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# Production of acid whey hydrolysates applying an integrative process: Effect of calcium on process performance

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ABSTRACT

High ionic calcium concentration and the absence of casein macropeptides (CMP) in acid whey could influence the production of angiotensin-I-converting enzyme (ACE)-inhibitory hydrolysate and its bioactivity through the application of the integrative process. Therefore, the aim of the present study was to produce a hydrolysate from acid whey applying the integrative process. Process performance was evaluated based on protein adsorption capacity and conversion in relation to ACE-inhibitory activity (ACEi%) and ionic calcium concentration. Hydrolysates with high potency of their biological activity were produced ($IC_{50}$ = 206–353 μg mL$^{-1}$). High ionic calcium concentration in acid whey contributed to ACE-inhibitory activity. However, low β-lactoglobulin adsorption and conversion was observed. Optimisation of the resin volume increased the adsorption of β-lactoglobulin significantly but with lower selectivity. The changes in conversion value were not significant even at higher concentration of enzyme. Several ACE inhibitors derived from β-lactoglobulin that were identified before in sweet whey hydrolysates such as, IIAEKT, IIAE, IVTQ, LIVTQ, LIVTQT, LDAQ and LIVT were found. New peptides such as, SNICNI and ECCHGD derived from α-lactalbumin and BSA respectively were identified.

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## 1. Introduction

Whey is a liquid material produced as a by-product of cheese production. There are two types of whey; sweet whey and acid whey. Common sweet whey is produced using chymosin. The proteolysis of κ-casein in milk produces cheese with calcium paracaseinate and whey containing casein macropeptides (CMP). Acid whey is produced by acidification of milk to the isoelectric point of casein (p*I* = 4.6) [1]. Precipitation of casein releases high concentration of ionic calcium in the acid whey as opposed to sweet whey. Different production procedures affect whey composition. For example, acid whey tends to have slightly lower protein and higher ash level as compared to sweet whey [2,3]. In addition, acid whey does not contain CMP [4].

Revalorisation of whey is therefore necessary to reduce environmental impact and make the dairy industry more competitive [5]. The major whey protein such as β-lactoglobulin can be a precursor for peptides with biological activity. However, these valuable proteins are still being regarded as waste and underutilised. β-Lactoglobulin and α-lactalbumin have been reported to be precursors of several potential bioactivities such as antibacterial, opioid activity and the most studied activity, the angiotensin-converting enzyme (ACE)-inhibitory activity [4,6,7]. Given the fact that β-lactoglobulin is easily isolated from whey, this makes β-lactoglobulin an interesting substrate for the production of peptides with vast potential of health benefits [8].

The enzyme ACE (EC 3.4.15.1) is one of the main regulators of blood pressure through the formation of vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. In contrast, ACE-inhibitory peptides inhibit the catalytic process of the ACE resulting in hypotensive effect in hypertensive subjects. Natural dietary sources such as milk and whey have been extensively used to produce these bioactive peptides through enzymatic proteolysis and bacterial fermentation [9–13]. These peptides have been shown to lower blood pressure in animal and clinical studies [7,14–18]. The prospective of ACE-inhibitory peptides is increasingly acknowledged due to recurrent condition of hypertension [19]. These peptides are considered to be milder and safer without the side effects associated with other common antihypertensive drugs [20]. Different synthetic ACE inhibitors such as captopril, imidapril and enalapril are being extensively used to treat critical hypertension [21,22]. Therefore, ACE-inhibitory peptides derived

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from milk and whey represents a potential natural dietary approach to control hypertension.

Calcium is another component of the milk that has shown antihypertensive activity [23,24]. It has been reported that calcium, potassium and magnesium have an independent effect on blood pressure when fermented milk was consumed and that these minerals intensified the antihypertensive effects of IPP and VPP [25]. Gonzalez-Gonzalez et al. [26] investigated the effect of ionic calcium concentration on ACE activity which showed an activation of ACE (130%) at concentration between 0.1 and 1 mM and above 1 mM had an inhibition effect. During acid whey production, ionic calcium is released; therefore it will be interesting to investigate the effect of ionic calcium on ACE inhibition. To date, there are no studies reporting the effect of ionic calcium on the ACE-inhibitory activity in acid whey.

Numerous techniques have been suggested for the separation and production of bioactive peptides in a laboratory scale. Separation methods based on membrane filtration in a conventional or continuous batch reactor and chromatographic methods have been applied to isolate a desired protein from a protein mixture. Enzymatic production of bioactive peptides either using digestive enzymes such as pepsin, trypsin, chymotrypsin or microbial enzymes such as proteinase K, protease N and thermolysin have proven to produce specific peptide sequences with high bioactivity [27–29]. High cost of operation, membrane fouling and loss of enzyme activity during hydrolysis, prove to be economical disadvantages for industrial scale production [30,31]. Interestingly, there is a plausible solution for cost reduction by simplifying the production through process integration.

In our group, we have developed an integrative process combining ion exchange and microfiltration [31,32]. Its application using sweet whey produced hydrolysates with potent ACE-inhibitory activity with peptides derived from β-lactoglobulin and CMP. Furthermore, Welderufael and Jauregi [31] demonstrated that the process required no purification or enrichment step since hydrolysates produced were of high potency. The different composition of acid whey as compared to sweet whey such as, high concentration of calcium and the absence of CMP in acid whey could lead to hydrolysates of different composition and/or potency. Therefore, the aim of this study was to investigate the production of hydrolysates with ACE-inhibitory activity from acid whey by applying the integrative process. In addition, the effect of ionic calcium concentration on ACE-inhibitory activity of acid whey and product hydrolysate was also investigated.

## 2. Materials and methods

### 2.1. Materials and reagents

All reagents and chemicals were of analytical grade including, bovine β-lactoglobulin, N-Hippuryl-L-His-Leu (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®, TNBS (2,4,6-trinitrobenzene sulfonic acid), L-leucine, sodium dodecyl sulphate (SDS) were obtained from Sigma–Aldrich, Dorset, UK. Potassium phosphate, potassium mono-phosphate, potassium di-phosphate, di-sodium hydrogen phosphate dehydrates, sodium di-hydrogen phosphate monohydrate, sodium chloride (NaCl), trifluroacetic acid (TFA), acetonitrile, hydrochloric acid were purchased from Fisher Scientific UK Limited, Loughborough; glycerol from BDH laboratory supplies, UK; Protease N Amano from *Bacillus subtilis* was obtained from Amano Enzyme Inc., Nagoya, Japan (191,000 U $g^{-1}$), where one unit of enzyme produces amino acids equivalent to 0.1 g of tyrosine in 60 min at pH 7 and a temperature of 55 °C. Amicon filtration cell was obtained from Amicon® (Millipore, Massachusetts, USA). Flat sheet microfiltration membranes (0.22 μm) and syringe driven PVDF filter (0.45 μm and 0.2 μm) were obtained from Milipore Corporation, Bedford, UK. Ultrospec 1100 pro UV/Visible spectrophotometer was from Biochrom Ltd., Cambridge, UK. Pasteurised skimmed bovine milk was obtained from a local supermarket and all other reagents and chemicals were also of analytical grade.

### 2.2. Preparation of acid whey

Milk was kept warm at 20 °C and pH was adjusted to pH 4.6 with 5 M of hydrochloric acid (HCl). Then, it was incubated at 20 °C for 30 min and casein precipitation was observed during the period. After the incubation, whey was separated from casein using cheesecloth. Produced whey was then centrifuged at 3200 × *g* for 30 min at 4 °C and filtered using microfiltration membrane. The pH was adjusted to 6.5 using 5 M NaOH followed by centrifugation and microfiltration. Prepared whey was stored at −20 °C until further use.

### 2.3. Integrative process for the production of hydrolysates

The integrative process developed for the production of hydrolysates is shown in Fig. 1 following the work from Welderufael and Jauregi [31] with several modifications. DEAE Sepharose® resin was prepared by equilibrating it using 10 mM potassium phosphate buffer at pH 6.5. Then, 100 mL of acid whey (pH 6.5) was fed directly to a stirred cell reactor (200 mL) fitted with microfiltration membrane sheet (0.22 μm) that contains an adsorption resin with resin to β-lactoglobulin ratio of 1:26 (v/v). The mixture was stirred for 10 min (Step 1). After the adsorption, the non adsorbed protein (MFP1) was filtered out by adding pressure ($\Delta P$ = 2 bar) to the stirred cell reactor through the microfiltration membranes (0.22 μm) (Step 2). The resin was washed using 10 mM potassium phosphate buffer pH 6.5 to further remove the non adsorbed proteins while β-lactoglobulin was adsorbed and retained. Adsorbed protein to the resin (MFR1) was resolubilised using 10 mM potassium phosphate buffer (30 mL) at pH 7. The solution was incubated in the thermostatically controlled stirred cell reactor (200 mL) until the temperature reached 45 °C. Protease N Amano at enzyme:substrate (E:S) of 1:100 (wt/wt) prepared in 10 mM potassium phosphate buffer (pH 7) was added to hydrolyse

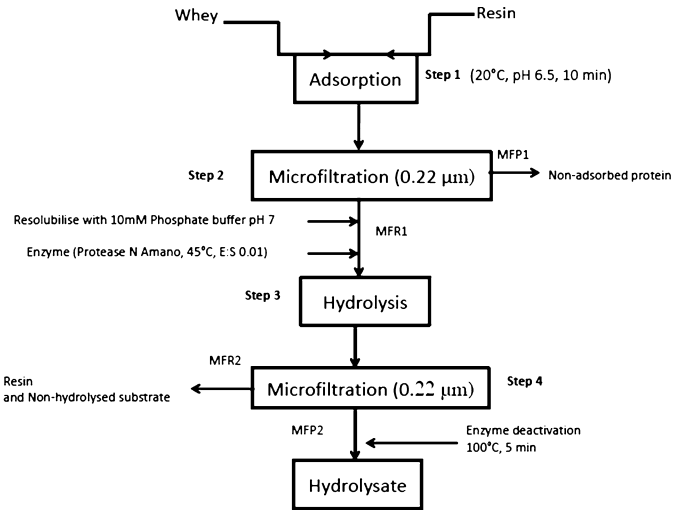


**Fig. 1.** Complete process of the integrative process for the production of bioactive peptides using acid whey.

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