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Characterisation of the hydrolytic specificity of *Aspergillus niger* derived prolyl endoproteinase on bovine β-casein and determination of ACE inhibitory activity.

Abbreviated title: An-PEP hydrolysis of β-casein

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Abstract

The hydrolytic specificity of *Aspergillus niger* prolyl endoproteinase (*An*-PEP) on purified β-casein (β-CN) was assessed. This analysis confirmed cleavage at the C-terminal side of Pro residues. *An*-PEP also had the ability to cleave at the C-terminal side of Ala, Glu, Gly, Ser, Lys and Leu. Incubation of purified β-CN with *An*-PEP resulted in the generation of highly potent angiotensin converting enzyme (ACE) inhibitory hydrolysates. The most potent hydrolysate was obtained after 24 h incubation (ACE IC_{50} = 16.41 ± 6.06 μg/mL). Fourteen β-CN derived C-terminal Pro-containing di-, tri, and tetrapeptides which were predicted *in silico* to be released following *An*-PEP hydrolysis or which were detected by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) in the 24 h hydrolysate were synthesised and characterised for their ACE inhibitory activity. The most potent inhibitory peptides were Ile-Gln-Ala (β-CN f187-189) and Val-Glu-Pro (β-CN f116-118) having ACE IC_{50} values of 32.9 ± 9.2 and 63.7 ± 12.0 μM, respectively. The hydrolysates generated appear to have the most potent ACE IC_{50} values reported for a food derived hydrolysate to date.

Keywords: *Aspergillus niger* derived prolyl endoproteinase; substrate specificity; bovine β-casein; ACE inhibition; LC-MS; bioactive peptides; food proteins.

1. Introduction

Food-derived peptides with biological activity have been the focus of much recent research. On release from their parent proteins, the bioactive sequences may act as regulators with hormone-like activity (Meisel, Walsh, Murray & FitzGerald, 2006). A large assortment of bioactive peptides (BAPs) have been reported with different bioactivities including peptides with angiotensin converting enzyme (ACE) inhibitory activity. ACE (EC 3.4.15.1) is a key enzyme in the regulation of peripheral blood pressure (BP). In the renin angiotensin system (RAS), ACE converts angiotensin I to the potent vasoconstrictor angiotensin II, while in the kinin nitric oxide system (KNOS) ACE inactivates the potent vasodilatory peptide bradykinin (Murray & FitzGerald, 2007). Therefore, inhibition of ACE may ultimately lead to a reduction in BP. Many food derived ACE inhibitory peptides have been reported to lead to significant reductions in BP during the course of animal and human trials (for review see Norris & FitzGerald, 2013). The most extensively studied are the milk derived tripeptides, Ile-Pro-Pro (β-CN f74-76; κ-CN f108-110) and Val-Pro-Pro (β-CN f84-86; Boelsma & Kloek, 2008). Thus, foods enriched with ACE inhibitory peptides with proven hypotensive effects *in vivo* have potential as antihypertensive functional foods.

An understanding of the relationship between a peptide and its bioactivity allows for the targeted release of potentially potent peptide sequences. Although structure-activity studies on ACE inhibitory peptides to date are somewhat limited, some common structural features have been recognised. Most ACE inhibitory sequences are short peptides generally in the region of 2-12 amino acids (Murray & FitzGerald, 2007). One feature common to many potent ACE inhibitory peptides is the presence of Pro residues at one or more positions in the C-terminal tripeptide region (Murray & FitzGerald, 2007). Bioavailability is a key property if a peptide is required to reach its target organ. While many short Pro-containing peptides are potent inhibitors of ACE, they are also known to be resistant to cleavage by human digestive

enzymes (Williamson, 1994). Therefore, the targeted release of Pro-containing peptides from Pro-rich substrates could yield potent and bioavailable inhibitors of ACE.

However, most proteinases and peptidases do not have the ability to cleave proteins at Pro residues (Walter Simmons & Yoshimoto, 1980). A prolyl endoproteinase (PEP) activity from *Aspergillus niger* (*An*-PEP) has recently been described (Edens, van der Hoeven, Deen, de Roos & Floris, 2005). Unlike PEP activities previously reported in other microbial or mammelian sources, *An*-PEP has the ability to hydrolyse large protein molecules (Edens *et al*., 2005)). Thus, *An*-PEP may be used to hydrolyse intact proteins without the requirement for a pre-hydrolysis step with another broad specificity proteinase activity. While *An*-PEP is reported to be active over a large pH range (pH 2-8), it has an optimal pH of 4.3-4.5 and an optimal temperature of 50-55°C (Edens *et al*., 2005; Edens, Roos & Van Platerlink, 2009). *An*-PEP's main cleavage preference is at the C-terminal side of Pro and hydroxy Pro residues but it has also been reported to cleave post Ala residues (Edens *et al*., 2005). However, the substrate specificity of *An*-PEP has yet to be extensively characterised using actual food protein substrates.

A detailed characterisation of the cleavage specificity of *An*-PEP on food proteins requires an isolated protein substrate. Therefore, bovine β-CN was chosen for these studies as it represents an important source for many potent BAPs including ACE inhibitory peptides. It is a 209 residue protein, containing 35 Pro residues. The objectives of this study were (i) to subject purified β-CN to hydrolysis with *An*-PEP, and to determine the ACE inhibitory activity of the subsequent hydrolysates, (ii) to determine the substrate specificity *An*-PEP on β-CN using ultra performance liquid chromatography-electrospray ionisation mass spectrometry (UPLC-ESI MS) and MS/MS, and (iii) to determine the ACE inhibitory activity of short β-CN derived peptides predicted to be released on incubation with *An*-PEP or detected in the hydrolysate by LC-MS.

2. Materials and Methods

2.1. Materials

Brewer's Clarex™ (*An*-PEP specific activity: 37 x 10^{-3} U/mg) was supplied as a gift from Dutch State Mines (DSM, Heerlen, Netherlands). Acid caseinate (89 % (w/w) protein) was supplied by Kerry Ingredients (Kerry, Ireland). The synthetic peptides Leu-Pro, Val-Glu-Pro, Lys-Tyr-Pro, His-Gln-Pro, Ile-Ile-Val, Asn-Ser-Leu-Pro, Phe-Leu-Gln-Pro, Val-Arg-Gly-Pro, Val-Leu-Gly-Pro, Lys-His-Pro, Asn-Pro, Ile-Thr-Pro and Trp-Ile-Gln-Pro were obtained from Thermo Fisher Scientific (Ulm, Germany). *o*-Aminobenzoylglycl-*ρ*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-Phe-(NO_2)-Pro) and o-aminobenzoylglycine (Abz-Gly-OH) were from Bachem GmbH (Hegenheimer, Germany). HPLC-grade water and acetonitrile, and dialysis tubing (high retention seamless cellulose tubing, molecular weight cut-off 12,400 Da) were from VWR International Ltd. (Dublin, Ireland). Trinitrobenzenesulfonic acid (TNBS) was from the Medical Supply Co. (Dublin, Ireland). Sodium tetraboratedecahydrate (borax), formic acid, sodium phosphate monobasic, sodium phosphate dibasic and all other analytical-grade general lab chemicals were from Sigma Aldrich (Dublin, Ireland).

2.2. Purification of β-casein from acid caseinate

A protein fraction enriched in β-CN was obtained following the purification procedure described by Kalyankar (2011). The procedure uses a combination of differential solubility of the individual caseins in the present of calcium and anion exchange fast protein liquid chromatography (FPLC). This resulted in a β-CN preparation that was both electrophoretically and chromagraphically pure (data not shown).

2.3. Hydrolysis of β-casein with *An*-PEP

Purified β-CN (20 mL, 5 % (w/v)) was dissolved in dH_2O and gently stirred for 30 min at 55°C. Although the optimum pH for *An*-PEP has been reported to be between pH 4.3-4.5, hydrolysis was carried out in this instance at pH 6.0 to facilitate β-CN solubilisation. The pH of the solution was adjusted to 6.0 using 1 M HCl. Brewer's Clarex™ was then added at a final *An*-PEP activity of 4 units/gram of protein and the solution was left to incubate for 24 h at 55°C. This corresponded to an enzyme to substrate ratio of 2.5 % (w/w). Aliquots were taken at 4 and 24 h and the enzyme was inactivated by heating at 85°C for 20 min. The samples were then lyophilised using a Free Zone, 4.5 freeze-dryer system (Labconco, Kancas City, USA) and the dry sample was stored at -20°C. The hydrolysis reaction was carried out in duplicate.

2.4. Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) of the β-CN *An*-PEP digests was analysed using the TNBS method (Adler-Nissen, 1979).

2.5. ACE inhibition assay

ACE inhibitory activity was determined using the fluorometric microtitre assay of Sentandreu and Toldrá, (2006) with some modifications as described in Norris, Casey, FitzGerald, Shields & Mooney (2012). Hydrolysates were assayed at final concentrations of 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1.000 and 10.000 mg/mL. The synthetic peptides

were assayed at concentrations ranging from 0.01, 0.05, 0.10, 0.50, 1.00, 10.00, 50.00, 100.00, 250.00, 500.00, 800.00 μM during determination of ACE IC_{50} values, i.e., the concentration of peptide inhibitor required to inhibit ACE by 50 %. IC_{50} values were calculated using GraphPad® Prism 4.0 from sigmoidal dose response plots of inhibitor concentration (μM) versus % inhibition. The values were expressed as the mean IC_{50} ± standard deviation of individual duplicates, assayed in triplicate.

2.6. Reversed–phase high performance liquid chromatography

Analytical reversed-phase RP-HPLC was carried out as described by Spellman, O' Cuinn & FitzGerald (2009) using a Waters HPLC system (Waters, Dublin, Ireland) Samples (0.8 % w/v) were dissolved in mobile phase A (0.1% TFA) and 20 μL was injected onto a Phenomenex Jupiter (C18, 250 x 4.6 mm ID, 5 mm particle size, 300 Å pore size) separating column. Peptides were eluted with an increasing gradient of mobile phase B (0.1% TFA, 80 % acetonitrile) at a flowrate of 1.0 mL/min. The gradient used was as follows: 0-4 min 0 % B, 4-69 min 0-80 % B, 69-70 min 80-100 % B, 70-80 min 100 % B, 80-85 min 100-0 % B, 85-100 min 0 % B. Detector response was monitored at 214 nm.

2.7. Gel permeation high performance liquid chromatography analysis

GPC was carried out as described by Spellman *et al.* (2009) using a Waters HPLC system (Waters, Dublin, Ireland). Samples were dissolved in mobile phase (30 % acetonitrile, 0.1 % TFA) at a concentration of 0.25% w/v and 20 μL was injected onto a TSK gel G2000 SW GPC column (600 mm x 7.5 mm ID). Separation was carried out at 1 mL/min and detector response was monitored at 214 nm. The molecular weights of peptides were calculated using a calibration curve prepared from the average retention times of standard proteins and peptides.

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158 ## 2.8. UPLC-ESI-MS and MS/MS analysis

159 The 24 h *An*-PEP β-CN hydrolysate was analysed by UPLC-ESI-MS and MS/MS.
160 The freeze-dried sample was reconstituted to a concentration of 1 mg/mL in UPLC mobile
161 phase A (0.025 % formic acid in MS-grade H_2O). The digest was separated using an
162 ACQUITY UPLC equipped with a binary solvent delivery system, on-line degasser, column
163 thermostat and dual wavelength UV detector (Waters, Milford, MA, USA) connected to a
164 Bruker micrOTOF Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). The
165 sample (7 µl; 1 mg/mL) was injected onto an ACQUITY BEH 300 C18 RP column (2.1 x 50
166 mm, 1.7 µm; Waters, Dublin, Ireland) using the partial loop mode of injection. The sample
167 was run at a flow rate of 0.2 mL/min. Separation was achieved by isocratic elution for 5 min
168 to 3 % mobile phase B (80 % acetonitrile and 0.025 % formic acid in MS-grade dH_2O),
169 followed by gradient elution to 80% mobile phase B from 5 to 30 min. Column temperature
170 was maintained at 25°C. Detector response was monitored at both 214 and 280 nm.

171 Prior to MS analysis, the MicrOTOF Q II was calibrated with ESI low molecular
172 mass tune mix (Agilent Technologies, Cork, Ireland). The MS experiments were controlled
173 using MicrOTOF control software (version 2.3.0, Bruker Daltonics). Mass spectra were
174 acquired in positive ion mode and full scans were performed for Auto MS/MS between 100-
175 2500 m/z. Analysis was achieved using the following ESI conditions: collision energy gas
176 was nitrogen; capillary voltage was 4500 V; collision energy was 10 eV or 30 eV; transfer
177 time was 120 s; nebulizer pressure was 1.8 bar; drying gas flow rate was 8 L/min and dry
178 heater temperature was 220°C.

179 Data were analysed using Bruker Data analysis software version 4 (Bruker Daltonics).
180 Since all components from the injected sample had passed through the UPLC column within

22 min, the data identification area was selected from between 0 and 22 min. PEAKS Studio software version 6 InChorus option (Bioinformatics Solutions Inc, Waterloo, Canada) in combination with integrated MASCOT 2.3 (Matrix Science, London, UK) were used for peptide identification which searched against the UniProt/SwissProt database through the *Bos taurus* species. Additional peptide sequences were identified using ProteinProspector (http://prospector.ucsf.edu/prospector/mshome.htm). Peptide sequences with mass error >20 ppm were discarded and all other peptides were verified by the SmartFormula function of the Data Analysis program (Bruker Daltonics).

2.9. Statistical analysis

Results were analysed by one-way analysis of variance (ANOVA) using the Bonferroni test at a significance level of $P = 0.05$ with GraphPad® Prism 4.0 software.

3. Results and discussion

3.1. Hydrolysis of β-casein and determination of ACE inhibitory activity

The purified β-CN was subjected to hydrolysis with *An*-PEP for 24 h. The RP-HPLC profiles of the samples taken during the hydrolysis can be seen in Figure 1. The intact purified β-CN eluted after ~52 min (Figure 1a). After 4 h incubation with *An*-PEP (Figure 1b), some of the intact protein was digested but it was evident that a significant amount of intact β-CN still remained. The digestion products eluted between 15 - 53 min with no peptides eluting within the first 15 min. A large proportion of the peptide material eluted between ~43 - 53 min. This represents the presence of highly hydrophobic peptides. Most of

the intact β-CN was broken down after 24 h incubation with *An*-PEP. The hydrophobic peptides which eluted between ~43 – 50 min after 4 h were digested and the intensities of the peaks eluting between ~15 – 32 min increased after 24 h incubation (Figure 1c). GP-HPLC analysis showed that after 4 h hydrolysis with *An*-PEP, 32 % of the peptide in the digest were ≤ 1000 Da, while after 24 h hydrolysis, 54% of the peptides were ≤ 1000 Da (data not shown).

The DH and ACE IC_{50} values for the β-CN hydrolysates are summarised in Table 1. As expected, the highest DH value was obtained for the 24 h *An*-PEP hydrolysate (8.78 ± 0.30 %), with the largest incremental increase in DH occurring during the first 4 h of hydrolysis. The theoretically expected DH when considering cleavage post Pro residues is 15.3 %. Therefore, this suggests that *An*-PEP was unable to hydrolyse all possible cleavage sites in β-CN. Digestion of the purified β-CN with *An*-PEP resulted in potent ACE inhibitory hydrolysates. However, no significant difference ($P > 0.05$) in ACE inhibitory potency was seen between the 4 and 24 h hydrolysates. However, it is evident from the RP-HPLC profiles that a significant number of new peptide sequences were generated following 24 h incubation with *An*-PEP (Figure 1c). Therefore, it may be that the most potent ACE inhibitory peptides were formed within the first 4 h of hydrolysis and that these peptides were stable to the prolonged incubation with *An*-PEP. Alternatively, it is also possible that further degradation of the potent sequences was balanced by a continued formation of new ACE inhibitory peptides. Similar findings were reported in a study which evaluated the effect of hydrolysis time on the ACE inhibitory activity of a number of different milk protein substrates. It was reported that the most potent ACE inhibitory activity was obtained after 3 h incubation with different enzyme preparations and that there was no significant increase in ACE inhibitory activity on further incubation for 24 h (Otte, Shalaby, Zakora, Pripp & El-Shabrawy, 2007).

The IC_{50} values obtained for the *An*-PEP hydrolysate in this study were among the most potent in comparison to the IC_{50} values reported in the literature for both casein-derived hydrolysates and other food-derived peptides (Mullally, Meisel & FitzGerald, 1997; Byun & Kim, 2001; Jung *et al.*, 2006; Tsai, Chen & Pan, 2008; Wang *et al.*, 2008; Zhao *et al.*, 2009; Wang, Tian & Eang, 2011; Samarakoon *et al.*, 2013). This indicates the key role of the hydrolytic specificity in the release of potent ACE inhibitory peptides. However, it must be noted that variations in the reported IC_{50} occur depending on the assay conditions employed. These include differences in the substrate, the source of ACE activity (Vermeirssen, van Camp & Verstraete, 2002) and the units of ACE activity used in the assay (Murray, Walsh & FitzGerald, 2004). Therefore, direct comparison with literature ACE IC_{50} values cannot be made. A limited number of studies have been carried out on the ACE inhibitory activity of isolated β-CN digests (Otte *et al.*, 2007; Stressler, Eisele & Fischer, 2013). However, many studies have focused on the hydrolysis of casein and these have identified specific ACE inhibitory peptides originating from β-CN (Yamamoto, Akino & Takano, 1994; Pihlanto-Leppälä, Rokka & Korhonen, 1998; Saito, Nakamura, Kitazawa, Kawai & Itoh, 2000; Gómez-Ruiz, Ramos & Recio, 2002; Hernández-Ledesma, Miralles, Amigo, Ramos & Recio, 2005; Contreras, Carrón, Montero, Ramos & Recio, 2009; Rojas-Ronquillo *et al.*, 2012; Wu, Pan, Zhen & Cao, 2012).

3.2. UPLC-ESI MS and MSMS analysis

LC-MS analysis was carried out on the 24 h β-CN hydrolysate in order to investigate the substrate specificity of *An*-PEP on purified β-CN and to identify the peptides which contributed to the observed ACE inhibitory activity. Separation of the β-CN hydrolysate was achieved following RP-UPLC, the chromatogram for which can be seen in Supplementary

Figure 1a. Furthermore, distinct peaks were observed in the base peak chromatogram (BPC) for the β-CN hydrolysate (Supplementary Figure 1b). This chromatogram showed maximal elution of peaks between ~2 and 18 min, with one additional peak eluting at 28 min.

Following MS analysis, 21 peptides were identified using PEAKS software, with 11 of these peptides also being identified by MASCOT analysis following MS/MS at a collision energy of 10 eV. Figure 2a shows the fragmentation spectrum for Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro (f125-136) which was detected using both MASCOT and PEAKS analysis. The identified peptide sequences, ions selected for MSMS, calculated monoisotropic molecular masses, experimental shift in mass (ppm) and MASCOT and/or PEAKS scores in the 24 h *An*-PEP β-CN digest are given in Table 2a. Four additional peptides were identified by MASCOT or PEAKS at a collision energy of 30 eV (Table 2a). This highlights that the MS/MS conditions employed are crucial in optimising the detection of peptides in a given hydrolysate. Eight additional short peptides were found in the 24 h β-CN hydrolysate using ProteinProspector analysis. The short sequences were verified using the SmartFormula function of the Data Analysis program (Bruker Daltonics; Table 2b). These short sequences went undetected using the database driven search engines. As the search engines use probability-based protein identification by searching sequence databases using mass spectrometry data, the protein source of low molecular weight peptides is more difficult to verify. This is due to the probability of these short peptide sequences being present in more than one different protein being much greater. Thus, peptides corresponding to more than one entry in the protein sequence database can be falsely identified (false positives) or go undetected as they are not statistically validated by the search engine (Nesvizhskii *et al.*, 2003). Figure 2b shows the fragmentation spectrum for Val-Leu-Gly-Pro (f197-200). In total, 29 peptides were identified in the 24 h β-CN hydrolysate giving a sequence coverage of 49 %.

276 It was found that 26 out of the 29 peptides identified contained C-terminal Pro
277 residues and Pro was also found adjacent to the N-terminal amino acid residue in 18 peptides.
278 These results confirm that *An*-PEP mainly cleaves at the C-terminal side of Pro residues. The
279 MS results also show some cleavage at Ala(101; 189), Glu(121), Ser(124; 142), Leu(125;
280 127; 139), Gln(141) and Lys(99) residues (Table 2a and b). The substrate specificity of *An*-
281 PEP has previously been analysed using various proteins including the bee hemolymph
282 peptide apidaecin 1A, cytochrome c, lysozyme, myoglobin, carbonic anhydrase 2, serum
283 albumin, alcohol dehydrogenase and beta-amylase as substrates. The results demonstrated
284 that *An*-PEP has a preference for cleavage at Pro residues, with minor cleavage at Ala, Glu,
285 Gln, Leu, Lys, Arg, Ser and Tyr (Šebela *et al.*, 2009). Therefore, although *An*-PEP has the
286 greatest specificity for the C-terminal side of Pro, the present study confirms that the enzyme
287 also has the ability to cleave at the C-terminal side of Ala, Glu, Gln, Ser, Lys and Leu in β-
288 CN. To our knowledge, this is the first report identifying the peptides generated following
289 *An*-PEP hydrolysis of β-CN.

290 Table 3 shows the theoretically expected cleavage sites for *An*-PEP on β-CN. The
291 secondary preference for the C-terminal side of Ala residues (Edens *et al.*, 2005; Edens *et al.*,
292 2009; Šebela *et al.*, 2009) has also been taken into account in the predicted cleavage sites.
293 The Table also shows which predicted Pro and Ala C-terminal peptides were detected in the
294 24 h β-CN hydrolysate. From a total of 36 predicted peptides, 11 peptides were detected in
295 the hydrolysate. This highlights that theoretical cleavage predictions of specific proteinases
296 can be used for the targeted release of sequences which may be known or thought to be
297 bioactive. Interestingly, (Pro)-Gln-Arg-Asp-Met-Pro-(Ile) (f182-186) was not detected, while
298 (Gln)-Arg-Asp-Met-Pro-(Ile) (f183-186) was detected. This demonstrated that *An*-PEP
299 cleaved at the C-terminal side of Gln with a Pro residue at the P_2 position to the scissile bond.
300 Hydrolysis of β-CN with *An*-PEP at the C-terminal side of Pro residues should theoretically

yield a range of short peptides (Table 3). These theoretically expected cleavages could generate 8 dipeptides (f62-63; f64-65; f66-67; f111-112; f137-138; f173-174; f180-181; f205-209), however, none of these dipeptides were detected under the MS/MS conditions employed. Out of 5 tripeptides predicted to be released by *An*-PEP (f113-115; f116-118; f145-147; f151-153; f207-209), only three were found in the digest (f113-115; f145-147; f151-153). Two of the four tetrapeptides predicted to be generated by *An*-PEP hydrolysis of β-CN were detected (f87-90; f201-204). All the low molecular mass peptides were detected using ProteinProspector and were verified by SmartFormula. Further studies are ongoing in relation to the detection of these peptides.

The amino acids located at the N- (P_4 P_3 P_2 P_1) and C-terminal side (P_4' P_3' P_2' P_1') of the scissile bond may also need to be taken into account as proteinases have preference for different amino acid residues in these positions. To the best of our knowledge, there are currently no detailed studies reporting on the influence of amino acid residues up- and downstream from the scissile bond on *An*-PEP activity. Our results indicate that *An*-PEP did not cleave at Pro_{206}. This Pro has a Phe at the P_2 and an Ile at the P_1' position. *An*-PEP was shown to cleave at a Pro residue with Ile at the P_1' position in this study with f183-186, but there were no other detected peptides with Phe at the P_2 position. Thus, it may be possible that *An*-PEP may not have the ability to cleave bonds with Phe at this position. Another reason for this apparent missed cleavage may be that *An*-PEP cannot cleave the C-terminal tripeptide and may require an amino acid at the P_4' position for hydrolysis of a bond.

MASCOT and PEAKS analysis also showed some post-translation modifications in the peptides identified (Table 2a). These include oxidation of Met residues (Met_{93}, Met_{151}) and the presence of pyroglutamic acid (Glu_{161}).

3.3. ACE inhibitory activity of β-casein derived synthetic peptides

As many potent ACE inhibitory peptides have low molecular masses, it was decided to assess the ACE inhibitory activities of a selected number of short peptide sequences identified or predicted *in silico* to be present in *An*-PEP digests of β-CN. This approach was taken as it was possible that some short peptides predicted to be present may not have been identified by the LC-MS protocol employed. Therefore, the ACE inhibitory potency was determined for 14 selected synthetic di-, tri- and tetrapeptides derived from β-CN. Table 4 summarises all the di-, tri- and tetrapeptides identified in or predicted to be released with the ACE IC_{50} values determined herein along with the IC_{50} values reported in the literature for those short peptides not synthesised. As can be seen from the table, many of the short sequences identified in the β-CN 24 h hydrolysate have not been previously characterised for ACE inhibitory activity. Consequently, these sequences were synthesised and characterised in the present study. Leu-Pro-Pro (f151-153), a peptide that was both predicted to be released and which was detected in the hydrolysate has a reported ACE IC_{50} value of 9.6 μM (Maruyama, Miyoshi, Kaneko & Tanaka, 1989). The most potent of the peptides derived from β-CN was Met-Ala-Pro (f102-103), which has a reported IC_{50} value of 0.4 μM (Edens *et al*., 2009). Of the new peptides analysed in the present study, Ile-Gln-Ala (f187-189; 32.9 ± 9.2 μM), Val-Glu-Pro (f116-118; 63.7 ± 12.0 μM) and Phe-Leu-Gln-Pro (f87-90; 68.7 ± 23.9 μM) were the most potent sequences. These three peptides have very hydrophobic amino acids at their amino terminus. Structure-activity studies have found that hydrophobic residues at this position is a feature favoured by ACE and thus, this may have contributed to the observed potent ACE inhibitory activities (for a recent review of the structure activity features of ACE inhibitory peptides, see Norris & FitzGerald, 2013). Seven of the peptides showed low ACE inhibitory potency with IC_{50} values >800 μM. The remaining peptides analysed in this study had IC_{50} values of moderate or low potency (IC_{50} values ranging from

134.7 ± 19.1 to 755.1 ± 64.2 μM). Some of the most potent ACE inhibitory peptides derived from β-CN include the lactotripeptides Il-Pro-Pro (β-CN f74-76; IC_{50} = 5 μM) and Val-Pro-Pro (β-CN f84-86; IC_{50} = 9 μM) released by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* and Lys-Val-Leu-Pro-Val-Pro (β-CN f169-174; IC_{50} = 5 μM) and Ser-Leu-Val-Leu-Pro-Val-Pro-Glu (β-CN f57-64; IC_{50} = 39 μM), which were generated on incubation of casein with *Lactobacillus helveticus* CP790 (Nakamura *et al*., 1995; Yamamoto *et al*., 1994). The peptide HLPLPLL (β-CN f134-140; IC_{50} = 34 μM) was identified in a pepsin hydrolysate of casein (Contreras *et al*., 2009).

4.0. Conclusion

Incubation of purified β-CN with *An*-PEP resulted in the generation of highly potent ACE inhibitory peptide hydrolysates, with an IC_{50} value of 16.41 ± 6.06 μg/mL for the 24 h hydrolysate. This appears to be the most potent ACE IC_{50} value reported for a food derived hydrolysate to date in the literature. These results emphasise the role of enzyme specificity in the generation of potent ACE inhibitory food protein hydrolysates. UPLC-ESI MS and MS/MS analysis of the 24 h β-CN *An*-PEP digest confirmed a cleavage preference at the C-terminal side of Pro residues. *An*-PEP also had the ability to cleave β-CN at the C-terminal side of Ala, Glu, Ser, Lys, Gly and Leu residues. In total, 29 β-CN peptides were identified in the 24 h *An*-PEP β-CN hydrolysate using the LC-MS approach employed in the present study. Eleven of the 36 peptides that were theoretically predicted to be released on incubation of β-CN with *An*-PEP on the basis of hydrolysis post Pro and Ala were detected in the 24 h hydrolysate. New ACE inhibitory peptide sequences were identified in β-CN with IC_{50} values ranging from 63.7 ± 12.0 and 755.1 ± 64.2 μM. Hydrolysis of Pro-rich food protein substrates with PEP shows significant potential in the development of highly potent ACE inhibitory

hydrolysates for application as hypotensive functional food ingredients. *In vivo* studies are required to validate the hypotensive effects of these hydrolysates.

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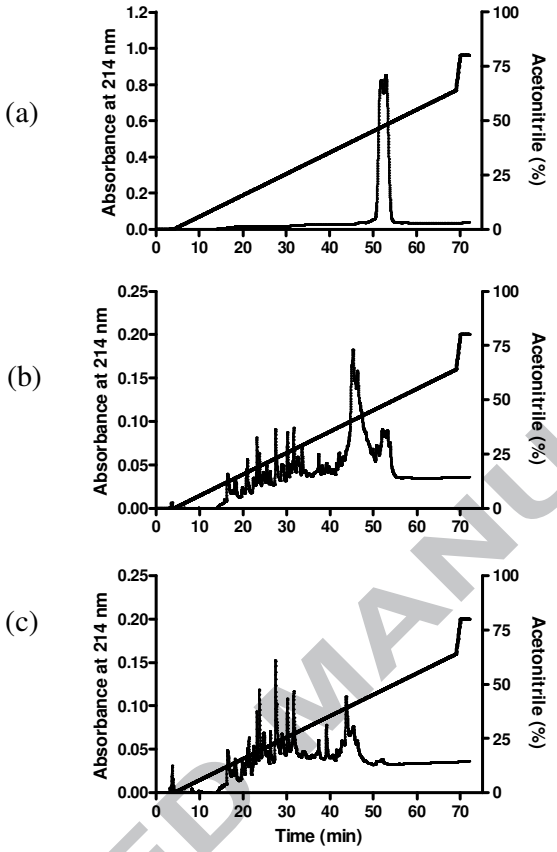

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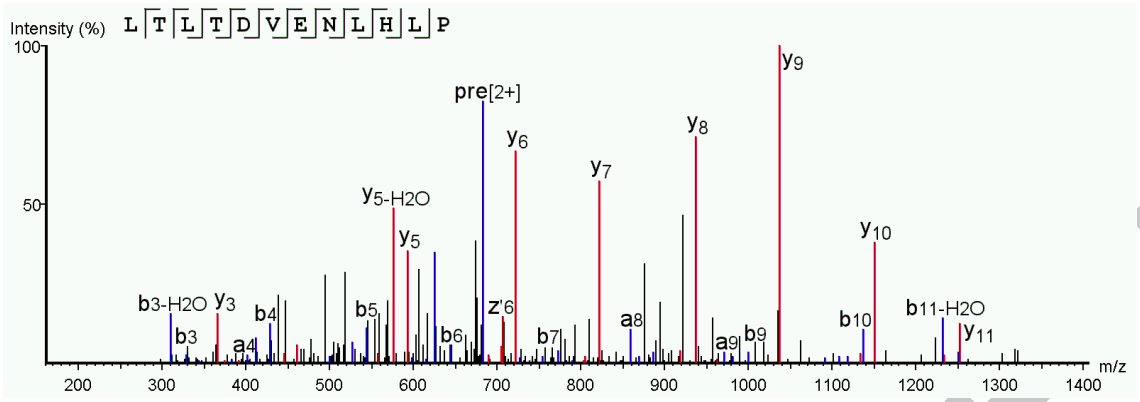
(a)



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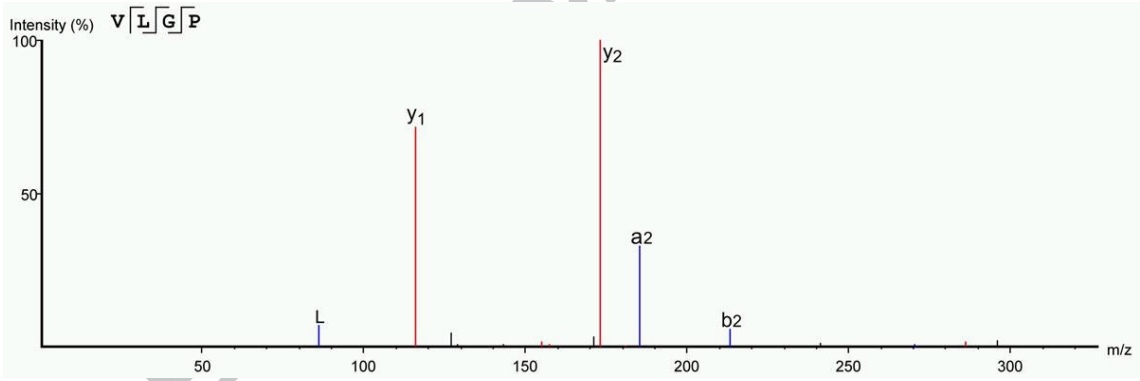

Figure 2

Figure 2. Mass fragmentation spectra for the β-casein derived peptides (a) Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro (m/z 682.87) and (b) Val-Leu-Gly-Pro (385.24). The y

558 axis represents the relative intensity of the precursor ion (pre) and each of the fragment ions.
559 The x axis shows the m/z region in which fragment ions were detected. Ions generated by
560 fragmentation are detailed (y, a and b ions) and the deduced sequence is shown on the upper
561 left.

562

Table 1. Incubation of bovine β-casein with *Aspergillus niger* derived proline endoprotease (*An*-PEP) and determination of the angiotensin converting enzyme (ACE) inhibitor concentration (IC_{50}) values and degree of hydrolysis (DH) values of the hydrolysates.

Incubation time (h)	DH (%)	ACE IC_{50}* (µg/ml)
0	---	> 10 mg/ml
4	6.62 ± 0.57[a]	16.94 ± 6.60[a]
24	8.78 ± 0.30[b]	16.41 ± 6.06 [a]

DH and IC_{50} values are reported as mean ± standard deviation. ACE IC_{50} values are the mean of duplicate hydrolysis samples, assayed in triplicate. DH values are the mean of duplicate hydrolysates samples, assayed in quadruplet.

Different letters within a column represents significantly different IC_{50} values at $p<0.05$.

* ACE IC_{50} values represent the concentration of hydrolysate that inhibits 50 % of ACE activity.

Table 2(a). Sequences of the longer peptides identified using the mass spectrometry search engines MASCOT and/or PEAKS upon incubation of β-casein with *Aspergillus niger* derived prolyl endoproteinase (*An*-PEP) for 24 h. The amino acid residues (R) adjacent to the C- and N-terminal residues in the peptides are given in brackets.

β-casein fragment	(R)-peptide sequence-(R)	Retention time (min)	Ions selected for MSMS	Calculated mass	Experimental shift in masses (ppm)	PEAKS score	MASCOT score	Post-translation modification
f91-99	(P)-EVM(+15.99)GVSKVK-(E)	4.05	496.77	991.5372	-12	28.53	31.72	Oxidation (M)
f91-101	(P)-EVM(+15.99)GVSKVKEA-(M)	8.45	596.82	1191.6169	-7.2	50.01	47.76	Oxidation (M)
f116-136	(P)-VEPFTESQSLTLTDVENLHLP-(L)	17.05	790.4	2368.1799	-0.7	50.24	44.39	-
f119-136	(P)-FTESQSLTLTDVENLHLP-(L)	16.35	1022.51	2043.0161	-5.2	40.4	113.47	-
f119-136	(P)-FTE(+21.98)SQSLTLTDVENLHLP-(L)	16.35	689.34	2064.9983	0	42.69	-	Sodium adduct (E)
f122-136	(E)-SQSLTLTDVENLHLP-(L)	16.35	833.93	1665.8574	-7.2	78.27	86.13	
f122-136	(E)-SQSLTLTD(+21.98)VENLHLP-(L)	15.95	563.62	1687.8394	-0.6	95.38	46.88	Sodium adduct (D)
f125-136	(S)-LTLTDVENLHLP-(L)	16.25	682.87	1363.7346	-6.7	54.88	58.18	-
f126-136	(L)-TLTDVENLHLP-(L)	14.85	626.33	1250.6506	-4.1	43.97	31.62	-
f128-136	(L)-TDVENLHLP-(L)	13.35	519.26	1036.5188	-13	36.55	-	-
f139-147	(P)-LLQSWMHQP-(H)	13.35	570.28	1138.5593	-12	22.3	-	-
f140-147	(L)-LQSWM(+15.99)HQP-(H)	10.25	521.74	1041.4702	-4.6	27.71	-	Oxidation (M)
f142-147	(Q)-SWM(+15.99)HQP-(H)*	8.95	381.1881	800.3275	-5.9	27.89	22.14	Oxidation (M)
f143-147	(S).WM(+15.99)HQP-(H)*	8.35	714.29	713.2955	-17.9	17.81	-	Oxidation (M)
f154-159	(P)-TVM(+15.99)FPP-(Q)*	13.75	707.34	706.3359	-4.5	24.81	-	Oxidation (M)
f160-172	(P)-Q(-17.03)SVLSLSQSKVLP-(V)	15.35	684.89	1367.7661	-0.4	56.15	-	Pyroglutamic acid (Q)
f187-196	(P)-IQAFLLYQEP-(V)	15.65	611.33	1220.644	-1.2	35.55	47.70	-
f187-196	(P)-IQAFLLYQE(+21.98)P-(V)	15.65	622.31	1242.626	-17	40.56	-	Sodium adduct (E)
f187-209	(P)-IQAFLLYQEPVLGPVRGPFPIIV	19.35	856.16	2565.4719	-5.3	56.13	68.42	-
f190-196	(A)-FLLYQEP-(V)*	14.55	909.47	908.4643	-1.7	12.06	-	-
f197-204	(P)-VLGPVRGP-(F)	10.65	397.75	793.481	-5.6	27.01	-	-

Table 2(b). Sequences of the short peptides identified using the mass spectrometry program ProteinProspector upon incubation of β-casein with *Aspergillus niger* derived prolyl endoproteinase (*An*-PEP) for 24 h. The amino acid residues (R) adjacent to the C- and N-terminal residues in the peptides are given in brackets.

β-casein fragment	(R)-peptide sequence-(R)	Retention time (min)	Calculated mass	Experimental shift in masses (ppm)	MS-tag score**
f87-91	(P)-FLQP-(E)*	11.05	504.2817	-3.3	4.5
f105-110	(P)-KHKEMP-(F)	4.05	769.4025	-12.7	-0.9
f113-115	(P)-KYP-(V)	2.50	407.2289	2.7	3.9
f145-147	(P)-HQP-(L)	1.20	381.1881	5.0	6.2
f151-153	(P)-LPP-(T)	5.05	326.2074	7.9	-5.3
f169-172	(S)-KVLP-(V)	9.05	456.318	4.3	0.4
f183-186	(Q)-RDMP-(I)	6.05	518.3291	1.7	0.5
f201-204	(P)-VRGP-(F)	9.25	428.2616	-3.7	10.2

** MS-tag represents the ProteinProspector score. The peptides were verified by BrukerDaltonics SmartFormula software.

Single letter amino acid codes are used.
Peptides were detected at collision energies of either 10 or 30(*) eV.

Table 3. Theoretically expected cleavage sites for *Aspergillus niger* derived proyl endoproteinase (*An*-PEP) incubated with bovine β-casein and actual peptides detected in a 24 h digest. The amino acid residues (R) adjacent to the C- and N-terminal residues in the peptides are given in brackets.

Location in β-casein	(R)-peptide sequence-(R)	Peptide detected*
f1-9	RELEELNVP-(G)	No
f10-51	(P)-GEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHP-(F)	No
f52-53	(P)-FA-(Q)	No
f54-61	(A)-QTQSLVYP-(F)	No
f62-63	(P)-FP-(G)	No
f64-65	(P)-GP-(I)	No
f66-67	(P)-IP-(N)	No
f68-71	(P)-NSLP-(Q)	No
f72-76	(P)-QNIPP-(L)	No
f77-81	(P)-LTQTP-(V)	No
f82-86	(P)-VVVPP-(F)	No
f87-90	(P)-FLQP-(E)	Yes
f91-101	(P)-EVMGVSKVKEA-(M)	No
f102-103	(A)-MAP-(A)	No
f104-110	(P)-KHKEMP-(F)	Yes
f111-112	(P)-FP-(K)	No
f113-115	(P)-KYP-(V)	Yes
f116-118	(P)-VEP-(F)	No
f119-136	(P)-FTESQSLTLTDVENLHLP-(L)	Yes
f137-138	(P)-LP-(L)	No
f139-148	(P)-LLQSWMHQP-(H)	Yes
f148-150	(P)-HQP-(L)	Yes
f151-153	(P)-LPP-(T)	Yes
f154-159	(P)-TVMFPP-(S)	Yes
f160-172	(P)-QSVLSLSQSKVLP-(V)	Yes
f173-174	(P)-VP-(Q)	No
f175-177	(P)-QKA-(V)	No
f178-179	(A)-VP-(Y)	No
f180-181	(P)-YP-(Q)	No
f182-186	(P)-QRDMP-(I)	No
f187-189	(P)-IQA-(F)	No
f190-196	(A)-FLLYQEP-(V)	Yes
f197-200	(P)-VLGP-(V)	Yes
f201-204	(P)-VRGP-(F)	Yes
f205-206	(P)-FP-(I)	No
f207-209	(P)-IIV	No

* Peptides identified by LC-MS/MS in the 24 h β-casein digest

Table 4. β-Casein derived di- tri- and tetrapeptides theoretically predicted to be released and/or detected in the 24 h *Aspergillus niger* derived proyl endoproteinase (*An*-PEP) hydrolysate

Peptide	Location in β-casein	Predicted to be released	Detected by MS	ACE IC_{50}* (μM)	Reference(s)
Phe-Ala	f(52-53)	Yes	No	487.1 ±	This study
Phe-Pro	f(62-63)	Yes	No	315.0	Abubakar, Saito, Kitazawa, Kawai & Itoh, 1998
	f(111-112)				
	f(205-206)				
Gly-Pro	f(64-65)	Yes	No	252.6	Byun & Kim, 2002
Ile-Pro	f(66-67)	Yes	No	130.0	Cheung, Wang, Ondetti, Sabo & Cushman,1980
Asn-Ser-Leu-Pro	f(68-71)	Yes		755.1 ± 64.2	This study
Phe-Leu-Gln-Pro	f(87-90)	Yes	Yes	68.7 ± 23.9	This study
Met-Ala-Pro	f(102-103)	Yes	No	0.4	Edens, Roos & Platerlink, 2009
Lys-Tyr-Pro	f(113-115)	Yes	Yes	363.9 ± 41.2	This study
Val-Glu-Pro	f(116-118)	Yes	No	63.7 ± 12.0	This study
Leu-Pro	f(137-138)	Yes	No	> 800.0	This study
His-Gln-Pro	f(148-150)	Yes	Yes	134.7 ± 19.1	This study
Leu-Pro-Pro	f(151-153)	Yes	Yes	9.6	Maruyama, Miyoshi, Kaneko & Tanaka, 1989
Lys-Val-Leu-Pro	f(169-172)	No	Yes	>1000.0	This study
Val-Pro	f(173-174)	Yes	No	420.0	Cheung *et al*., 1980
	f(178-179)				
Gln-Lys-Ala	f(175-177)	Yes	No	>1000.0	This study
Tyr-Pro	f(180-181)	Yes	No	720.0	Lantz, Glamsta, Talback & Nyberg, 1991
Arg-Asp-Met-Pro	f(183-186)	No	Yes	>1000	This study
Ile-Gln-Ala	f(187-189)	Yes	No	32.9 ± 9.2	This study
Val-Leu-Gly-Pro	f(197-200)	Yes	Yes	153.5 ± 11.4	This study
Val-Arg-Gly-Pro	f(201-204)	Yes	Yes	179.7 ± 35.2	This study
Ile-Ile-Val	f(207-209)	Yes	No	> 800.0	This study

* ACE IC_{50} values represent the concentration of hydrolysate that inhibits 50 % of ACE activity.

Legend for Figures

Figure 1. Reverse phase high performance liquid chromatography profiles for purified bovine β-casein incubated with *Aspergillus niger* derived prolyl endoprotease (*An*-PEP) at 55°C for (a) 0 h, (b) 4 h and (c) 24 h.

Figure 2. Mass fragmentation spectra for the β-casein derived peptides (a) Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro (m/z 682.87) and (b) Val-Leu-Gly-Pro (385.24). The y axis represents the relative intensity of the precursor ion (pre) and each of the fragment ions.

21 The x axis shows the m/z region in which fragment ions were detected. Ions generated by
22 fragmentation are detailed (y, a and b ions) and the deduced sequence is shown on the upper
23 left.

24 Supplementary Figure 1. (a) Reverse phase ultra performance liquid chromatography (RP-
25 UPLC) profile for purified β-casein hydrolysed with *Aspergillus niger* derived prolyl
26 endoproteinase (*An*-PEP) for 24 h. Detector response was monitored at both 214 (▬) nm and
27 280nm (▬). (b) The base peak chromatogram obtained during mass spectrometry analysis.

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29

FOODCHEM-D-13-04518 – Revised Highlights

- The hydrolytic specificity of prolyl endoproteinase (*An*-PEP) was assessed using β-casein.
- Incubation of β-casein with *An*-PEP generated a highly potent ACE inhibitory hydrolysate.
- Peptides contributing to the ACE inhibitory activity were identified using LC-MS/MS.
- Synthetic peptide studies allowed identification of new ACE inhibitors.