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

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ABSTRACT

Whey proteins are a rich source of angiotensin converting enzyme I (ACE) inhibitory peptides. The ACE inhibition is one of the key mechanisms by which reduction in blood pressure is exerted in the body so some of these peptides have also shown a blood pressure reduction effect ie, antihypertensive activity. They can therefore be part of a dietary intervention to treat this health condition. The aim of this work is to develop a simplified integrative process using a combination of adsorption and microfiltration steps for the production of ACE inhibitory (ACEi) peptides by selectively separating and immobilizing these two proteins, β-lactoglobulin and casein derived peptides from rennet whey. Unlike the conventional methods, this approach integrates the selective separation of the target proteins and their in-situ hydrolysis which results in the production of partially pure, less complex peptides with higher bioactivity, free from the non hydrolysed proteins. In this work protease N Amano was added directly to the adsorbed proteins at different enzyme to substrate ratios (E: S) in a thermostatically controlled membrane reactor operated in a batch mode. The hydrolysates were analysed by HPLC, total protein was measured using the BCA method and ACE activity was determined applying the widely used enzyme assay which is based on the quantification of hipuric acid by HPLC; the bioactivity was measured in terms %ACE inhibition and as IC_{50} which is the peptide concentration required to reduce the ACE activity to 50%. One of the most important outcomes of this research is that applying the immobilisation method we can produce two different hydrolysates with high ACE inhibitory activity starting from the same feedstock: (i) mainly composed of casein derived peptides with IC_{50}=280µg/ml and (ii) mainly composed of β-lactoglobulin derived peptides with IC_{50}=128µg/ml. Furthermore separation of the smaller peptides from the enzyme and larger peptides was achieved with a 1 kDa molecular weight cut off (MWCO) ultrafiltration membrane which allows also recycling of the larger peptides and enzyme.

Keywords: Anion exchange resin; hydrolysate; Angiotensine converting enzyme (ACE); β-lactoglobulin (β-Lg); casein derived peptides.

INTRODUCTION

Whey is the liquid remaining after the removal of casein protein as a result of the milk curd formation in the production of cheese. Only a small fraction of the total world production of whey is industrially utilised and processed into higher added value products such as, whey powder, whey proteins and whey protein fractions [1]. Peptides encrypted in the whey proteins have important biological functionalities with health enhancing results. Particularly peptides derived from β-lactoglobulin and casein peptides have proven to possess high angiotensin converting enzyme I (ACE) inhibitory activity in-vitro which in some cases leads to antihypertensive effect in-vivo [2, 3]. Therefore there is increasing demand for the production and enrichment of these bioactive peptides from natural sources. Different technologies have been applied such as, conventional batch membrane reactors [4], continuous membrane reactors [5] and ion exchange chromatography [6]. Recently electro-filtration and electro dialysis have been investigated both in a laboratory and pilot plant scale [7]. Moreover simulated moving bed (SMB) size exclusion chromatography has been also studied for the enrichment of casein derived ACE inhibitory peptides [8]. However all these production and enrichment technologies are either limited to laboratory scale or expensive for industrial scale applications. The aim of our research is to develop a simplified process for the production of hydrolysates with high ACE inhibitory activity. The process developed here is based on the integration of the separation of proteins from whey and their hydrolysis which results in the selective hydrolysis of β-lactoglobulin and casein derived peptides (CDP) and consequently, in less complex hydrolysates with high ACE inhibitory activity.
MATERIALS & METHODS

Materials and reagents

Bovine β-lactoglobulin, N-Hippuryl – L – Histidyle – L – Leucine (HHL), α-lactalbunin, bovine serum albumin (BSA), hippuric acid (HA), angiotensin converting enzyme (ACE; EC 3.4.15.1), captopril, bicinehonicinic acid solution (BCA), copper-sulfate solution and DEAE sepharose® were bought from Sigma, Steinhein, Germany; Flat sheet microfiltration membranes (0.45 mm), sodium chloride (NaCl), trifluoroacetic acid (TFA), acetonitrile, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific UK Limited; Glycerol from BDH laboratory supplies, England; Protease N ‘Amano’ of Bacillus subtilis 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. Syringe driven PVDF filter (0.45 mm and 0.2 mm) was obtained from Millipore Corporation, Bedford, UK. Ultrospec 1100 pro UV/Visible spectrophotometer was from Biochrom Ltd., Cambridge, England. Skimmed milk was obtained from a local shop and all other reagents and chemicals were also analytical grade.

Methods

Integrative process for the production of hydrolysates

The integrative process for the production of hydrolysates is described in Figure 1. In brief the process consists of three unit operations: (1) ion exchange adsorption (2) microfiltration and (3) ultrafiltration; the three unit operations were carried out at a laboratory scale in two reaction vessels in 5 steps: I) a reactor fitted with a microfiltration membrane that contains an anion exchange resin and II) ultrafiltration membrane. In the first process step, 100 ml rennet whey (pH 6.6 ±0.1) was fed directly to a thermostatically controlled stirred cell filtration device fitted with a microfiltration membrane that contained 10 ml of the anion exchanger adsorbent (DEAE sepharose). The mixture was then incubated for 10 minutes in order for the adsorption of casein derived peptides and β-lactoglobulin to take place followed by a filtration step where the non-adsorbed whey proteins are filtered out. In the third process step the hydrolysate takes place after resolubilising the bound proteins with 10mM potassium phosphate buffer at pH 7 and 45 °C, and protease N Amano was added directly into the solution. The hydrolysate mixture was microfiltered (4th step) to separate the hydrolysate from the non hydrolysed protein and peptides bound to the resin. Finally the microfiltered hydrolysates were ultrafiltered through the 1kDa ultrafiltration membrane (5th step) to separate the most active smaller peptides from the enzyme and larger peptides, and stored at -20 °C until further analysis. The IC50 value (concentration of hydrolysates to inhibit the original ACE by 50%) was determined by plotting the inverse of hydrolysate concentration (1/1, 1/2.5, 1/5, 1/10, 1/25 and 1/50) against the inverse of their ACEi% and expressed as µg/ml.

Total protein determination

The total protein content of hydrolysates was determined based on the bicinehonicinic acid (BCA) assay. In brief, two ml of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50) was mixed with 100µl of sample. The mixture allowed standing for 30 min at 37° C and the absorbance reading was taken at 562 nm using ultrospec 1100 pro UV/ visible spectrophotometer. Bovine serum albumin was used as a standard to quantify the total protein content of the samples.

Statistical analysis

The analysis of variance for the results of the above experiments was computed using Gen statistical software package for statistical comparison among groups of different treatments, with P<0.5 indicating significant difference. All the above results were expressed as ±S.E.M.
RESULTS & DISCUSSION

ACEi and IC\textsubscript{50} of ultrafiltered hydrolysates of active fractions and sub fractions

Hydrolysates obtained from rennet whey free in solution as well as hydrolysates obtained using the ion exchange resin following the process developed here were tested for ACE inhibitory activity (see Figure 2). The main outcomes of this research were:

- After 2 hours hydrolysis Protease N Amano targeted the immobilised casein derived peptides as opposed to β-lactoglobulin; almost complete hydrolysis of casein derived peptides was achieved whilst only 4.5% β-lactoglobulin was hydrolysed.

- ACEi activity of hydrolysates increased about ten times after two hours hydrolysis using the immobilised approach as opposed to carrying out the hydrolysis on whey free in solution. However extending the hydrolysis time up to six hours did not result in an increase in ACEi activity (see Figure 2).
Interestingly, by filtering the 2 hrs hydrolysate and extending the hydrolysis time of the retentate further for 6 hours and adding fresh enzyme, a less complex hydrolysate is obtained (see Figure 4 a & b). This is also reflected in the lower IC₅₀ values obtained for the ‘2+6-hydrolysate’ than the ‘2-hydrolysate’ (Figure 3). Furthermore the conversion value of β-lactoglobulin increased significantly by about two fold (70-80%) compared to that obtained at 6 hrs hydrolysis (45%). This is because in the 6 hour hydrolysate both proteins were adsorbed to the resin and therefore hydrolysis of CDP competed with that of β-lactoglobulin, whereas at 2+6 hours the CDP were removed and subsequently a higher enzymatic conversion of β-lactoglobulin was achieved (70% for the ‘2+6’ and 80% for both ‘2+6*’ and ‘2+6**’).

Incorporating the 1kDa ultrafiltration membrane enables to separate the most active smaller peptides from the less active and larger peptides with a further increase, up to 5 times, in bioactivity.

Figure 2
IC₅₀ and ACEi activity of hydrolysates of casein derived peptides and β-lactoglobulin selectively adsorbed in ionic exchanger from sweet whey and hydrolysed at pH 7 and temperature of 45°C using protease N amino at an E:S ratio of 1:100.

Figure 3
IC₅₀ and ACEi activity of hydrolysates of three different E:S ratio in which 2+6, 2+6* and 2+6** are hydrolysates of β-lactoglobulin adsorbed on the ion exchange resin from sweet whey for an additional 6 hrs after removing hydrolysates of the casein d.
Enzyme to substrate ratio also shows to play a major role in the bioactivity of β-lactoglobulin hydrolysates (see Figure 4 b, c & d) as an increase in enzyme concentration results in an increase in ACE inhibitory activity with the production of the same major peptides but different relative abundance. Mass spectrometry analysis of the 2hrs hydrolysate shows the presence of the most potent milk derived ACE inhibitory reported up to now, the antihypertensive tripeptide Ile-Pro-Pro (result not shown).

**Figure 4**

RP-HPLC peak of casein derived peptides and β-lactoglobulin selectively adsorbed from rennet whey and hydrolysed at a pH of 7 and a temperature of 45°C. (a) hydrolysates mainly from casein derived peptide hydrolysed for 2 hrs using E: S ratio of 1: 100 (b) (c) and (d) hydrolysates mainly from β-lactoglobulin after removing the casein derived peptide hydrolysate at 2 hrs and extending the hydrolysis for another 6 hrs using E: S ratio of 1: 100, 1: 50 and 1:25 respectively.

**Conclusion**

Applying an integrative approach enabled the production of hydrolysates with 15 times higher ACE inhibitory activity than those obtained from whey free in solution. Furthermore, it was possible to produce from the same feedstock two different hydrolysates with high bioactivity. First this approach allowed targeting the selective hydrolysis of casein derived peptides and producing a hydrolysate mainly composed of CDP including the well known antihypertensive tripeptide Ile-Pro-Pro. Secondly the immobilisation method allowed to separate this hydrolysate from the non-hydrolysed and bound β-lactoglobulin which was then
hydrolysed by extending the hydrolysis for 6 hrs with the subsequent production of a less complex hydrolysate with increased potency.

REFERENCES