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Development of an Integrative Process for the Production of Bioactive Peptides from Whey by Proteolytic Commercial Mixtures

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The aim of this work is to develop an integrative process based on the use of an ion exchange resin for the enzymatic hydrolysis of β-lactoglobulin to produce peptides with angiotensin converting enzyme (ACE) inhibitory activity using whey as a feed stock. Unlike the conventional methods, the main advantage of this approach is that by integrating the selective separation of β-lactoglobulin from whey and its hydrolysis less complex mixtures of peptides are produced. Furthermore, peptides of similar charge as β-lactoglobulin remain adsorbed achieving further purification. In this work, the enzyme protease N Amano at an enzyme to substrate ratio of 1/100 (wt/wt) was added directly to the adsorbed proteins in a thermostatically controlled membrane reactor operated in batch mode. Separation of the smaller peptides from the enzyme and larger peptides was achieved with a 1 kDa molecular weight cut-off ultrafiltration membrane. Also, this step enables the recycling of non-hydrolyzed substrates, large peptides, and enzyme. The adsorbed protein was re-solubilised in a 10 mM potassium phosphate buffer (pH 7 and 45°C). The different fractions were assayed for their bioactivity in terms of angiotensin converting enzyme inhibition percentage (ACEi%) and IC_{50} which is the concentration of peptides that can inhibit the ACE activity by 50%. Results show that permeates of 2 and 6 hrs hydrolysis have the highest bioactivity with $IC_{50} = 67$ and 98 μg/ml respectively.

Keywords ACE inhibition; β-lactoglobulin; hydrolysate; ion exchanger; Protease N "Amano"

INTRODUCTION

Sweet whey is produced as a result of chymosin coagulation of the milk for cheese making (1). Only 10–20% of the raw milk is utilized in cheese production and the other 80–90% yields whey as a waste product (2). A vast amount of whey is produced worldwide annually and estimated about 115 million metric tons. Out of this only 53% are used as whey products in food and the rest of the 47% are dumped as a waste into the environment (2–4). This vast amount of waste product consists of abundant valuable proteins (see Table 1). The constituents of these proteins have important biological functionalities with health-enhancing results. Particularly peptides derived from β-lactoglobulin (5–8) and casein have proved to possess high ACE inhibitory activity *in-vitro* which in some cases leads to antihypertensive effect *in-vivo* (9,10).

Numerous techniques have been suggested for the separation and production of these bioactive peptides in a laboratory as well as at industrial scale. However, due to the lack of suitable large-scale technologies, its commercial production has been limited. The most widely used approach for their production is the batch reactor. However, this approach is very expensive due to the large quantities of enzymes needed as a result of enzyme inactivation at the end of hydrolysis process (11). Immobilized enzyme reactors have also been applied; however, the high cost of the immobilization procedure and significant losses in activity and leakage of enzymes limits the applicability of this system (12). The growing interest of bioactive peptides attracts the integration of membrane process in bioreactors for their continuous separation (13). Korhonen and Pihlanto-Lappalla (14) applied an enzyme membrane reactor (EMR) in which enzymes were immobilized on the membrane or were freely circulating inside the reactor. In both cases enzymes were retained and selectively separated from the end product by the help of the membrane which increases the utilization of enzymes (14). Gaudix et al. (15), designed a stable membrane reactor for the production of whey protein hydrolysate with low antigenicity; however, they found inactivation of enzymes due to temperature.

The potential use of purified individual proteins has encouraged the development of ionic exchange chromatography to replace the traditional methods such as acid precipitation (16) that causes denaturation as well as low level of purity (17). Ion exchange chromatography has been applied to the enrichment of the parent proteins and bioactive peptides from milk. Ellegård et al. (18) developed a process scale chromatography for the isolation of bioactive peptides produced by tryptic hydrolysis with an excellent

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TABLE 1
Physico-chemical characteristics of whey proteins (35,36)

Whey proteins	Concentration (mg/ml)	Isoelectric point (PI)	Molecular weight (Kg/mol)
β-lactoglobulin	3.4	5.2–5.4	18.3
α-lactoalbumin	1.2–1.5	4.2	14.2
Serum albumin	0.3–0.6	4.9–5.1	66
IgG, IgM, IgA	0.6–0.9	5.8–7.3	150.0–900.0
Lactoperoxidase	0.06	9.6	78
Lactoferin	0.05	8	78

product yield of 40 Kg casein phosphopeptides enriched with sodium or calcium. Bouhallab et al. (19) evaluated ionic exchange chromatography using a highly hydrophilic ion exchange for the separation of small cationic bioactive peptides prepared by tryptic hydrolysis of caseinomacropeptide in a membrane reactor as a function of pH and salt gradient. However, regardless of their efficiency, these techniques are expensive and limited only to a laboratory scale. Recio and Visser (20) used ion exchange membrane chromatography for the in-situ hydrolysis of lactoferrin and at the same time separating lactoferricin-B from cheese whey. They suggested that this membrane-based process has several economic advantages which are rapid and can be easily scaled up to gram or even kilograms of quantities. However, its low adsorption capacity per unit area and shorter life time, limits its application to laboratory scale (21). Following a similar approach in our lab we have investigated the use of an anion exchange resin for the production of ACE inhibitory peptides from pure β-lactoglobulin. In this study we found that we could produce a potent ACE inhibitory peptide Ser-Ala-Pro-Leu-Arg-Val-Tyr with an IC_{50} (the concentration of peptides that can inhibit the original ACE by 50%) of 8 μM (8). This peptide is the most potent peptide reported so far from β-lactoglobulin. Furthermore, in that study we found that the hydrolysate produced from immobilized β-lactoglobulin had twice more ACE inhibitory activity than the hydrolysate from the protein free in solution under the same conditions.

The focus of the present work is to develop this process further by applying it to whey and test if selective immobilization of β-lactoglobulin and similar bioactivity as that obtained from pure β-lactoglobulin can be achieved. The advantage of the proposed process as opposed to those in which the enzyme is immobilized is that the non-hydrolyzed β-lactoglobulin and peptides with similar charge as the protein will remain adsorbed to the anion exchanger while other peptides (the most active ones) pass through the microfiltration membrane thus achieving in one step the separation of the product from the substrate and partial fractionation of peptides. In addition, the application of a second fractionation step is investigated by a 1 kDa ultrafiltration membrane to separate the smaller peptides from the bigger ones and the enzyme.

MATERIALS AND METHODS

Materials and Reagents

All reagents and chemicals were analytical grade including, bovine β-lactoglobulin, N-Hippuryl – L – Histidyle – L – Leucine (HHL), hippuric acid (HA), angiotensin converting enzyme (ACE; EC 3.4.15.1), captopril, Bovine serum albumin (BSA), bicinchoninic acid solution (BCA), copper-sulfate solution, DEAE sepharose®. Bromophenol Blue were obtained from Sigma-Aldrich, Dorset, UK; Acrylamide, SDS, Tris-HCl, Glycine, TEMED were bought from Sigma, Steinhein, Germany; Potassium phosphate, sodium chloride (NaCl), methanol, trifluoroacetic acid (TFA), acetic acid, acetonitrile, hydrochloric acid, were purchased from Fisher Scientific UK Limited; Glycerol from BDH laboratory supplies, England; Protease N 'Amano' from Bacillus subtilitis was obtained from Amano Enzyme Inc., Nagoya, Japan (191,000 units/gm), where one unit of enzyme produces amino acids equivalent to 0.1 gm of tyrosine in 60 min at pH 7 and a temperature of 55°C. Amicon filtration cell was obtained from amicon® a Grace company. Regenerated cellulose Ultrafiltration membranes (1 kDa), microfiltration (0.45 μm) membranes and syringe driven PVDF filter (0.45 μm and 0.2 μm) were obtained from Millipore Corporation, Bedford, UK. Ultrospec 1100 pro UV/Visible spectrophotometer was from Biochrom Ltd., Cambridge, England.

Methods

Production of β-Lactoglobulin Hydrolysate by Integrative Process

The integrative process is described in Fig. 1. Bovine sweet whey was used as a feed stock and fed directly to a stirred cell filtration device (200 ml) fitted with a micro filtration membrane that contains an adsorption resin at a resin: β-lactoglobulin ratio of 1:26 (v/v) for 10 minutes. After the adsorption process, the mixture was filtered and then loosely bound proteins and intrinsically trapped materials were washed twice while β-lactoglobulin was adsorbed and retained. Protease N Amano at an enzyme: substrate ratio of 1/100 (wt/wt) in a 10 mM potassium phosphate buffer was directly added into the adsorbed protein at pH 7 and 45°C for hydrolysis to start. At the end of hydrolysis, the mixtures were then filtered through and the loosely bound proteins and intrinsically trapped materials were washed twice with a 10 mM of potassium phosphate buffer (10 ml) at pH 6.6 while β-lactoglobulin was adsorbed and retained. The adsorbed protein was then re-solubilized in 10 mM potassium phosphate buffer (30 ml) at pH 7 and

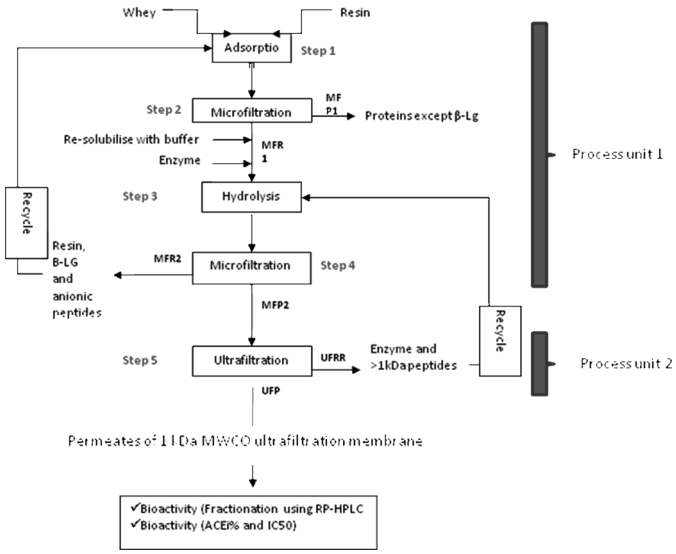

FIG. 1. Whole process of bioactive peptide production from immobilised β-lactoglobulin using whey as a feed stock at an adsorption temperature of 20°C and pH 6.5, followed by re-solubilisation of the adsorbed protein using 10 mM potassium phosphate buffer at pH 7 and a temperature of 45°C and addition of protease N 'Amano' at an enzyme; substrate ratio of 0.01. Where: MFP is permeate of microfiltration step; MFR is retentate of microfiltration step; UFP is permeate of 1 kDa ultrafiltration step; UFR is retentate of 1 kDa ultrafiltration step.

45°C. Hydrolysis was started by adding protease N 'Amano' at an enzyme: substrate ratio of 1/100 wt/wt and incubated at 2, 4, and 6 hrs. At the end of hydrolysis, the mixtures were filtered by applying pressure ($\Delta P = 2$ bar) and enzyme activity in the permeate was stopped by boiling at 100°C for 5 minutes, then passed to a stirred cell device fitted with the 1 kDa ultrafiltration membrane. In addition, those peptides adsorbed to the resin were eluted by applying a 10 mM potassium phosphate buffer (10 ml) at pH 6.6 that contains 1 M NaCl then was filtered through a filtration device that was fitted with 1 kDa ultrafiltration membrane. The different fractions of the samples (permeate, retentate, elute permeate, and elute retentate) were then stored at −20°C until further analysis.

Adsorption Equilibrium and Adsorption Kinetics

β-lactoglobulin solutions (3 mg/ml) were prepared in nine volumetric flasks using 10 mM potassium phosphate buffer at pH 6.5 with a final volume of 30 ml at room temperature. The anion exchanger DEAE sepharose® was pre-equilibrated to adsorption condition by washing twice with 10 mM potassium phosphate buffer at pH 6.5. After washing, it was reconstituted at 50% (V/V) with 10 mM potassium phosphate buffer pH 6.5. Then 0.5 ml of the resin was added to each volumetric flask. The mixtures were incubated at different adsorption times (0, 10, 30, 45, 60, 75, 90, 105, and 120 minutes) with stirring. At the end of incubation, the ion exchanger was allowed to settle. The supernatants were carefully collected, filtered with a syringe driven 0.45 μm PVDF filter, and protein concentrations were determined with a UV spectrophotometer at 280 nm. The concentration of protein adsorbed to the DEAE sepharose® at each data point was calculated from a mass balance on protein in solution and adsorption capacity (Q) and adsorption kinetics were determined.

Whey Preparation

Pasteurized skimmed milk was bought from the local supermarket and heated at 35°C. Then commercial rennet was added at 0.3 ml per liter and stirred gently for a further one minute. The milk was left for 1 hour and then the casein coagulum was cut vertically (25 × 25 mm) with a knife to drain the lactoserum. Incubation then was extended for 20 minutes, after which the whey was scooped from the vessel and filtered using cheese cloth. The collected whey was then stored at −20°C until use for further experiments.

Quantification of the Major Whey Proteins

The major whey proteins (β-lactoglobulin, α-lactalbumin and BSA) were quantified using RP-HPLC adapted from Thoma et al. (22) with some modification. The samples were filtered with a 0.45 μm PVDF filter and analyzed in a Dionex that contains P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC100, PDA-100 photodiode array detector with a C18 column (250 × 4.6 mm). A gradient of solvent A which is 0.1% trifluoroacetic acid in HPLC grade water and solvent B of 80% acetonitrile and 0.555% trifluoroacetic acid in water was used. Solvent B was 43–47% over 8 min, 47–52% over 8 min, 52–57% over 6 min, and 57–58% over 1 min, and then 58–100% over 5 min. The column temperature was kept at 40°C. Analysis was carried out using an injection volume of 50 μl, flow rate of 1 ml/min, and the peak areas were monitored at 226 nm The injection volume was 50 μl and the peak areas were monitored at 226 nm. β-lactoglobulin model protein was used as a standard.

β-Lactoglobulin Conversion Value Determination

The conversion values of β-lactoglobulin were calculated based on RP-HPLC method described in section 1.2.3 by Thoma et al. (22) with some modification by taking the peak area of the proteins at a given time as follows:

$$[\%\beta - \mathrm{Lg}]_{Conv} = \left[\frac{(A_{\beta-Lg})_{t=0} - (A_{\beta-Lg})_{t=x}}{(A_{\beta-Lg})_{t=0}}\right] \times 100\% \quad (1)$$

where: $[\%\beta - \mathrm{Lg}]_{\mathrm{Conv}}$ is the percentage of β-lactoglobulin converted into peptides at a given time. $(A_{\beta-\mathrm{Lg}})_{t=0}$ is the

peak area of β-lactoglobulin at zero time hydrolysis; $(A_{\beta-Lg})_{t=x}$ is the peak area of the β-lactoglobulin left unhydrolyzed at a given time.

Hydrolysates Peak Profile Determination using RP-HPLC

The hydrolyasate peak profiles were determined based on semi-prep RP-HPLC method using a Dionex that contains P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC100, PDA-100 photodiode array detector with a C18 column (250 × 4.6 mm). The mobile phase was a gradient system that contains eluent 'A' which is 0.1% TFA in water and eluent 'B' is 0.08% TFA in acetonitrile. The flow rate was 4 ml/min and the column and the sampler temperature was kept at 25 and 4°C consecutively; eluent B was 0 to 45% for 60 min, 45 to 70% for 5 min, 70% for 10 min and then eluent A was 100% for 15 min. The injection volume was 250 µl and the peak areas were monitored at 214 and 280 nm.

ACE Inhibition Determination

Enzymatic Assay with ACE. The enzyme incubation assay was adapted from (23). 0.1 M potassium phosphate buffer pH 8.3 that contains 0.3 M NaCl and 5 mM Hippuryl-Histidyle-Leucine (HHL) was prepared. Each 325 µl assay contains 225 µl of 5 mM HHL; 25 µl of inhibitor and 75 µl of 60 mU ACE. The mixture that contains both 25 µl of inhibitor and 225 µl of HHL was pre-incubated for 10 minutes at 37°C and 75 µl of ACE was also pre-incubated separately before mixing for 10 minutes at 37°C and then re-incubated for a further one hour by mixing all solutions (225 µl of 5 mM HHL, 25 µl sample and 75 µl ACE) at 37°C. The hydrolysis was stopped by adding 20 µl of 5 M HCl and filtering through 0.2 µm PVDF filters.

HPLC Method to Determine ACEi%. The reverse-phase high performance liquid chromatography (RP-HPLC) method for the determination of ACE inhibition was adapted from Mehanna and Dowling (24) based on the liberation of hippuric acid from N-Hippuryl – L – Histidyle – L – Leucine (HHL) hydrolysed by ACE with some modifications. The hippuric acid liberated was determined using a Dionex RP-HPLC system that contains P680 HPLC pump; ASI-100 Automated sample injector; thermostatted column compartment TCC-100 and PDA-100 photodiode array detector. The mobile phase was isocratic with 20% (v/v) acetonitrile and 0.1% trifluoroacetic acid (TFA) in HPLC grade water and flow rate of 0.8 ml/min for 25 min. The injection volume of the sample was 50 µl and the hippuric acid liberated was detected at 228 nm on a symmetry C18 column (Ace5 C18, 250 × 4.6 mm). The column and the sampler temperature were 25 and 4°C respectively.

Calculation of % ACE Inhibition. The percentage of ACE inhibition is calculated based on the hippuric acid liberated using the inhibitor and control as follows:

$$[ACEi\%] = \left[\frac{(HA)_C - (HA)_S}{(HA)_C}\right] \times 100\% \qquad (2)$$

where: [ACEi%] is the percentage of angiotensin-I-converting enzyme inhibition, $(HA)_C$ is the area of the hippuric acid liberated without the addition of any inhibitor, which is considered as 100% HA production and $(HA)_S$ is the area of the HA liberated in the sample with inhibitors.

Determination of IC_{50} Values. Permeate were chosen for further analysis to determine IC_{50} values and expressed as µg/ml. Permeates were diluted to different concentration (1/1, 1/2.5, 1/5, 1/10, 1/20 dilutions) and their ACEi% and total protein content determined. The IC_{50} values for the hydrolysates were determined from the linear equation that resulted from the inverse of ACEi% versus the inverse of total protein concentration.

Fractionation of Hydrolysates by Semi-Preparative RP-HPLC

Hydrolysates were fractionated into eight fractions using RP-HPLC (see Fig. 3) and assayed for their ACE inhibitory activity. The hydrolysates were concentrated three times before fractionation using a rotary evaporator at 50°C. These concentrated hydrolysates were then fractionated using the Dionex system with semi-preparative reverse phase column (Ace 5 C18, 250 × 10 mm, 5 µm, 100 Å, Ace 5 C18 guard cartridge; Advanced chromatography Technologies) with gradient is described in section titled "Hydrolysates Peak Profile Determination using RP-HPLC."

Determination of Total Soluble Protein Content

The total soluble protein contents of ultrafiltered fractions (permeates, retentate, elute permeate, and elute retentate) were determined and compared using a bicinchoninic acid (BCA) assay. 100 µl of the sample was mixed with 2 ml of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50), the mixture was allowed to stand for 30 min at 37°C and the absorbance was measured at 562 nm with ultrospec 1100 pro UV/Visible spectrophotometer. Bovine serum albumin was used as a standard and the assay was conducted in duplicate.

Statistical Analysis

The results were expressed as ±S.E.M. Analysis of variance used for statistical comparisons among groups of different treatments, with a value of $P < 0.05$ indicting significance difference and were computed using Gen statistical software package.

RESULTS AND DISCUSSION

Adsorption Equilibrium and Kinetics

β-lactoglobulin has an isoelectric point (Ip) of 5.2 hence it becomes negatively charged at pH 6.5 (pH of whey) (25) and at these conditions it adsorbs to the anionic resin. The kinetics of the adsorption of β-lactoglobulin to the anionic exchanger DEAE sepharose® were determined (see Table 2) which shows a very fast adsorption during the first 10 minutes and slows down after this time; there was an increase only of around 3% between 10 and 120 minutes. Therefore the adsorption of β-lactoglobulin to the resin was fast and equilibrium was reached after 10 min with a maximum binding capacity of 276 mg of protein/ml of resin.

Based on the above analysis, the selective adsorption of β-lactoglobulin from sweet whey (pH 6.5 ± 0.2) was conducted directly without any pre-treatment using a DEAE sepharose®. The protein composition of the supernatant and eluent (see Table 3) show that β-lactoglobulin adsorbs preferentially to this anion exchanger. The elution was conducted with 10 mM potassium phosphate buffer that contains 1 M NaCl which was found to be more efficient than reducing the pH to create electrostatic repulsion (26). In this elution step, no other major proteins were de-adsorbed. Before elution, the loosely bound proteins were washed twice with the adsorption buffer only small amounts of β-lactoglobulin, α-lactalbumin and BSA were washed out. These results are in agreement with other research carried out in our lab using adsorptive membrane and colloidal gas aphrons (27,28). The adsorption capacity at 10 minutes decreased when whey was used (29 mg of

TABLE 2
Data points of adsorption equilibrium of β-lactoglobulin (90 mg/30 ml) using 0.5 ml (50% v/v) DEAE sepharose® in a 10 mM potassium phosphate buffer at pH 6.5 and 20°C at different adsorption time (min). Total protein concentration was quantified using UV spectrophotometer at 280 nm. Where Q is adsorbed β-lactoglobulin per ml of resin and Y is total protein in solution. Yield is calculated from a mass balance on protein in solution

Adsorption time (min)	Q (mg protein/ml of resin)	Y (mg/30 ml)	Yield (%)
0	0	90	0
10	264.9 ± 0.0	23.8 ± 0.0	74
30	267.2 ± 0.8	23.2 ± 0.2	74
45	270.2 ± 1.0	22.5 ± 0.3	75
60	271.2 ± 0.2	22.2 ± 0.1	75
75	272.6 ± 0.2	21.8 ± 0.1	76
90	273.1 ± 0.8	21.7 ± 0.2	76
105	274.8 ± 0.4	21.3 ± 0.1	76
120	276.1 ± 0.4	21.0 ± 0.1	77

$n = 2 \pm$ S.E.M.

TABLE 3
Summary of data for isolation of β-lactoglobulin (β − Lg) from rennet whey (pH 6.6) using 10.5 ml DEAE sepharose® (100%) in which individual proteins were determined by RP-HPLC and total protein was determined by BCA assay. Deviation % is mass balance deviation %

Steps	Volume (ml)	Total protein (mg)	β-lactoglobulin (mg)	α-lactalbumin (mg)
Whey	100	856.2 ± 8.3	289.5 ± 18.2	130.6
Supernatant	100	515.4 ± 10.8	13.12 ± 0.84	93.3 ± 0.9
Wash	20	26.38 ± 0.12	0.19 ± 0.01	7.11 ± 0.34
Elute 1	50	327.1 ± 6.5	251.9 ± 11.4	17.09 ± 0.34
Elute 2	20	35.75 ± 0.04	18.18 ± 0.34	3.33 ± 0.08
Elute 3	20	4.20 ± 0.20	1.135 ± 0.12	0.17 ± 0.01
Deviation (%)		6.15	1.72	7.38

$n = 2 \pm$ S.E.M.

β-lactoglobulin/ml of resin) as compared to standard β-lactoglobulin (264 mg of β-lactoglobulin/ml of resin). This decrease could be caused by the presence of ions such as sulfates and phosphates in whey (27) and other peptides such as the negatively charged glycomacropeptide (1.2–1.5 mg/ml in whey) that are generated from k-casein during the renneting of whey (29).

Hydrolysis of β-Lactoglobulin by Protease N Amano

Suitable enzyme selection is important in protein hydrolysis process for the production of bioactive peptides. Cheison et al. (31) evaluated four different enzymes for protein hydrolysis of which one was the less known Protease N 'Amano' and reported to have greater superiority in the quality of protein hydrolysate production. Furthermore, in previous work in our lab, we proved that we could produce strong ACE inhibitory peptides from β-lactoglobulin standard protein using this enzyme (8).

Temperature and pH are also some of the factors that contribute to the conformational structural change of β-lactoglobulin which could also contribute to the extent of hydrolysis. This experiment was conducted at 45°C and pH 7. According to the manufacturers the enzyme maximum activity is found at 55°C. Research carried out in our lab at four different temperatures (45, 50, 55, and 60°C) at an enzyme: substrate ratio of 0.002 with protein free in solution shows a conversion value of 76 and 84% at 45 and 55°C respectively with no significant difference in bioactivity. However, at 55°C, elution of β-lactoglobulin from the ion exchanger was difficult (32). Therefore, taking the effects of temperature on β-lactoglobulin (4,5,31,33), protease N Amano and DEAE sepharose® (31) into consideration, 45°C was chosen as the optimum temperature for further experiments.

The comparison of conversion values obtained for β-lactoglobulin standard protein free in solution and β-lactoglobulin immobilized from whey with the same enzyme and under the same conditions are shown in Table 4. A higher conversion value was obtained for β-lactoglobulin free in solution than for immobilized β-lactoglobulin. When the hydrolysis time increases from 2 to 6 hrs, the conversion value increases for immobilized β-lactoglobulin from 4.5–45% (see Fig. 2) while for free in solution from 94–100% at an enzyme: substrate ratio of 0.01. This lower conversion value for immobilized β-lactoglobulin may be partly due to the protein being bound to the resin and thus becoming less accessible to the enzyme as previously observed in our lab (31). In addition casein peptides which are produced by the action of chymosin called caseinomacropeptides (CMP) can competitively adsorb to the anion exchanger as they are also strongly negatively charged (29) and this could result in a reduction of β-lactoglobulin hydrolysis rate as this protein may not be the only substrate of the hydrolysis reaction.

TABLE 4
Percentage of conversion value of β-lactoglobulin (3 mg/ml in a 10 mM potassium phosphate buffer at pH 7) free in solution and immobilized from whey (100 ml) as a function of hydrolysis time (hrs) using food grade protease N 'Amano' at an E/S ratio (wt/wt) of 0.01. Where E/S is enzyme to substrate ratio

Hydrolysis time (hrs)	Conversion value of β-lactoglobulin (%)	
	Free in solution	Immobilized
2	94 ± 0.10	4.54 ± 0.08
4	100 ± 0.20	34.3 ± 4.09
6	100 ± 0.00	45.5 ± 3.91

$n = 2 \pm$ S.E.M.

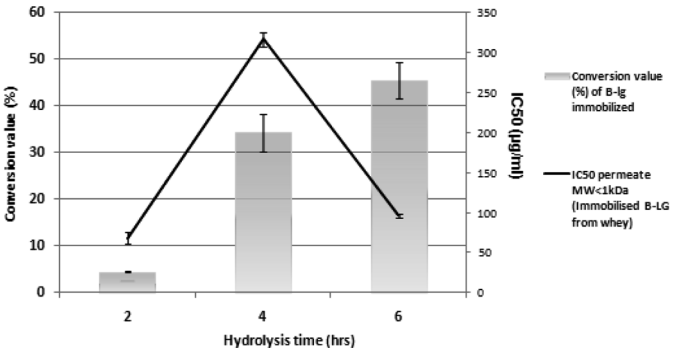

FIG. 2. Conversion value of beta lactoglobulin adsorbed from sweet whey and IC_{50} of hydrolysates of a 1 kDa MWCO ultrafiltration permeates as a function of time (hrs) using protease N Amano at an enzyme substrate ratio of 1:100 (wt/wt) in a 10 mM potassium phospate buffer at pH 7.

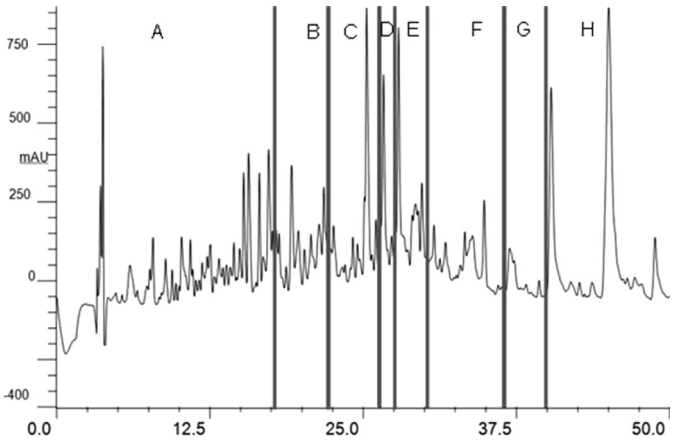

FIG. 3. Chromatogram of fractions of the 6 hrs hydrolysate permeate of 1 kDa MWCO ultrafiltration membrane by semi-preparative RP-HPLC.

TABLE 5
ACE inhibitory activity of fractions produced from ultrafiltration of hydrolysates from immobilized protein. Where P: Permeate, R: Retentate; EP: Eluted permeate; Eluted retentate; ND: Not determined; IC_{50}: Concentration of hydrolysate needed to inhibit the original ACE activity by 50%

Hydrolysis time (hrs)	Sample	E/S ratio	Total protein concentration (μg/ml)	ACE inhibition (%)	IC_{50} (μg/ml)
2	P	0.01	510.0 ± 11.7	67.8 ± 2.3	66.61
	R	0.01	599.2 ± 7.5	15.3 ± 0.7	N/D
	EP	0.01	833.3 ± 12.5	40.5 ± 0.3	N/D
	ER	0.01	1052 ± 0.003	N/D	N/D
4	P	0.01	329.2 ± 0.8	51.0 ± 0.6	323.05
	R	0.01	441.7 ± 3.3	21.7 ± 0.47	N/D
	EP	0.01	181.25 ± 2.08	N/D	N/D
	ER	0.01	1056.2 ± 10.4	N/D	N/D
6	P	0.01	635.8 ± 1.7	86.7 ± 0.3	98.1
	R	0.01	630.4 ± 0.4	43.1 ± 0.4	N/D
	EP	0.01	862.5 ± 4.2	59.3 ± 0.1	N/D
	ER	0.01	397 ± 0	N/D	N/D

$n = 2 \pm$ S.E.M.
$p < 0.001$.

ACE Inhibitory Activity and IC_{50} of Immobilized Protein Hydrolysates

The different ultrafiltration fractions produced from hydrolysates (permeate, retentate, elute permeate, and elute retentate) obtained at different hydrolysis times were tested for ACE inhibitory activity (Table 5). ACEi% of 68 and 87% were achieved for permeates at 2 and 6 hrs of hydrolysis respectively with an IC_{50} value of 67 and 98 μg/ml respectively, while eluted permeates (except eluted permeate at 6 hours of hydrolysis) as well as retentates possess a lower percentage of ACE inhibitory activity (see Table 5). So those peptides negatively charged (and non-hydrolyzed protein) and which remain adsorbed to the resin have lower bioactivity than those peptides that do not adsorb and hence permeate through the membrane. In this way effective fractionation of the most potent peptides is achieved. Furthermore, these results show that molecular weights of the peptides have a significant effect on the ACE inhibitory activity and larger peptides (>1 KDa) have lower bioactivity than the smaller ones which is in agreement with other reported works (34). Moreover, a lower IC_{50} was obtained ($IC_{50} = 67$ μg/ml) at 2 hrs from whey as a feed stock than from standard β-lactoglobulin protein (108 μg/ml). This could be a result of the production of peptides within the casein macropeptide sequence such as the well known tripeptide with potent ACE inhibitory activity, IPP ($IC_{50} = 5$ μM) (10).

The IC_{50} value of permeates obtained by Pihlanto-Laeppala et al. (34) of a tryptic β-lactoglobulin hydrolysate (hydrolysis time = 3 hrs) and permeated through a 1 KDa MWCO membrane was 237 μg/ml which is about three times higher than the IC_{50} value obtained in this experiment ($IC_{50} = 67$ μg/ml). Therefore using the ion exchange resin allows the integration of several process steps such as the separation of proteins from whey, their hydrolysis, and the fractionation of hydrolysis product which results in hydrolysates enriched in potent peptides, hence the increased bioactivity.

Fractionation of Hydrolysates using Semi-Preparative Column in RP-HPLC

The 6 hrs hydrolysate permeate was further fractionated into eight fractions which were assayed for their antihypertensive activity. All fractions except fraction D and G had

TABLE 6
Percentage ACE inhibitory and IC_{50} of fractions of a 1 kDa MWCO ultrafiltered permeate at 6 hrs

Fractions	Total protein concentration (μg/ml)	ACE inhibition (%)	IC_{50} (μg/ml)
A	818.8 ± 4.6	82.56 ± 0.36	71.4
B	466.7 ± 1.7	71.70 ± 0.16	70.2
C	454.2 ± 2.5	61.13 ± 0.02	326
D	225.4 ± 2.9	4.69 ± 0.28	N/D
E	242.1 ± 1.3	22.94 ± 0.19	
F	141.3 ± 2.9	17.63 ± 0.03	
G	102.1 ± 2.1	1.47 ± 0.25	
H	166.3 ± 2.1	18.19	

$n = 2 \pm$ S.E.M.

high ACE inhibitory activity, and those fractions that eluted first from RP-HPLC (fractions A, B, and C) showed the highest percentage ACE inhibition with an IC_{50} value of 71.4, 70, and 372 μg/ml respectively (Table 6). This suggests that the more polar peptides are more active than the less polar ones.

CONCLUSIONS

The in-situ hydrolysis of immobilized whey proteins with protease N Amano generates peptides with strong ACE inhibitory activity. The most potent ACE-inhibitory peptides corresponded to those in permeate fractions ($MW < 1000$ Da). Even if only 4.5% of β-lactoglobulin had been hydrolyzed at 2 hours, this work shows that by applying the immobilization method to sweet whey we can produce high bioactivity at reduced time, $IC_{50} = 67$ μg/ml at $t = 2$ h. This is most probably due to hydrolysis of caseinomacropeptides (CMP) that can competitively adsorb with β-lactoglobulin on the ion exchanger as CMP possesses strong ACE inhibitory sequences such as IPP. However when the hydrolysis time was extended to 6 h, about 45% of β-lactoglobulin was hydrolyzed with a slight decrease in bioactivity. This could be because some of the potent peptides that are generated at 2 h become hydrolyzed. Overall these results show that two different hydrolysates with similar bioactivities but different peptide compositions can be produced by changing hydrolysis time: at 2 h bioactivity is generated mainly from the hydrolysis of CMP whilst after 6 h peptides from β-lactoglobulin are also generated and contribute towards ACE inhibitory activity. Furthermore, the use of the ion exchanger allows partial purification of hydrolysates as peptides bound to the resin are less bioactive than those not bound. Thus immobilizing the substrate is advantageous compared to immobilizing the enzyme. The process developed in this work is very efficient since it integrates the purification of β-lactoglobulin (90% purity in relation to BSA and α-lactalbumin), its hydrolysis and partial fractionation of peptides; moreover, further fractionation with 1 kDa UF membrane shows that peptides in the permeate are more potent that those in the retentate. Furthermore, when fractionation of the permeates was carried by RP-HPLC we found that the more polar fractions had higher bioactivity than the less polar ones.

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