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Chemical characterisation and determination of sensory attributes of hydrolysates produced by enzymatic hydrolysis of whey proteins following a novel integrative process

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ABSTRACT

The overall aim of this work was to characterize the major angiotensin converting enzyme (ACE) inhibitory peptides produced by enzymatic hydrolysis of whey proteins, through the application of a novel integrative process. This process consisted of the combination of adsorption and microfiltration within a stirred cell unit for the selective immobilization of β -lactoglobulin and casein derived peptides (CDP) from whey. The adsorbed proteins were hydrolyzed in-situ which resulted in the separation of peptide products from the substrate and fractionation of peptides. Two different hydrolysates were produced: (i) from CDP ($IC_{50} = 287 \mu\text{g/mL}$) and (ii) from β -lactoglobulin ($IC_{50} = 128 \mu\text{g/mL}$). IC_{50} is the concentration of inhibitor needed to inhibit ACE by half. The well known antihypertensive peptide IPP and several novel peptides that have structural similarities with reported ACE inhibitory peptides were identified and characterized in both hydrolysates. Furthermore, the hydrolysates were assessed for bitterness. No significant difference was found between the control (milk with no hydrolysate) and hydrolysate samples at different concentrations (at, below and above the IC_{50}). The IC_{50} is the concentration of peptide needed to inhibit ACE by half.

Keywords: ACE inhibitory peptides, β -lactoglobulin, Casein derived peptides, ion exchange resin, protease N 'Amano', bitterness.

1. Introduction

ACE inhibitory peptides derived from natural sources such as whey proteins could be used to prevent and help to treat hypertension by dietary intervention. Several animal model and human trial studies have demonstrated the antihypertensive effect of ACE

inhibitory peptides derived from milk proteins (Abubakara, Saito, Kitazawa, Kawai & Itoh, 1998). Some fermented milk products and hydrolysates containing ACE inhibitory peptides from milk proteins are already in the market (Korhonen & Pihlanto, 2006).

In recent years, several sequences of bioactive peptides from natural sources including peptides from β -lactoglobulin and casein derived peptides that can inhibit ACE activity have been discovered (Jauregi, 2008; Jauregi & Welter, 2010; Ortiz-Chao et al., 2009; Pihlanto-Leppala, Koskinen, Piilola, Tupasela & Korhonen, 2000). ACE, which is a constituent part of the rennin-angiotensin system, is a widely accepted enzyme and is considered as the first line of therapy to treat hypertension (Coppey et al, 2006). The first ACE inhibitory peptide was isolated from snake venom (Ferreira, 1965) and most antihypertensive drugs such as, captopril, lisinopril and enalapril that can block the ACE mediated production of angiotensin II were designed based on the snake venom peptide scaffold. The inactivation of ACE also results in an increase in the nonapeptide bradykinin which is a vasodilator. Because of these dual vascular and endothelial protective mechanism of ACE inhibition, the production of nitric oxide is stimulated, vascular smooth muscle is relaxed and fibrinolysis is increased (Ceconi, Francolini, Olivares, Comini, Bachetti & Ferrari, 2007).

ACE has two biologically active substrates: a decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) and a nonapeptide bradykinin (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg). Cheung and co-authors (1980) reported the importance of the C-terminal sequences of ACE inhibitory peptides by studying the binding of two

peptides Hippuryl-Histidyle-Leucine (Hip-His-Leu) and Hippuryl-Phenylalanine-Arginine (Hip-Phe-Arg) that have similar C-terminal di-peptides with that of angiotensin I and bradykinin. They reported that these two peptides had similar binding affinity trends as angiotensin I and bradykinin, hence indicating substrate specificity of ACE.

ACE has specificity for smaller peptides up to 12 amino acids in length with hydrophobic and positively charged amino acids at the C-terminal end (Cheung et al., 1980; Mullally, Meisel & FitzGerald, 1997b; Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995). Wu and co-authors (2006) also recently reported the most favorable structure-function relationship of di- and tri-peptide sequences for potent ACE inhibition; di-peptides with amino acids with bulky and hydrophobic side chains are more favorable while tri-peptides with aromatic amino acids at the carboxyl end, hydrophobic amino acids in the amino terminus and positively charged in the middle are more favorable. In addition to their amino acid composition and sequences, peptides have to be able to resist the gastrointestinal digestion in order to be absorbed and pass to the circulatory system so that they can reach the peripheral organ in active form and exert its biological effect (Quiro's, Contreras, Ramos, Amigo & Recio, 2009).

Several processes have been proposed for the production of hydrolysates with ACE inhibitory activity based on fermentation and enzymatic hydrolysis of food proteins. However most of these either produce hydrolysates with complex mixtures of peptides or use further purification steps for enrichment purposes (see Table 1). We have developed an integrative process for the production of ACE inhibitory peptides from β -lactoglobulin and CDP (Welderufael and Jauregi, 2010; Welderufael, Gibson

and Jauregi, 2012). This process has several advantages: it is simple as it avoids subsequent purification and enrichment steps; less complex hydrolysates are produced with high potency (i.e., low IC₅₀); using the ion exchanger increases enzyme stability; it enables enzyme recycling. Moreover, it is well known that hydrolysis of casein results in many bitter peptides (Kilara & Panyam, 2003) and often those amino acid sequences with high bioactivity are responsible for increased bitterness. Especially hydrophobic peptides with smaller molecular weight, less than 3 kDa, are the reason for this undesirable taste that hinders their incorporation into food products (Cheung & Li-Chan, 2010).

The purpose of the current work was to characterise, identify and evaluate the structure-function relationship of the major peptides in the hydrolysates produced by the integrative process. The *in-vitro* stability of the hydrolysates was assessed by simulating the gastrointestinal digestion using pepsin and corolase PP. Moreover, the *in silico* digestion of the major peptides was carried out with pepsin, trypsin and chymotrypsin. Finally the sensory attributes of the hydrolysates in milk drinks were evaluated at different hydrolysate concentrations at, below and above their IC₅₀.

2. Materials and Methods

2.1. Materials and reagents

Bovine β -lactoglobulin, N-Hippuryl – L – Histidyle – L – Leucine (HHL), α -lactalbumin, bovine serum albumin (BSA), hippuric acid (HA), angiotensin converting enzyme (ACE; EC 3.4.15.1), bicinchoninic acid solution (BCA), copper-sulphate solution and DEAE sepharose® were purchased from Sigma (Steinheim, Germany). Flat sheet microfiltration membranes (0.45mm), potassium mono-

phosphate, potassium di-phosphate, 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). Food grade sodium mono-phosphate, sodium di-phosphate, sodium chloride (NaCl) were purchased from Meridian star, United Kingdom. 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. The preferred hydrolysis of this enzyme is at the C-terminus of threonine, cysteine, methionine, phenylalanine and leucine (Ortiz Chao 2008). Amicon filtration cell was obtained from Amicon a Grace company. Syringe driven PVDF filter (0.45 µm and 0.2 µm) was obtained from Millipore Corporation, Bedford, UK. Skimmed milk was obtained from a local retailer and all other reagents and chemicals were analytical grade.

2.2. Methods

2.2.1. Whey preparation

Pasteurised skimmed milk was bought from the local supermarket and heated at 35 °C. Then commercial rennet was added at 0.3 mL/L and stirred gently for 1 min. The milk was left for 1 h and then the casein coagulum was cut vertically (25 X 25 mm) with a knife to drain the lactoserum. Incubation was extended for 20 min after which the whey was scooped from the vessel and filtered using cheese cloth. The collected whey was stored at -20 °C until used for further experiments.

2.2.2. Hydrolysate production

130

131 ***Lab scale production of hydrolysates for chemical characterisation***

132 The hydrolysates were produced from β -lactoglobulin and CDP in sweet whey
133 following an integrative approach as described in our previous work (Welderufael
134 Gibson and Jauregi 2012). The integrative process consists of three main unit
135 operations: (1) ion exchange adsorption (2) hydrolysis and (3) microfiltration. These
136 unit operations were integrated within a stirred cell (200 ml) fitted with a
137 microfiltration membrane where 10 mL of ion exchange adsorber were mixed with
138 100 mL of whey during 10 minutes. The non-adsorbed whey proteins were filtered.
139 Then selective hydrolysis of the bound proteins (CDP and β -lactoglobulin) was
140 carried out followed by microfiltration of the first hydrolysate product with peptides
141 mainly from CDP (4th step). Two hydrolysates were produced from CDP, one after 2
142 hours hydrolysis and the second one after 6 hours hydrolysis (Fig 1). One β -
143 lactoglobulin hydrolysate product was formed by first hydrolysing the bound proteins
144 for 2 hours, then filtering the resulting hydrolysate and extending the hydrolysis for a
145 further six hours with fresh enzyme (2+6 h hydrolysis in Figure 1) followed by
146 microfiltration and recovery of the hydrolysate. All hydrolysis reactions were carried
147 out with Amano N enzyme at pH 7, 45 °C and E:S ratio of 1:100 and for the the β -
148 lactoglobulin derived hydrolysate we also investigated the effect of reducing the E:S
149 ratio to 1:50. At the end of the hydrolysis the hydrolysates were fractionated using 1
150 kDa ultrafiltration membrane which yield two different hydrolysate products: P1 and
151 P2 with peptides derived from CDP. These hydrolysate products were further
152 fractionated with semi-prep RP-HPLC prior to MS analysis; the hydrolysate derived
153 from β -lactoglobulin (P3) was less complex hence it was directly injected into the
154 LC/MS/MS system.

Scaled up production of hydrolysates for sensory evaluation

The hydrolysate production was scaled up to process 2 L of whey. The process was the same as that describe above and in Figure 2 but was carried out in the pilot plant with the larger equipment used for each unit operation. The ion exchange resin (200 mL) and whey (2 L) were mixed for 10 min with continuous stirring (400 rpm at room temperature) in a stirred tank with a heating water jacket through which water is circulating (2.5 L, Applicon Biotechnology, Holland) at room temperature. The mixture was transferred to a feed tank and from there it was pumped into the filtration unit which consisted of a cross flow filtration system (PCI Midi ultrafiltration Plant, UK, Whiteley, Fareham) fitted with 0.22 µm nitrocellulose microfiltration membrane (Millipore Corporation, UK, Watford) by applying a pressure of 100 psi at an average flow rate of 10 mL/min. The non-adsorbed proteins went through and removed while the retentate containing the resin, CDP and β-lactoglobulin were recycled back to the reaction vessel. The hydrolysis process was carried out exactly at the same conditions as in the lab scale experiments (see above). After 2 hours hydrolysis the mixture was passed through the cross flow filtration system and the first hydrolysate product containing mainly CDP derived peptides was collected in the permeate. The retentate was recycled back to the reaction vessel and hydrolysis was resumed for 6 more hours (2+6) by adding fresh enzyme at an E:S ratio of 1:50 (as in lab scale process) to produce the second hydrolysate containing mainly β-lactoglobulin derived peptides.

180

181 **2.2.3. *Micro QTOF electrospray ionisation tandem mass spectrometry (ESI***
182 ***MS/MS) for amino acid sequencing of peptides derived mainly from CDP at***
183 ***2 h***

184

185 The freeze dried hydrolysate fractions were taken up in 100µl of HPLC grade water
186 and then an aliquot diluted 1:1 in water containing 0.1% formic acid for infusion into
187 the mass spectrometer. The mass spectrometer used was a Bruker MicroTOFQ II
188 (Bruker Daltonic, Bremen, Germany) equipped with electrospray ionisation source.
189 The samples were infused into the source, at 3µL/min, using a Harvard syringe pump
190 equipped with a 100µL syringe. The mass spectrometer had previously been
191 calibrated over a mass range of m/z 300-3000 using Agilent Tunemix™.

192 As each spectrum appeared a major peptide mass was isolated and fragmented in the
193 collision cell using sufficient energy to reduce the precursor ions to about 10% and to
194 produce an intense product ion spectrum. The accurate mass of each precursor ion
195 was then used to predict a formula to within approximately 20ppm accuracy and that
196 was used in combination with the MS/MS spectrum to obtain a partial or full amino
197 acid sequence. Software used was Bruker sequence editor and Mass Analysis Peptide
198 Sequence Prediction (MAPSP).

199

200 **2.2.4. *LC-ESI/MS method for peptide identification in β-lactoglobulin hydrolysate***

201

202 Peptides of β-lactoglobulin hydrolysate and peptides in sweet whey were analysed by
203 LC-MS. Samples were reconstituted in HPLC grade water (1:100) and 2 µL injected.
204 The peptides were separated using reverse phase liquid chromatography (RP-HPLC)

with an Agilent 1100 HPLC system (Agilent Technologies, CA, USA). The column used was a Nova-Pak C18 column (150 × 2.1 mm *i.d.*). A gradient solvent system was applied with eluent “A” as 0.1% formic acid in water and eluent “B” 0.1% formic acid in acetonitrile. The flow rate was 0.2 mL/min and, the column and the auto sampler temperature were kept at 25 and 10 °C respectively. Eluent “A” was 98 to 55% for 45 min, 55 to 30% for 5 min and then kept at 30% for 5 min, 30 to 98% for 5 min and 98% for 15 min. The peaks were identified using a Bruker MicroTof QII high resolution TOFMS equipped with an electrospray ionisation (ESI) source. The ion spray voltage was held at 4500V in positive ion mode. The nebuliser gas was nitrogen at a pressure of 1.0 bar; drying gas and temperature were 8 L/min and 180 °C respectively. The instrument was interfaced to a computer running Bruker Data Analysis software version 4 and data acquired over a mass range of 50-3000 Da.

2.2.5. Determination of ACE inhibitory activity

The ACE inhibitory activity of hydrolysates was measured following the HPLC based method by Hyun and Shin (2000) with some modifications as described in Welderufael and Jauregi (2010). The enzymatic assay in this method was based on the hydrolysis of the substrate, 5mM HHL in a 0.1M sodium phosphate buffer (pH 8.2) with 0.3M NaCl by the ACE (60mU) which resulted in the production of hippuric acid (HA). The hippuric acid was determined by RP-HPLC) as described in Welderufael and Jauregi (2010) using a Dionex HPLC system (Camberley, UK) that consisted of, a P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC-100 and PDA-100 photodiode array detector and a C18 column (Ace5 250 x 4.6 mm).

The percentage of ACE inhibitory activity (ACEi %) was calculated based on the hippuric acid liberated in the hydrolysate sample in relation to that in the control sample (water).

The IC₅₀ of hydrolysates was determined by making serial dilutions (1/1, 1/2.5, 1/5, 1/10, 1/25 and 1/50) of the hydrolysate and plotting the inverse of their ACEi% versus the inverse of their total protein concentration. The IC₅₀ was determined from the resulting linear equation and expressed as µg/mL.

2.2.6. Sensory evaluation

The sensory evaluation of the products was reviewed and approved by the University of Reading Ethics Committee. All participants gave written informed consent prior to taking part in the study. Sensory discrimination tests were carried out for the two different whey protein hydrolysates: (i) hydrolysate from CDP, and (ii) hydrolysate from β-lactoglobulin. Prior to the sensory testing, the IC₅₀ values of both hydrolysates were measured as 145 µg/mL for β-lactoglobulin derived hydrolysate and 288 µg/mL for CDP derived hydrolysate. The hydrolysates were spray dried and incorporated into pasteurised semi skimmed milk based at three different concentrations related to their IC₅₀: 100, 150 and 200 µg/mL for the β-lactoglobulin derived hydrolysate and 200, 300 and 400 µg/mL for the CDP derived hydrolysate. Samples were then stored at 4 °C for 24 h. Volunteers (n=39, untrained, age 18-60) were recruited from the students and staff of the University of Reading. The sensory tests were carried out in individual sensory booths under artificial daylight conditions. Samples (10 mL) were coded with three-digit random number and held in plastic cups (30 mL). The discrimination test followed a forced choice triangle test methodology, each panellist

receiving a triad of either two samples and a control (milk without hydrolysate) or vice versa, sample presentation orders were balanced. Panellists were asked to move the sample around the mouth and then expectorate. They were asked to select the sample that was different out of the three and to describe the difference(s) perceived. Water and crackers were used for palate cleansing in between the samples.

2.2.7. Stability study

2.2.7.1. Gastrointestinal digestibility study

The *in-vitro* stability of hydrolysates was studied by simulating the gastrointestinal environment and subjecting them to the action of pepsin at pH 2 and E: S ratio of 1:50, temperature 37 °C for 90 min as previously described by Ortiz-Chao (2008). After this incubation, the hydrolysate was adjusted to pH 7 and the hydrolysis was started by adding corolase PP at E/S ratio of 1:25 for 150 min in a water bath with shaking. The reaction mixture was stopped by heating at 95 °C for 15 min and then centrifuged for half an hour at 15000 x g. Finally the supernatant was stored in the freezer at – 20 °C until further analysis.

2.2.7.2. In-silico digestibility study

In silico digestion of the peptides identified in this work was performed using a combination of pepsin, trypsin and α -chymotrypsin to simulate *in-vitro* human gastrointestinal digestion with the software PeptideCutter proteomics tool (<http://www.expasy.org>). The preferred hydrolysis sites of these three gastrointestinal digestive enzymes are: N- and C-terminus of phenylalanine, tyrosine, tryptophan and

leucine for pepsin; C-terminus of arginine and lysine for trypsin; and C-terminus of tryptophan, tyrosine, phenylalanine, leucine, methionine and histidine for α -chymotrypsin.

2.2.7.3. Prediction of ACE inhibitory activity of main peptides in hydrolysates and their *in silico* digests

The ACE inhibitory activity of main peptides identified in the hydrolysate fractions was predicted using BIOPEP software (http://www.uwm.edu.pl/biochemia/index_en.php) and also published works. Furthermore using the same software the ACE inhibitory activity of the *in-silico* digested peptide fragments was predicted

2.2.8. Total protein analysis

The total protein content of hydrolysates was determined based on the bicinchoninic acid (BCA) assay. In brief, two ml of the BCA working reagent (copper sulphate solution: BCA solution at a ratio of 1:50) were mixed with 100 μ l of sample. The mixture was incubated for 30 min at 37 °C and the absorbance reading was taken at 562 nm using ultrospec 1100 pro UV/ visible spectrophotometer. Serial dilutions of bovine serum albumin were used as standard.

300

301 **2.2.9. Statistical analysis**

302 The analysis of variance for the results of the above experiments was computed using
303 Genstat statistical software package for statistical comparison among groups of
304 different treatments, with $P < 0.05$ indicating significant difference. All the above
305 results were expressed as Mean \pm standard error of mean (S.E.M). Data from the
306 sensory analysis was analyzed using the binomial probability model at a significant
307 level of 0.05 (Difftest version 2.0, 2002, www.difftest.co.uk).

308

309 **3. Results and discussion**

310

311

312 Mass spectrometry analysis of peptide compositions in sweet whey confirmed the
313 presence of ten peptides ranging from 1881.1 to 20978.7 Da (see Figure 2). Several
314 studies also indicated the presence of CDP in whey during cheese manufacturing as a
315 result of proteolysis of casein proteins by chymosin and endogenous milk enzymes
316 (Fox, 1993). Particularly, plasmin activity could be high at conditions of rennet whey
317 preparation (35 ± 2 °C and pH of milk) (Bastian and Brown, 1996).

318

319

320 In our previous works (Welderufael and Jauregi, 2010 and Welderufael et al 2012) we
321 demonstrated that applying the integrative approach to whey resulted in the
322 production of two different hydrolysates, one from CDP and the other one from β -
323 lactoglobulin. In the present work we carried out the chemical characterisation of each
324 hydrolysate using mass spectrometer techniques in order to identify the main peptides
325 contributing to the ACE inhibitory activity measured in these hydrolysates.

326

327 **3.1. Identification and characterisation of major peptide composition derived from**
328 **active fractions of CDP**

329 The structure-function relationship of ACE inhibitory peptides is not fully
330 characterised however some information exists and quantitative structure activity
331 relationships (QSAR) have been established (Pripp, Isaksson, Stepaniak, Sørhaug, &
332 Ardö, 2005 and Wu, Aluko, & Nakai, 2006) which, enable prediction of ACE
333 inhibitory activity from a knowledge of amino acid sequences. ACE cleaves di-
334 peptides from the C-terminal sequences of peptide substrates and particularly the three
335 amino acids from the C-terminal are very important for their binding to ACE (Lopez-
336 Fandino et al., 2006). Furthermore, this enzyme prefers small peptides up to 12 amino
337 acids in length with hydrophobic amino acids (aromatic: phenylalanine, tryptophan
338 and tyrosine; and branched chain amino acids: valine, leucine and isoleucine) in one
339 of the three sequences of the C-terminal (Cheung et al., 1980; Mullally et al., 1997b).
340 The positively charged amino acids such as arginine and lysine at the C-terminal
341 sequences were also reported to contribute towards ACE inhibitory or
342 antihypertensive effect (Cheung et al., 1980).

343
344 The mass spectrometry analysis of the 2 and 6 h hydrolysates which had permeated
345 through the 1kDa ultrafiltration membrane show that both hydrolysates contain almost
346 the same peptide composition with peptides mainly from casein derived peptides (see
347 Table 2 for peptide composition of the 2 h hydrolysate).

348
349
350 These two hydrolysates were fractionated into 8 fractions (see 2 h hydrolysate
351 chromatogram in Fig 3) and the three polar fractions A, B and C showed the highest
352 bioactivity. Particularly, fraction “B” in both, the 2 (see Table 2 and Figure 4) and 6 h

353 hydrolysates (data not shown) contained IPP. This peptide has been identified as the
354 most potent ACE inhibitor from milk protein and it is derived from casein with an
355 IC_{50} of $5\mu M$ ($1.6\mu g/mL$) (Nakamura et al., 1995). In recent years, several human
356 trials and animal studies have demonstrated the antihypertensive activity of this
357 tripeptide (Ehlers, Nurmi, Turpeinen, Korpela, & Vapaatalo, 2011). In addition, this
358 fraction, in both hydrolysates, contained the peptide His-Leu-Pro (HLP) which could
359 be a contributor to ACE inhibitory activity as it is a fragment of the β -casein derived
360 hexapeptide Leu-His-Leu-Pro-Leu-Pro (LHLPLP). This hexapeptide has been
361 reported to have an $IC_{50} = 5.5 \pm 0.5\mu M = 3.8\mu g/mL$ and has also shown a significant
362 blood pressure reduction effect at a dosage of 2 mg/kg (Quiros et al., 2009).

363

364 Fraction “C” of the 2 h hydrolysate contained a β -lactoglobulin derived peptide Leu-
365 Asp-Ile-Gln-Lys (LDIQK) while it was absent in the 6 h one. This pentapeptide is a
366 fraction of the ACE inhibitory hexapeptide Gly-Leu-Asp-Ile-Gln-Lys (GLDIQK)
367 which has been reported as a potent ACE inhibitor with an IC_{50} of $27.5\mu M$ or 18.5
368 $\mu g/mL$ (Schlothauer, Schollum, Singh & Reid, 1999). This same fraction, in both
369 hydrolysates, also contained an octapeptide Gln-Asp-Lys-Thr-Glu-Ile-Pro-Thr
370 (QDKTEIPT). In order to identify the main peptides responsible for the high
371 bioactivity of fraction C, the fraction of the 6 h hydrolysate was further fractionated
372 into four sub-fractions. This led to a fraction containing almost solely the octapeptide
373 QDKTEIPT and its IC_{50} was $17.5\mu g/mL$ (see Figure 5 and 6). So this proves that the
374 octapeptide was one of the main contributors to the IC_{50} measured in fraction C (113
375 $\mu g/mL$). Furthermore this peptide holds some structural similarities to other reported
376 ACE inhibitory peptides. Quirós et al. (2005) reported an ACE inhibitory peptide
377 Leu-Val-Tyr-Pro-Phe-Thr-Gly-Pro-Ile-Pro-Asn (LVYPFTGPIPN) from caprine kefir

with an IC_{50} of $27.9 \pm 2.3 \mu\text{g/mL}$. This peptide has the same two penultimate sequences Iso-leucine and Proline at the end of the C-terminal as the octapeptide (QDKTEIPT). Moreover both peptides have uncharged and polar amino acids such as, asparagine and threonine at the C-terminal end. Therefore, taking into account all the above evidence both peptides LDIQK and QDKTEIPT are most likely to be the main contributors to the high potency of fraction “C” of the 2 h hydrolysate. The octapeptide QDKTEIPT is the main contributor to the potency of the 6 h hydrolysate.

The β -lactoglobulin derived tetrapeptide Lys-Ile-Pro-Ala (KIPA) in fraction “B” has a very similar peptide sequence to a tripeptide reported by Abubakar and co-authors (1998), the Ile-Pro-Ala (IPA), which was found to be a potent ACE inhibitor with $IC_{50} = 141 \mu\text{M}$ ($42 \mu\text{g/mL}$).

Fraction “A” also showed a high ACEi activity. Six peptide sequences Leu-Arg, Met-Ala-Pro-Lys, Ala- Met-Ala-Pro-Lys, Ile/Leu-Gln-Lys, Val-Ser-Lys and Thr-Val-Lys were among the peptides identified in this fraction (see Figure 7). These peptides, like the other well characterised ACE inhibitory peptides, also had some structural features of ACE-inhibitors, *e.g.*: the presence of charged followed by hydrophobic amino acid residues in one of the penultimate sequences at the C-terminal sequences. Interestingly, the tripeptide Thr-Val-Lys found in this fraction contained the same C-terminal sequence as the ACE inhibitory peptide Val-Lys extracted from buckwheat with an IC_{50} value of $13 \mu\text{M} = 3.17 \mu\text{g/mL}$ (Vermeirssen, Bent, Camp, Amerongen & Verstraete, 2004).

3.2. Identification and characterization of major peptides in the hydrolysate containing peptides derived from β -lactoglobulin

Selective hydrolysis of the immobilised β -lactoglobulin after removing the 2 h hydrolysates of casein derived peptides by adding two different concentrations of fresh enzyme at an E:S ratio of 1:100 and 1:50 resulted in a hydrolysate with a composition different to that obtained at 2 h, with peptides mainly from β -lactoglobulin and with increased bioactivity. Even if the peptide composition of these two β -lactoglobulin derived hydrolysates was similar, the relative abundance of individual peptides was different (see Figure 8a and b). Almost all these major peptides contained either charged or hydrophobic amino acids in one of the three C-terminal sequences (see Table 3).

Interestingly, the relative abundance of the tetrapeptide Ile-Ile-Ala-Glu (IIAE) increased with an increase in an E: S ratio from 1:100 to 1:50. This peptide was one of the two most abundant peptides in the hydrolysate of the 1:50 E:S ratio and shared similarities with a potent microalgae derived tripeptide Ile-Ala-Glu (IAE). This tripeptide was reported to have an $IC_{50} = 34.7\mu M = 11.5\mu g/mL$ (Suetsuna & Chen, 2001). The other major peptide was Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu (KPTPEGDLEILL) and it increased with an increase in E:S ratio (see Figure 8a and 8b) which resulted in an increase in ACE inhibitory activity. This peptide contained three hydrophobic amino acids at the end of the C-terminal sequence. Therefore these two peptides IIAE and KPTPEGDLEILL could be the major contributors for the high ACE inhibitory activity measured in this hydrolysate. Furthermore the two amino acids at the N-terminal sequence of KPTPEGDLEILL

were similar to the dipeptide Lys-Pro (KP) that was isolated from anchovy and bonito. This dipeptide is known for its high ACE inhibitory activity, $IC_{50} = 22 \mu M = 5.3 \mu g/mL$ and an animal study showed a significant blood pressure reduction effect (Toshiaki, Jianen, Duong & Susumu, 2003). A tripeptide Val-Phe-Lys (VFK) was also identified in this hydrolysate and it has been reported to have ACE inhibitory activity with $IC_{50} = 1029 \mu M = 402.6 \mu g/mL$ (Pihlanto-Leppala et al., 2000).

Peptides IVTQ, VAGT, LDAQ, RL, IIAE, VFK LIVTQ and FK were also identified in the hydrolysate of a standard β -lactoglobulin solution (not immobilized) obtained at the same experimental conditions in previously carried out work by our group (Ortiz-Chao et al., 2009). Out of these eight peptides, all except LIVTQ were identified in the mixtures of the most active fractions. Therefore the hydrolysis process shows good reproducibility. An additional novel peptide KPTPEGDLEILL was identified in the current work that could be a potential contributor to ACE inhibitory activity. Besides these interesting findings, the most interesting outcome of this work is that unlike the complex peptide mixture produced from the standard β -lactoglobulin solution, the hydrolysates produced here were less complex and with comparable IC_{50} values ($IC_{50} = 128 \mu g/mL$ for the immobilized whey whereas $102 \mu g/mL$ for the standard β -lactoglobulin solution).

3.3. Impact of simulated gastrointestinal digestion on ACE inhibitory activity

Some food protein derived ACE inhibitory peptides may lose some of their ACE inhibitory activity after oral administration due to further hydrolysis by gastrointestinal enzymes (Walsh et al., 2004). In this work, the ACE inhibitory activity of CDP and β -lactoglobulin derived hydrolysates was further assessed after

the *in-vitro* simulated gastrointestinal digestion. The results showed no significant differences in ACE inhibitory activity before and after the *in-vitro* digestion (see Figure 9). This could be because either the potent peptides that contributed to the inhibitory activity might be resistant to digestion or the digestion resulted in partial hydrolysis and the active sequences were maintained intact with little impact on the bioactivity. This was tested further by carrying out *in-silico* digestion of major peptides in most active fractions.

3.4. *In-silico* digestibility study of CDP derived peptides

In order to assess the digestibility of specific peptides present in the hydrolysates, *in-silico* digestion of individual peptides was carried out following the method in section 2.2.7.2. Furthermore the ACE inhibitory activity of the peptides produced after digestion was predicted following method in 2.2.7.3. Out of 25 peptide sequences reported in the 2 h hydrolysate, 9 of the peptides were found to be resistant to digestive enzymes including the most potent ACE inhibitory tripeptide IPP while only 4 were completely digested. Twelve peptides were partially hydrolysed (see Table 4) but their C-terminal sequences remained intact. Furthermore, out of the ten peptides that were mainly derived from β -lactoglobulin, only two were completely digested, three were resistant to gastrointestinal digestion while five of the peptides were partially hydrolysed. The three peptides resistant to the digestive enzymes were: Ile-Ile-Ala-Glu, Ile-Val-Thr-Gln and Val-Ala-Gln-Thr (see Table 3).

The octa-peptide Gln-Asp-Lys-Thr-Glu-Ile-Pro-Thr that was predominant in the active fraction “Fraction C” (Table 2 and table 4) was digested into two peptides Gln-Asp-Lys and Thr-Glu-Ile-Pro-Thr. These two digests have some structural features of

ACE inhibitors. Both peptides contained charged or hydrophobic amino acids at the two penultimate sequences at the end of the C-terminal.

A peptide Leu-Thr-Gln-Thr-Pro-Val (LTQTPV) was predicted to lose L-leucine from the N-terminal sequence and produce TQTPV. This peptide shared similarities at the C-terminal sequence with an ACE inhibitory peptide Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val produced from fermented milk by *Lactobacillus delbrueckii* subspecies *bulgaricus* SS1 and *Lactobacillus lactis* subspecies *cremoris* FT4 with an IC_{50} of 173 μ M (Gobbetti, Ferranti, Smacchi, Goffredi & Addeo, 2000).

The octapeptide derived from caseinomacropeptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn (MAIPPCKN) was predicted to lose the amino acid asparagine from the C-terminal sequence resulting in MAIPPCK which is known for its vasodilatory effect. This peptide is a modest ACE inhibitor with IC_{50} of 4785 μ M however, in an animal study this peptide showed a significant blood pressure reduction effect at a dose level of 10mg/kg (Miguel, Manso, López-fandiño, Alonso & Salaices, 2007).

The peptide Ile-Ile-Ala-Glu-Lys-Thr (IIAEKT) was susceptible to partial digestion and was predicted to lose threonine from the end of the Carboxyl-terminal. This fragment, IIAEK (lactostatin) was reported to exhibit a greater hypercholesterolemia effect even when compared to the drug β -sitosterol as tested in rats (Nagaoka et al., 2001).

Interestingly the findings from the *in-vitro* and *in-silico* digestibility studies were in agreement as using both methods it was found that digestion of the hydrolysates resulted in no loss of ACE inhibitory activity.

3.5. Sensory evaluation

Sensory is one of the most important aspects that need to be given consideration after the production of hydrolysates. The release of bitter tasting peptides can significantly affect the sensory attributes of the resultant hydrolysate and subsequently alter the quality of products to which they are added. Therefore to assess whether the hydrolysate products developed in this study had any effect on a typical beverage, they were added into milk which was subjected to a sensory discrimination test. Milk with hydrolysate addition was compared to a control milk sample with no addition.

Overall, for the CDP and β -lactoglobulin derived hydrolysates no significant differences were found in perception between any of the three samples and the control milk samples (see tables 5 and 6). However the sample with the lowest level of hydrolysate was the most different to the control milk ($p=0.066$). With the relatively low number of assessors ($n=39$) used in the trials, although there was no significant differences in perception found, the samples could not be declared with confidence to be perceptually identical, as either the proportion of discriminators amongst the population was too high ($>30\%$), or at a risk level of 10 % (type II error) the probability of correctly identifying a sample from the control in any individual trial was too high (the upper bound limit), or the probability of obtaining a result higher than the upper bound limit was too high. The results of this pilot study must be scaled up and taken to a larger consumer trial in order to prove the samples with hydrolysate are perceptually identical to milk.

CDP derived hydrolysates: Three different concentrations of hydrolysate derived from CDP were tested for sensory attributes. The IC_{50} of CDP derived hydrolysate was 288 $\mu\text{g/mL}$. Therefore we used three different concentrations, (i) 200 $\mu\text{g/mL}$ (ii) 300 $\mu\text{g/mL}$ and (ii) 400 $\mu\text{g/mL}$.

Although there was no statistical significant difference between the control and the three different hydrolysate concentrations (see Table 5), comments given by the assessors were compiled. The 200 $\mu\text{g/mL}$ and the 300 $\mu\text{g/mL}$ were described as less sweet compared to the control, by 4 and 3 assessors respectively, and 3 assessors described the taste and odour of the 400 $\mu\text{g/mL}$ as less milky. However, there were no comments concerning off notes, taints or bitter taste. These findings are very interesting as they help to demonstrate the advantage of the integrative process in

that the hydrolysates produced here are enriched in specific peptides and partially fractionated which might have resulted in the removal of bitter fractions. Other authors have used ion exchange as a debittering method (Cheison, Wang & Xu, 2007). Bitterness of hydrolysates is mainly caused by the composition of amino acids in the peptide sequence. Smaller peptides less than 3 kDa and hydrophobic amino acids in the order of phenylalanine (F) \approx tryptophan (W) > Proline (P) > isoleucine (I) \approx tyrosine (Y) \approx histidine (H) are reported to be the main contributors to bitterness (Cheung et al., 2010; Linde, Junior, Faria, Colauto, Moraes & Zanin, 2009). In our hydrolysate, we have identified twenty five peptides from the active fractions that were permeated through the 1kDa ultrafiltration membrane. Out of the twenty five major peptides, only four peptides VSK, TVK, VQVT and TVQVT were free from the above mentioned bitterness causing amino acids. The other twenty one peptides contain at least one of these amino acids. However even if the majority of these peptides contained Phe, Tyr, Trp, Pro, Ile or His amino acids, their position in the peptide sequence plays a major role in the development of bitterness as reported by Otagiri et al. (1985). Otagiri and co-authors reported the above mentioned amino acids should be at the end of the C-terminal sequences. However, within our hydrolysate only four peptides PP, IPP, HLP and LTQTP have this structural feature while the rest contain those amino acids either at the N-terminus or within the peptide sequence. Furthermore, they found that peptides with arginine followed by proline had a strong bitter taste.

β -lactoglobulin derived hydrolysate: Three different concentrations were also tested for the β -lactoglobulin derived hydrolysate. The IC_{50} of this hydrolysate was 145 μ g/mL. Therefore three different concentrations were chosen in relation to its IC_{50} ; (i)

100 µg/mL, (ii) 150 µg/mL (iii) 200 µg/mL. The result showed statistically no significant difference between the three different concentrations and the control (see Table 6). From the compiled comments, only 2 assessors out of 39 reported adverse tastes; one reported sour for the 100 µg/mL sample and the other reported bitter for the 150 µg/mL. Moreover, only 4 assessors described the 300 µg/mL samples as less sweet compared to the control.

A total of ten major peptides were identified in this hydrolysate and out of the ten peptides, only three peptides VAGT, RL and LDAQ were free from the above bitterness causing amino acids. The other seven peptides FK, IIAE, IVTQ, KPTPEGDLEILL, LIVT, LIVTQ and VFK contain at least one of these bitterness causing amino acids ($F \approx W > P > I \approx Y \approx H$). All the identified peptides did not contain these amino acids at their ultimate C-terminal position. However only three peptides FK, IIAE and IVTQ contain phenylalanine and isoleucine at the ultimate N-terminus and four peptides KPTPEGDLEILL, LIVT, LIVTQ and VFK contained proline, isoleucine or phenylalanine within their sequences. Therefore, the majority of the peptides identified in the hydrolysate according to their chemical structure were expected not to be bitter which is in agreement with the results of the sensory analysis.

4. Conclusions

This study demonstrated that the production process applied in this work resulted in hydrolysates of high ACE inhibitory activity. The fractionation of hydrolysates by preparative HPLC and the use of MS techniques helped to identify major bioactive peptides. This together with available structure activity relationship data including QSAR enable identification of main peptides contributing to the ACE inhibitory

activity of hydrolysates. Among these potent peptides some novel sequences were identified such as, VSK, IIAE, QDKTEIPT, KPTPEGDLEILL and LDIQK . Also the well known ACE inhibitory peptide IPP was identified in the CDP derived hydrolysates. The *in-vitro* simulated gastrointestinal digestibility study showed that there was no significant change in the ACE inhibitory activity of the hydrolysates. This was also in agreement with the findings from the *in-silico* digestion study. The *in-silico* digestion of both hydrolysates predicted that most of the peptides were either resistant or only susceptible to partial hydrolysis and the resulting fragments were predicted to be ACE inhibitory. Hence, no overall loss of ACE inhibitory activity was predicted. The sensory evaluation of the hydrolysates showed no significant difference between the reconstituted hydrolysate products and the control. This might be due to low structural similarity of the peptides with that of bitterness causing peptides. Overall this work demonstrates the advantage of producing hydrolysates following the integrative approach as less complex hydrolysates with high potency and with positive sensory attributes can be produced.

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617 **References**

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FIGURE LEGENDS

Figure 1: Flowchart of production of ACE inhibitory peptides from CDP and β -lactoglobulin (β -Lg). Where P1 and P2 were the permeates of hydrolysates produced from CDP after 2 and 6 hours hydrolysis and P3 the hydrolysate from β -lactoglobulin after 2+6 h hydrolysis; P1 and P2 contained mainly CDP derived peptides and P3 β -lactoglobulin derived peptides.

Figure 2: Total ion current (TIC) of sweet whey extract with masses of peaks analysed by liquid chromatography coupled to mass spectrometry (LCMS).

Figure 3: Peptide peak profiles of the 2 h hydrolysate permeated through the 1kDa ultrafiltration membrane (P1) and peak profiles of the 8 fractions using semi-prep RP-HPLC

Figure 4: (a) MS/MS spectrum of fraction “B” of the 2 h hydrolysate after filtration through the 1kDa ultrafiltration membrane (P1) (b) MS-MS spectrum of ion m/z 326.2074 of IPP.

Figure 5: MS/MS spectrum of fraction “C” of the 2 h hydrolysate after filtration through the 1kDa ultrafiltration membrane (P1).

Figure 6. MS/MS spectrum of subfraction from fraction “C” of the 6 h hydrolysate (P2). The spectrum shows the ion m/z 931.4827 which corresponds to the doubly charged ion of the octapeptide QDKTEIPT (466.2418).

Figure 7: Micro-TOF/ESI-MS spectrometry of fraction “A” of the 2 h immobilised whey hydrolysate after filtration through the 1kDa ultrafiltration membrane (P1).

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838 **Figure 8:** Base peak chromatograms of hydrolysate with β -lactoglobulin derived
839 peptides (P3) at an enzyme to substrate (E:S) ratio of (a) 1:50 and (b) 1:100.

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843 **Figure 9:** Percentage of ACE inhibitory activity of hydrolysates before and after the
844 in-vitro digestion using gastrointestinal digestive enzymes (pepsin, corolase PP).
845 Where 2+6 (E:S=1:100), 2+6 (E:S=1:50) and 2+6 (E:S=1:25) are hydrolysates
846 produced after filtering the 2 h hydrolysate (hydrolysate mainly from CDP) and
847 extending the hydrolysis of β -lactoglobulin for further 6 hours at an E:S ratio of 1:100,
848 1:50 and 1:25.

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TABLES

Table 1:

Different ACE inhibitory hydrolysate production methods from whey proteins and from other natural sources and their IC₅₀ values.

Methods of production	Protein source	Hydrolysis time (hrs)	Enrichment	IC ₅₀ (µg/ml)	Reference
TCA precipitation then hydrolysis	✓ Ovine β-lactoglobulin from acid whey	24	Nil	117-278	(Hernandez-Ledesma et al., 2000)
	✓ Ovine β-lactoglobulin from sweet whey	24	Nil	38-296	(Hernández-Ledesma et al., 2000)
	✓ Caprine β-lactoglobulin from sweet whey	24	Nil	118-388	(Hernandez-Ledesma et al., 2000)
Batch	✓ WPC	4	Ultrafiltration (1kDa)	201	(Mullally, Meisel & Kinsella, 1998)
	✓ β-lactoglobulin	4	Ultrafiltration (1kDa)	160	(Mullally et al., 1997)
Batch	✓ CMP	3	Nil	477	(Otte, Shalaby, Zakaria & Kinsella, 2000)
Fermentation	✓ Milk	24-48	Nil	420-520	(Pihlanto, Virtanen & Kumpulainen, 1998)
Immobilized enzyme	✓ <i>Brassica carinata</i>	-	Nil	338	(Pedroche et al., 2000)
Fermentation	✓ Blue mussels	6 months	Filtered, desalted electrodialysed then lyophilized	1010	(Je, Park, Byun, Jung & Kwon, 2000)
	Our work (Integrative approach)				
	✓ CDP	2	Nil	287	(Welderufael et al., 2000)
			Ultrafiltration (1kDa)	67	(Welderufael & Jaurio, 2000)
	✓ β-lactoglobulin	8	Nil	128	(Welderufael et al., 2000)

Table 2

IC₅₀ (µg/mL) value and the major peptides of the three active fractions of the 2 h hydrolysate that were permeated through the 1kDa ultrafiltration membrane followed by fractionation using semi-prep RP-HPLC.

Fraction	Peptides	Protein Source	Observed Masses (Da)	IC₅₀ (µg/mL)
A	PE		245.1057	213
	L/IK	β-Lg	260.1928	
	L/IR	β-Lg	288.3398	
	VSK	β-casein	333.3596	
	TVK	Casein kinase	347.3637	
	PHL	k-casein	365.2534	
	I/LQK	β-Lg	388.2582	
	MAPK	β-casein	446.5118	
	LQPE	β-casein	486.2502	
	AMAPK	β-casein	517.4536	
B	PP	k-casein	213.3244	62
	IPP	k-casein	326.2074	
	HLP	β-casein	366.3774	
	VFK	β-Lg	393.5045	
	KIPA	β-Lg	428.5043	
	VQVT	k-casein	446.2622	
	LQPE	β-casein	486.2572	
	TVQVT	k-casein	547.3091	
	LTQTP	β-casein	559.3091	
	IASGEPT	k-casein	674.7145	
	MAIPPCKN	k-casein	449.7635 ⁺² (898.5197)	
C	MAIPPCKN	k-casein	449.7635 ⁺² (898.5197)	113
	QDKTEIPT	k-casein	466.2745 ⁺² (931.4801)	
	LIVTQ	β-Lg	573.3596	
	LTQTPV	β-casein	658.3603	
	LDIQK	β-Lg	616.3632	

Table 3

Major peptides identified in hydrolysates produced from β -lactoglobulin after filtering the 2 h hydrolysate and extending the hydrolysis for 6 more hours by adding fresh enzyme (P3). Where: ** stands for peptides that was commonly found at 2+6 h (E:S=1:50) and 2+6 h (E:S = 1:100) and ↓chymotrypsin, ↑pepsin, →Trypsin digesting sites of the peptides.

E: S ratio	Theoretical pepsin, trypsin & chymotrypsin Cleavage site	Protein Source	In silico predicted ACE inhibitors	Molecular weight (Da) ^a	Isoelectric point (Pi) ^a
1: 50	F↓↑K/K↑→F	β-LG		294.188	8.75
	IIAE**	β-LG	IA, IAE	445.2656	4.6
	IVTQ**	β-LG	TQ	460.2766	5.52
	L↓↑DAQ	β-LG	DA	446.2245	3.8
	L↓↑IVT**	β-LG		445.302	5.52
	L↓↑IVTQ**	β-LG	TQ	573.3606	5.52
	R→L/L↓R**	β-LG	RL	288.203	9.75
	VAGT**	β-LG	AG, GT	347.1925	5.49
	V↑F↓↑K	β-LG	VF, VFK	393.2496	8.72
	KPTPEGD↑L↓↑EI↑L↓↑L**	β-LG	GD, EG, KP, EI, PT	1324.7358	4.14
<hr/>					
1: 100	IIAEK→T	β-LG	IA, IAE, EK	674.4083	6

^a is the isoelectric point of peptides from ExPASy proteomics tool and applied only for the peptides before digestion.

Table 4

In silico digestion of the major peptides identified in the active fraction of the 2 h and 6 h hydrolysate permeated through the 1kDa ultrafiltration membrane (P1 and P2).

Where: * stands for peptides that were common to both hydrolysates at 2 and 6 h and ↓chymotrypsin, ↑pepsin, →Trypsin are cleavage sites of these enzymes.

Hydrolysate (h)	Theoretical pepsin, trypsin & chymotrypsin Cleavage site	In silico predicted ACE inhibitors ^b	Molecular Weight (Da) ^a	<i>Pi</i> ^a
2				8.80
E:S=1:100	AM↓APK	AP	517.2803	6.75
	H↓L↑P*/ H↓IP	HL	365.2136	4.00
	IASGEPT*	GEP, IA, GE, SG, GEP, PT	674.3355	5.52
	IPP*	IPP, IP, PP	326.2074	8.75
	L↓↑K/I→K*		260.1968	8.75
	I→QK/L↓↑QK*	QK, LQ	388.2554	8.75
	K→IPA*	IPA, IP	428.2867	8.75
	L↓↑DIQK*	LDIQK, QK	616.3664	5.84
	L↓↑IVTQ	TQ	573.3606	5.52
	L ↓QPE*	LQP, LQ	486.2558	4.60
	L↓↑R*/R→L*	RL	288.2030	9.75
	L↓TQTP*	TQ	559.3086	5.52
	L↓↑TQTPV	TQ, NIPPLTQTPV	658.3770	5.52
	M↓AIIPPK→K→N	IPP, <u>AIP, PK, IP,</u> <u>PPK, AIPP, PP, AI</u>	896.5179	10.0
	M↓APK*	AP	446.2431	8.50
	PE*		245.1132	4.6
	PH ↓L*	HL, PH	366.2136	7.17
	PP	PP	213.1233	
	QDK→TEIPT*	IP, EI, TE, PT	931.4731	4.37
	TVK*	VK	347.1925	5.19
	TVQVT*		547.3086	5.19
	V↓F↓↑K	VF, VFK	393.2496	8.72
	VSK*		333.2132	8.72
	VTST	VTSTAV	407.2136	5.49
	VQVT*		446.2609	5.49
6	EK→VT	EK	476.2715	6.10
E: S=1:100	F↓↑→AQT		466.2296	5.52
	IIAEK→T	IA, IAE, EK	674.4083	6.00
	K→VK→E	VK, KE	503.3187	8.59
	SL↑PQN	PQ	558.2882	5.24

^a Molecular weight and isoelectric points of peptides (before digestion) taken from ExPASy proteomics tool .

^b predicted peptide sequences using the BIOPEP software (http://www.uwm.edu.pl/biochemia/index_en.php).

Table 5

Sensory discrimination of CDP derived hydrolysate; where CDP200, CDP300 and CDP400 were hydrolysates of casein derived peptides at 200 µg/mL, 300 µg/mL and 400 µg/mL respectively. N is number of panellists.

Samples	N	Number of correctly identified samples	Significance (p value) ^a	Proportion of discriminators ^b	Upper bound ^c	Probability of obtaining a higher upper bound ^d
CDP200	39	18	0.066	0.406	0.604	0.033
CDP300	39	13	0.56	0.215	0.477	0.425
CDP400	39	13	0.56	0.215	0.477	0.425

^a Type I error : risk of finding a false difference

^b The maximum acceptable proportion of the population that can distinguish between the samples (typically an acceptable maximum set at 0.3 or 30 %)

^c The upper bound is the probability of a correct trial

^d The probability of obtaining a higher upper bound value in 39 trials (i.e. of obtaining a less satisfactory result)

Table 6

Sensory discrimination of β -lactoglobulin derived hydrolysate; where β -LG100, β -LG150 and β -LG200 were 100 $\mu\text{g/mL}$, 150 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ respectively. N is number of panellists.

Samples	N	Number of Correctly identified samples	Significance (p value) ^a	Proportion of discriminators ^b	Upper bound ^c	Probability of obtaining a higher upper bound ^d
β -LG100	39	10	0.885	0.094	0.396	0.80
β -LG150	39	16	0.196	0.332	0.555	0.118
β -LG200	39	17	0.118	0.369	0.579	0.066

^a Type I error : risk of finding a false difference

^b The maximum acceptable proportion of the population that can distinguish between the samples (typically an acceptable maximum set at 0.3 or 30 %)

^c The upper bound is the probability of a correct trial

^d The probability of obtaining a higher upper bound value in 39 trials (ie of obtaining a less satisfactory result)

FIGURES:

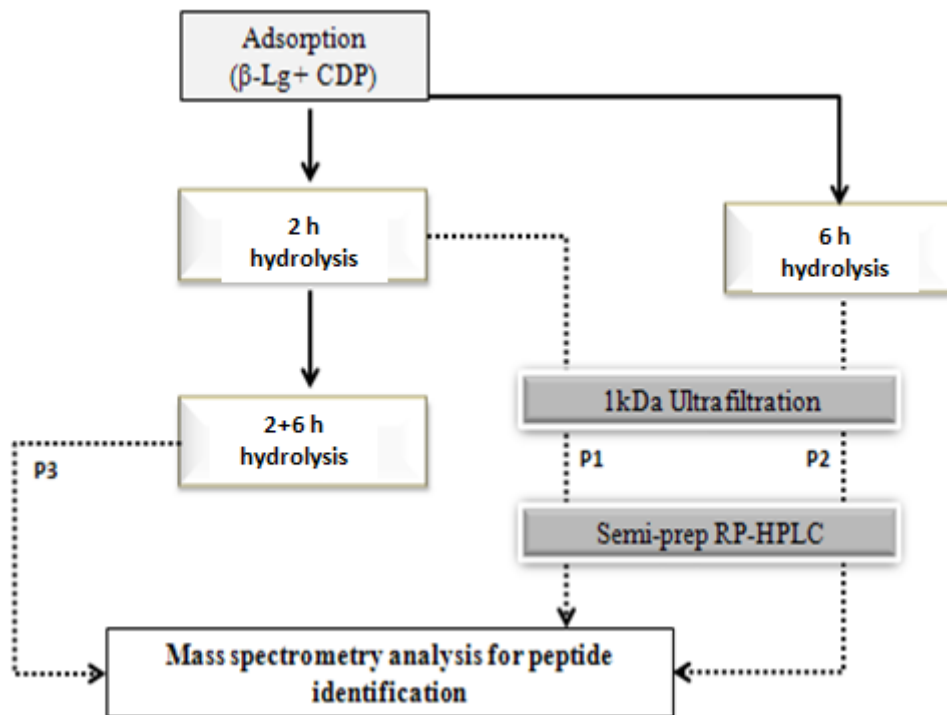


Figure 1

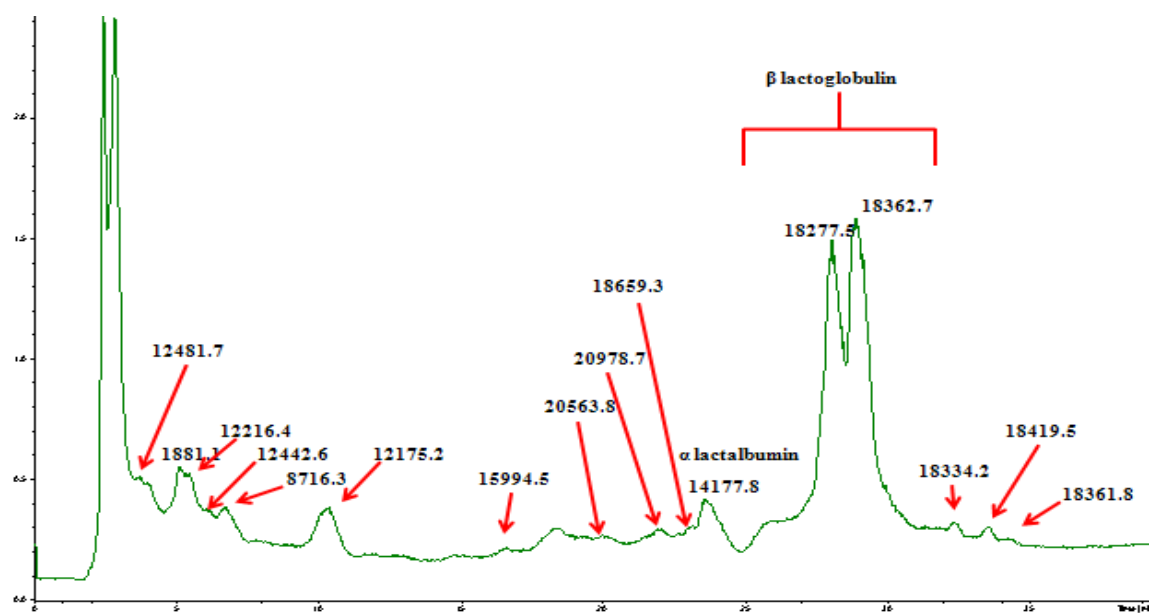


Figure 2

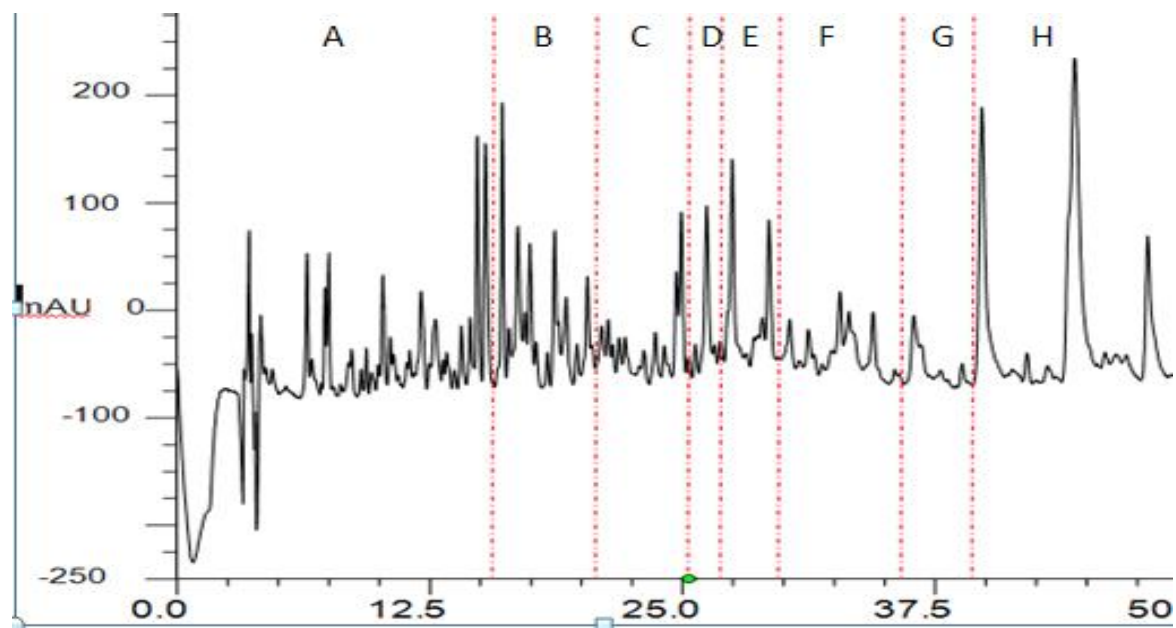


Figure 3

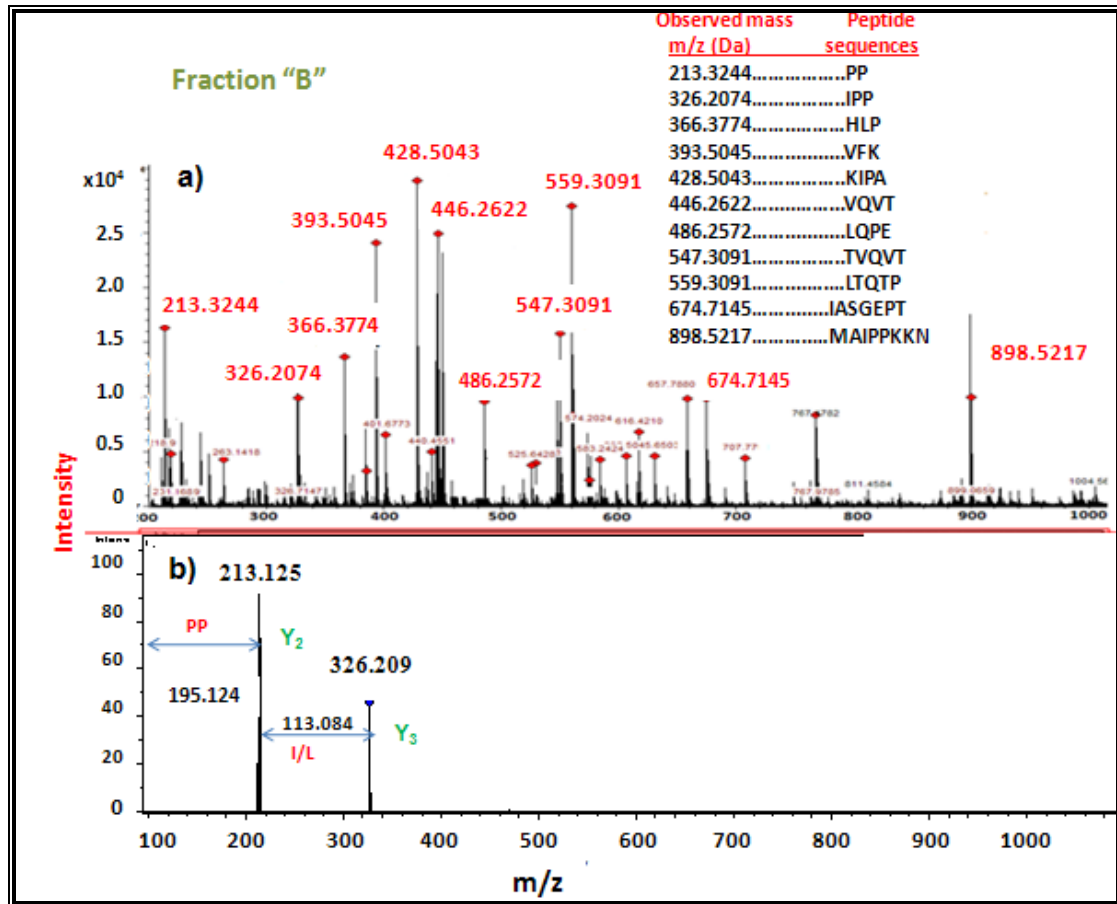


Figure 4

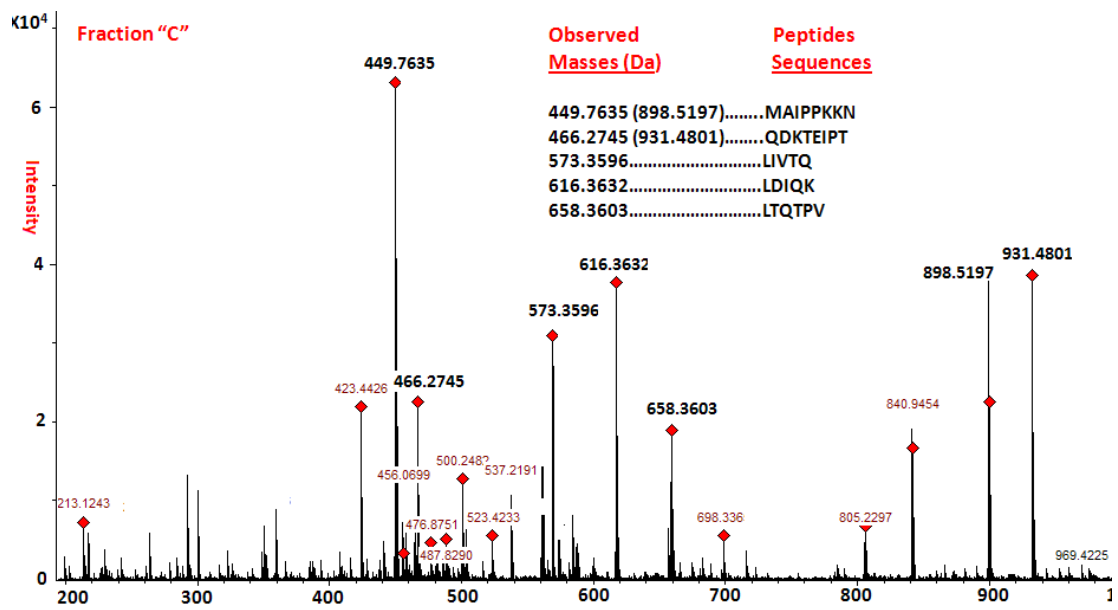


Figure 5

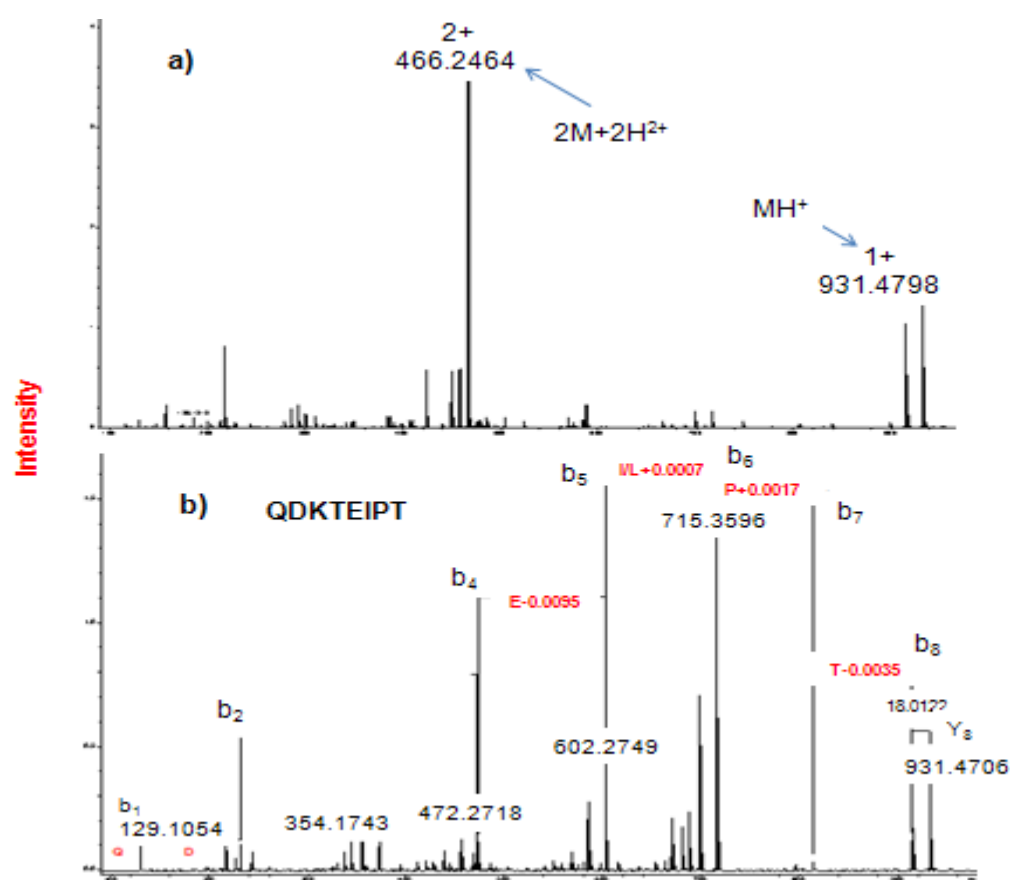


Figure 6

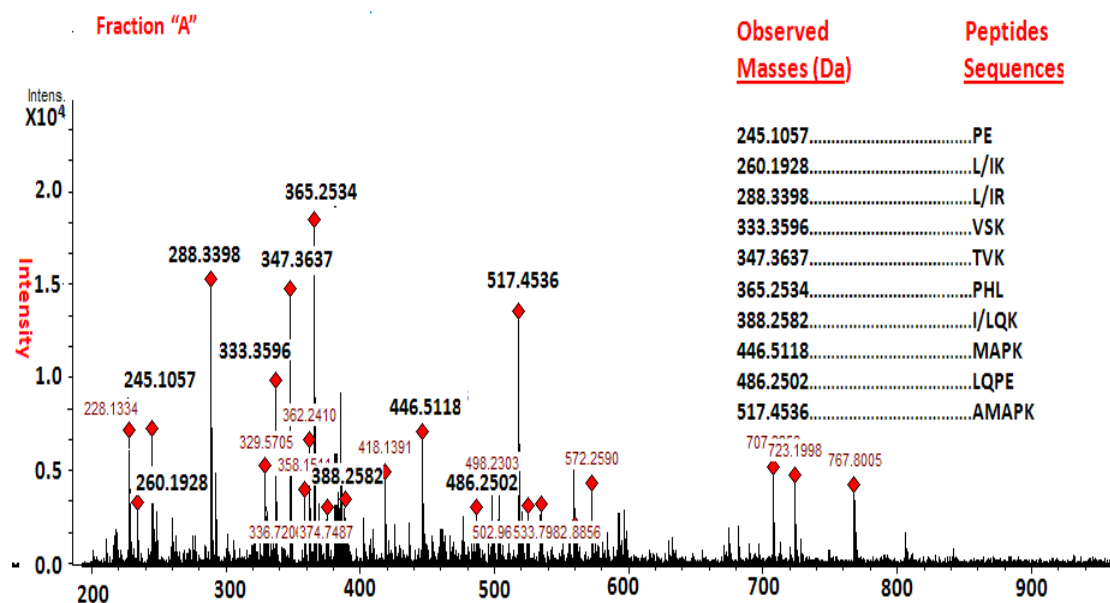


Figure 7

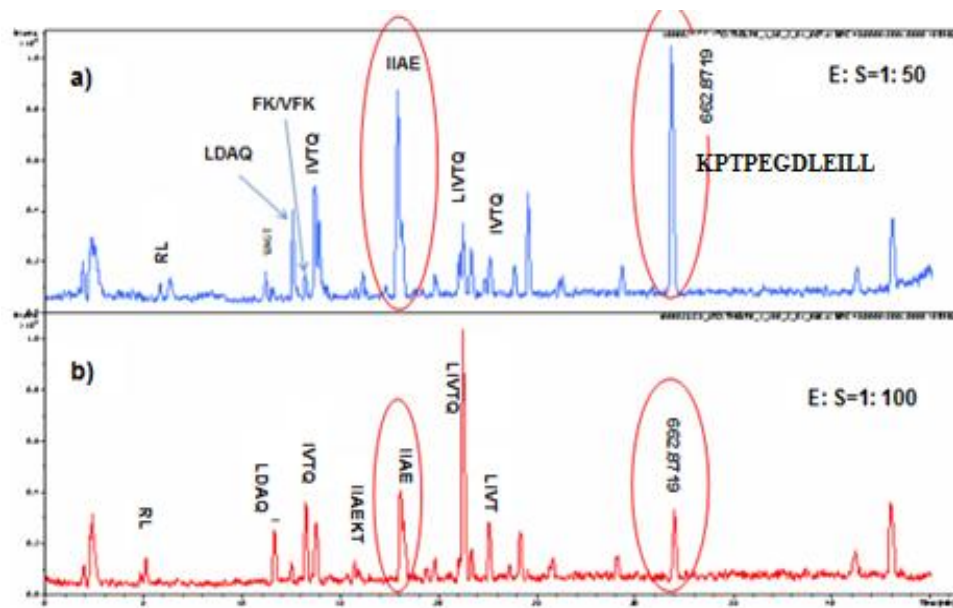


Figure 8

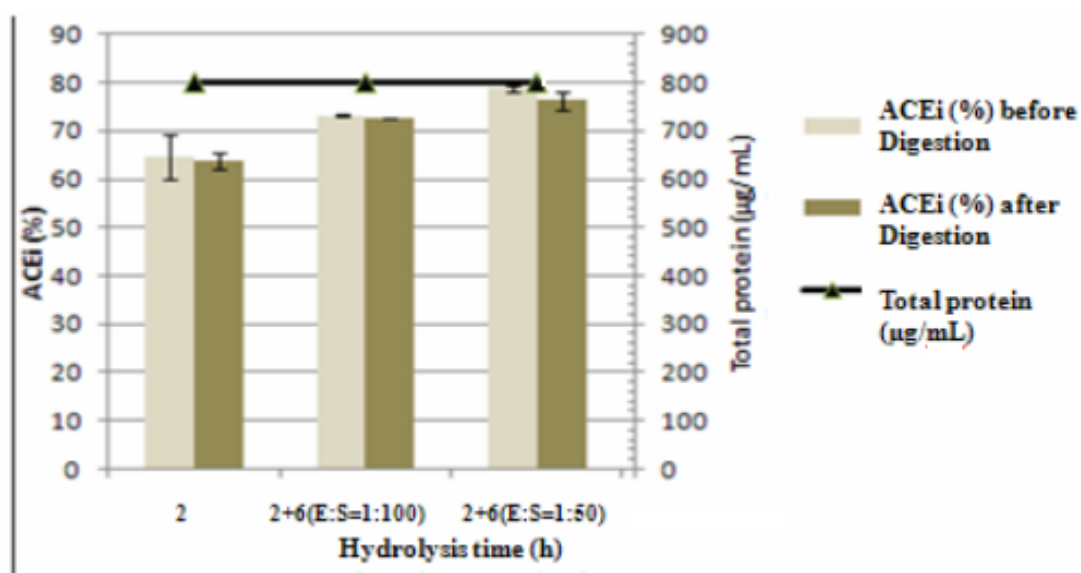


Figure 9