z.; Xu, S.-Y. Use of macroporous adsorption resin for simultaneous desalting and debittering of whey protein hydrolysates. *Int. J. Food Sci. Technol.* **2006**, DOI: 10.1111/j.1365-2621.2006.01461.x, in press.

(24) Mota, M. V. T.; Ferreira, I. M. P. L. V. O.; Oliveira, M. B. P.; Rocha, C.; Teixeira, J. A.; Torres, D.; Goncalves, M. P. Trypsin hydrolysis of whey protein concentrates: characterization using multivariate data analysis. *Food Chem.* **2006**, *94*, 278−286.

(25) Peña-Ramos, E. A.; Xiong, Y. L. Antioxidative activity of whey protein hydrolysates in a liposomal system. *J. Dairy Sci.* **2001**, *84*, 2577−2583.

(26) Cheison, S. C.; Wang, Z.; Xu, S.-Y. Multivariate strategy in screening of enzymes to be used for whey protein hydrolysis in an enzymatic membrane reactor. *Int. Dairy J.* **2007**, *17*, 393−402.

(27) Cheison, S. C.; Wang, Z.; Xu, S.-Y. Use of response surface methodology to optimise the hydrolysis of whey protein isolate in a tangential flow filter membrane reactor. *J. Food Eng.* **2007**, *80*, 1134−1145.

(28) Cheison, S. C.; Wang, Z.; Xu, S.-Y. Hydrolysis of whey protein isolate in a tangential flow filter membrane reactor: II. Characterisation for the fate of the enzyme by multivariate data analysis. *J. Membr. Sci.* **2006**, *286*, 322−332.

(29) Satake, K.; Okuyama, T.; Ohashi, M.; Shinoda, T. The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene 1-sulfonic acid. *J. Biochem. (Tokyo)* **1960**, *47*, 654−660.

(30) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256−1262.

(31) Svenning, C.; Brynhildsvold, J.; Molland, T.; Langsrud, T.; Vegarud, G. E. Antigenic response of whey proteins and genetic variants of $\beta$-lactoglobulin−the effect of proteolysis and processing. *Int. Dairy J.* **2000**, *10*, 699−711.

(32) Braitbard, O.; Glickstein, H.; Bishara-Shieban, J.; Pace, U.; Stein, W. Competition between bound and free peptides in an elisa-based procedure that assays peptides derived from protein digests. *Proteome Sci.* **2006**, *4*, 12.

(33) Huang, D.-J.; Ou, B.-X.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(34) AOAC. *Official Methods of Analysis*, 16th ed.; AOAC International: Gaithersburg, MD, 1997; Vol. II, revision 3.

(35) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin−phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(36) Smyth, M.; FitzGerald, R. J. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.* **1998**, *8*, 819−827.

(37) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int. Dairy J.* **2002**, *12*, 813−820.

(38) Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: London, U.K., 1986; p 427.

(39) Iametti, S.; Rasmussen, P.; Frokiaer, H.; Ferranti, P.; Addeo, F.; Bonomi, F. Proteolysis of bovine $\beta$-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur. J. Biochem.* **2002**, *269*, 1362−1372.

(40) Cho, M. J.; Unklesbay, N.; Hsieh, F.; Clarke, A. D. Hydrophobicity of bitter peptides from soy protein hydrolysates. *J. Agric. Food Chem.* **2004**, *52*, 5895−5901.

(41) Heddleson, R. A.; Allen, J. C. Relative immunogenicity of $\beta$-lactoglobulin, bovine serum albumin, and a model acid whey product. *Nutr. Res.* **1997**, *17*, 505−514.

(42) Exl, B. M.; Fritsche, R. Cow's milk protein allergy and possible means for its prevention. *Nutrition* **2001**, *17*, 642−651.

(43) Davis, P. J.; Williams, S. C. Protein modification by thermal processing. *Allergy* **1998**, *53*, 102−105.

(44) van Hoeyveld, E. M.; Escalona-Monge, M.; de Swert, L. F.; Stevens, E. A. Allergenic and antigenic activity of peptide fragments in a whey hydrolysate formula. *Clin. Exp. Allergy* **1998**, *28*, 1131−1137.

(45) Görtler, I.; Urbanek, R.; Forster, J. Characterization of antigens and allergens in hypo-allergenic infant formulae. *Eur. J. Pediatr.* **1995**, *154*, 289−294.

(46) Lee, Y. H. Food-processing approaches to altering allergenic potential of milk-based formula. *J. Pediatr.* **1992**, *121*, S47−50.

(47) Bonomi, F.; Fiocchi, A.; Frøkiaer, H.; Gaiaschi, A.; Iametti, S.; Poiesi, C.; Rasmussen, P.; Restani, P.; Rovere, P. Reduction of immunoreactivity of bovine $\beta$-lactoglobulin upon combined physical and proteolytic treatment. *J. Dairy Res.* **2003**, *70*, 51−59.

(48) Pihlanto, A. Antioxidative peptides derived from milk proteins. *Int. Dairy J.* **2006**, *16*, 1306−1314.

(49) Peña-Ramos, E. A.; Xiong, Y. L.; Arteaga, G. E. Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *J. Sci. Food. Agric.* **2004**, *84*, 1908−1918.

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