Fractionation of angiotensin I converting enzyme inhibitory activity from pea and whey protein in vitro gastrointestinal digests

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Abstract: In vitro gastrointestinal digestion of pea and whey protein produced high angiotensin I converting enzyme (ACE) inhibitory activity with IC50 values of 0.070 and 0.041 mg protein ml⁻¹ respectively. Ultrafiltration/centrifugation using a membrane with a molecular weight cut-off of 3000 Da decreased the IC50 value to 0.055 mg protein ml⁻¹ for pea permeate and 0.014 mg protein ml⁻¹ for whey permeate. Further fractionation by reverse phase HPLC gave IC50 values as low as 0.016 mg protein ml⁻¹ for pea and 0.003 mg protein ml⁻¹ for whey. Consequently, these purification steps enriched the ACE inhibitory activity of the pea digest more than four times and that of the whey digest more than 13 times. HPLC profiles after digestion and ultrafiltration indicate that high ACE inhibitory activity is due to short and more hydrophobic peptides. The results also suggest that potent ACE inhibitory peptides were present alongside low active peptides in whey hydrolysate, while all peptides had more or less the same ACE inhibitory activity in pea hydrolysate. In addition, the hydrolysates and enriched fractions will resist in vivo gastrointestinal digestion after oral administration. Hence these ACE inhibitory peptides, as part of functional foods, can play significant roles in the prevention and treatment of hypertension.

Keywords: angiotensin I converting enzyme inhibitory peptides; pea protein; whey protein; ultrafiltration; high-performance liquid chromatography

INTRODUCTION
Angiotensin I converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides may cause a lowering of the blood pressure by inhibition of the ACE enzyme, which results in a decrease in the vasoconstrictor angiotensin II and an increase in the vasodilator bradykinin.1 ACE inhibitory peptides have already been found in several food protein hydrolysates and ferments.2,3 Foods containing ACE inhibitory peptides have been shown to be effective in both the prevention and treatment of hypertension.4,5 As ACE inhibitory peptides have not yet revealed any side effects, they may represent an alternative or an additional treatment to antihypertensive drugs and may be applied in the prevention of high blood pressure.6

In a previous paper we reported high ACE inhibitory activity release upon in vitro gastrointestinal digestion of pea and whey protein.7 Whereas bioactive peptides have already been characterised from whey protein,8,9 they have not yet been studied from pea protein. Both animal whey protein and vegetable pea protein have a high nutritional value.10,11 In addition, whey is a by-product from milk during cheese making, and pea is an environmentally friendly crop presenting a non-GMO (genetically modified organism) alternative to soy as well as providing a symbiotic nitrogen fixation process. Purification of ACE inhibitory food protein hydrolysates may lead to more active fractions or ultimately a potent ACE inhibitory peptide. Ultrafiltration may be exploited to enrich ACE inhibitory peptides from whey proteins.12,13 Additional purification by ion exchange chromatography and reverse phase high—performance liquid chromatography (RP-HPLC) augments substantially the ACE inhibitory activity from wheat germ hydrolysate: the IC50 value of the digest is 0.67 mg ml⁻¹, while that of the pure peptide Ile-Val-Tyr is 0.48 μM or 0.189 μgm l⁻¹.14 The ACE inhibitory activity of an alcalase hydrolysate of chickpea protein is augmented upon gel filtration and RP-HPLC chromatography: the IC50 values of the isolated peptides are half that of the digest.15
The aim of this study was to obtain more ACE inhibitory active fractions from pea and whey protein hydrolysates by means of ultrafiltration and RP-HPLC. The HPLC profiles were used to characterise the hydrolysates.

**EXPERIMENTAL**

**Products**
The pea protein isolate Pisane HD (900 g protein kg\(^{-1}\) dry matter) and the whey protein isolate Lacprodan DI-9213 (900 g protein kg\(^{-1}\) dry matter) were obtained from Cosucra SA (Fontenoy, Belgium) and Acatris Belgium NV (Londerzeel, Belgium) respectively. Pepsin (P 6887), trypsin (T 1426), \(\alpha\)-chymotrypsin (C 4129), rat intestinal acetone powder (I 1630), trichloroacetic acid (TCA) solution (490-10), ACE reagent (305-10), ACE control-E (A 7040) and 1 kg kg\(^{-1}\) trifuluoracetic acid (TFA) solution (30203-1) were purchased from Sigma-Aldrich (St Louis, MO, USA). Non-specified products were of analytical grade and came from VWR International (Zaventem, Belgium).

**In vitro gastrointestinal digestion**
A 100 ml volume of 40 g l\(^{-1}\) pea or whey protein isolate solution was brought to pH 2 with 1 and 10 M HCl and NaOH under rigorous mixing for the stomach digestion. Pepsin (EC 3.4.23.1) was added at a ratio of enzyme to substrate of 1 to 250 kg kg\(^{-1}\), then incubation was started at 37 °C on a shaker. After 2 h the pH was set at 6.5 for the small intestine digestion, and trypsin (EC 3.4.21.4) and \(\alpha\)-chymotrypsin (EC 3.4.21.1) were both supplemented at a ratio of enzyme to substrate of 1 to 250 kg kg\(^{-1}\). Then the solution was again incubated at 37 °C for 2.5 h. When samples were taken at the start and the end of digestion, the pH was adjusted to 5. As this pH is close to the isoelectric point for both proteins (pea, pH 4.5; whey, pH 4–5), a clear separation was obtained by subsequent centrifugation. Samples were centrifuged at 10,000 × g for 15 min at 4 °C and the supernatant was frozen in liquid nitrogen and stored at −80 °C. The frozen samples were subsequently lyophilised to obtain a dry powder.

**Ultrafiltration/centrifugation**
The lyophilised digest (80 mg) was dissolved in 2 ml of MilliiQ water (Millipore, Bedford, MA, USA) and ultrafiltered/centrifuged in Centricon YM-3000 tubes (molecular weight cut-off (MWCO) 3000 Da; low-adsorption, hydrophilic, regenerated cellulose membranes) (Millipore) for 2 h at 7500 × g. The permeate and retentate were lyophilised for determination of the ACE inhibitory activity and HPLC analysis.

**Fractionation by preparative RP-HPLC**
The permeate was fractionated using preparative RP-HPLC by injecting repetitively 1 ml of a 35–40 mg protein ml\(^{-1}\) solution on a Vydac C\(_{18}\) column (250 mm × 10 mm) and ABI Model 783A gradient controller and absorbance detector at 210 nm with an ABI Model 400A solvent delivery system and Model 491 dynamic mixer/injector (Applied Biosystems, Foster City, CA, USA) operating at room temperature. The flow rate was 4 ml min\(^{-1}\). A linear gradient from 95% solvent A (H\(_2\)O + 1 g l\(^{-1}\) TFA) to 40% solvent B (acetonitrile + 0.085 g l\(^{-1}\) TFA) in 30 min was applied; thereafter the gradient increased linearly to 95% solvent A in 10 min and remained at 95% solvent A during the last 10 min. Commencing from 5 min after the start of elution, fractions were collected every 6 min. The five fractions obtained were lyophilised and dissolved in demineralised water to determine the ACE inhibitory activity.

**ACE inhibitory activity**
The lyophilised sample was dissolved in demineralised water at 10 mg ml\(^{-1}\) and analysed by an ACE inhibitory assay using the substrate furanacryloyl-Phe-Gly-Gly (ACE reagent) and ACE control-E as enzyme source.\(^7\) When the ACE inhibitory activity exceeded 80%, a dilution series was made to determine the 50% inhibitory concentration (IC\(_{50}\)) value. The IC\(_{50}\) value was obtained by fitting dose–response data to a four—parameter logistic model using the Marquardt–Levenberg algorithm (Sigmaplot 4.0, SPSS Inc, Chicago, IL, USA).

**Protein content**
Protein content was determined by the Bio-Rad DC protein standard assay (Bio-Rad Laboratories SA-NV, Nazareth Eke, Belgium) with BSA as standard, based on the method of Lowry,\(^{16}\) according to the procedures of the manufacturer.

**HPLC analysis**
HPLC profiles were obtained by analytical RP-HPLC by injecting the lyophilised sample at a concentration of 10 mg protein ml\(^{-1}\) on a Prophers 300 Å C\(_{18}\) column (250 mm × 4.6 mm, 5 μm) (Alltech Associates, Deerfield, IL, USA) and Dionex (Sunnyvale, CA, USA) HPLC with an ASI-100 autosampler, P580 pump, STH585 column oven, UVD340S detector at 210 nm and Chromeloeon 6.0 software. Elution was performed at 25 °C and a flow rate of 1 ml min\(^{-1}\) with solvents A (H\(_2\)O + 1 g l\(^{-1}\) TFA) and B (acetonitrile + 0.085 g l\(^{-1}\) TFA). The lyophilised sample (10 mg) was dissolved in 1 ml of MilliiQ water, and 20 μl of this solution was injected into the HPLC system for the protein and digest samples. Whey protein and digest were analysed using a linear gradient from 90% solvent A to 50% solvent B in 30 min, again to 90% solvent A in the next 20 min and remaining at 90% solvent A during the last 10 min. For pea protein and digest a linear gradient from 95% solvent A to 30% solvent B in 30 min was applied; subsequently the gradient increased linearly to 95% solvent A in the next 20 min and remained at 95% solvent A during
the last 10 min. A control solution containing 16 mg of pepsin, trypsin and α-chymotrypsin in 100 ml of MilliQ water, centrifuged for 15 min at 10 000 × g, was also eluted by the HPLC programmes for pea and whey respectively. Only an injection peak could be observed on these chromatograms. The permeates were analysed by the same programmes as for preparative RP-HPLC, and 100 μl of a 10 mg protein ml⁻¹ solution was injected.

**Statistical analysis**

All values are reported as mean ± standard error of the mean (nmin = 3). Only the logarithm of IC₅₀ is normally distributed. A Student t-test was used to compare the ACE inhibitory activity (log IC₅₀) of the permeate and the retentate with that in the digest, to investigate if the collected fractions exhibited a higher or lower ACE inhibitory activity than the permeate.

**RESULTS AND DISCUSSION**

### In vitro gastrointestinal digestion

Pea and whey protein solutions exhibited IC₅₀ values of 14 and 16 mg protein ml⁻¹ respectively (Table 1). The HPLC chromatograms of these soluble non-digested proteins are depicted in Figs 1 and 2 respectively. The separation by RP-HPLC is based on both molecular weight and hydrophobicity, with the higher-molecular-weight and more hydrophobic molecules eluting later in time. As the proteins are not yet digested, they appear at the end of the solvent gradient. For whey protein the peak of β-lactoglobulin was confirmed by elution of pure

**Table 1. Log IC₅₀ ± standard error of mean (mg protein ml⁻¹) of pea and whey protein, in vitro stomach digest, physiological digest, permeate and retentate and fractions I, II, III, IV and V collected after RP-HPLC fractionation of permeate (nmin = 3)**

<table>
<thead>
<tr>
<th></th>
<th>Pea</th>
<th>Whey</th>
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<tbody>
<tr>
<td>Protein</td>
<td>1.15 ± 0.05</td>
<td>1.21 ± 0.05</td>
</tr>
<tr>
<td>Stomach digest</td>
<td>−0.96 ± 0.03°</td>
<td>−0.40 ± 0.04**</td>
</tr>
<tr>
<td>Physiological digest</td>
<td>−1.16 ± 0.03</td>
<td>−1.39 ± 0.04</td>
</tr>
<tr>
<td>Permeate</td>
<td>−1.26 ± 0.03°</td>
<td>−1.86 ± 0.07**</td>
</tr>
<tr>
<td>Retentate</td>
<td>−1.06 ± 0.07</td>
<td>−1.23 ± 0.04</td>
</tr>
<tr>
<td>I</td>
<td>−0.92 ± 0.07***</td>
<td>−0.76 ± 0.02***</td>
</tr>
<tr>
<td>II</td>
<td>−1.40 ± 0.02**</td>
<td>−1.82 ± 0.03</td>
</tr>
<tr>
<td>III</td>
<td>−1.64 ± 0.03***</td>
<td>−2.54 ± 0.02***</td>
</tr>
<tr>
<td>IV</td>
<td>−1.79 ± 0.03***</td>
<td>−2.23 ± 0.04**</td>
</tr>
<tr>
<td>V</td>
<td>−1.24 ± 0.05</td>
<td>−2.20 ± 0.05**</td>
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Significantly different from physiological digest: °p < 0.05; °°p < 0.01.
Significantly different from permeate: *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 1. HPLC chromatograms of soluble fractions of non-digested pea protein and after in vitro stomach and complete physiological digestion.
β-lactoglobulin. An *in vitro* physiological digestion which simulated the conditions of protein digestion *in vivo* produced hydrolysates from pea and whey protein with high ACE inhibitory activity. The IC$_{50}$ values amounted to 0.070 and 0.041 mg protein ml$^{-1}$ respectively (Table 1), the latter value being lower than those reported in the literature for whey protein digested by gastrointestinal proteases.$^{12,13,18}$

ACE inhibitory activity from pea protein has not yet been reported by other authors. The *in vitro* digestion consisted of a simulation of the stomach digestion with pepsin and the small intestine digestion with trypsin and α-chymotrypsin. An *in vitro* digestion involving only the stomach phase was not as efficient as the physiological digestion in releasing ACE inhibitory activity from pea and whey proteins. The IC$_{50}$ values of the stomach digests were significantly higher than those of the physiological digests, and this effect was much more pronounced for whey protein (Table 1).

When the HPLC chromatograms of the stomach and physiological digests are compared for both proteins (Figs 1 and 2), a transition occurs from several peaks eluting after 20 min in the stomach digests to more peaks with lower elution times in the physiological digests. Apparently, a major breakdown of proteins and high-molecular-weight peptides still took place during the small intestine phase of digestion. β-Lactoglobulin, the major whey protein,$^{19}$ was mainly degraded by trypsin and α-chymotrypsin and hardly at all by pepsin. It is known that β-lactoglobulin resists pepsin and, largely, trypsin hydrolysis,$^{20}$ while it is hydrolysed by α-chymotrypsin to a limited extent.$^{21}$ Therefore the considerable increase in ACE inhibitory activity of the whey hydrolysate during the small intestine phase of digestion is probably caused by the release of potent bioactive peptides from β-lactoglobulin. It is reported that pepsin hydrolysis for 3 h at pH 2 and 37°C at a ratio of enzyme to substrate of 1 to 200 kg kg$^{-1}$ is insufficient to release ACE inhibitory peptides from α-lactalbumin and β-lactoglobulin.$^{13}$ Trypsin is necessary for the formation of high ACE inhibitory activity from both whey proteins.

**Ultrafiltration/centrifugation**

A first enrichment of ACE inhibitory peptides was obtained by ultrafiltration/centrifugation using a membrane with an MWCO of 3000 Da. The permeate, the hygroscopic low-molecular-weight fraction, of both pea and whey had a higher ACE inhibitory activity than the digest, while the IC$_{50}$ value of the retentate did not differ significantly from the corresponding digest IC$_{50}$ (Table 1). The former effect was more pronounced for whey, where the IC$_{50}$ value of 0.014 mg protein ml$^{-1}$ of the permeate was one-third of the IC$_{50}$ value of the digest. Pea permeate had an IC$_{50}$
value of 0.055 mg protein ml$^{-1}$. Compared with pea, whey hydrolysate seemed to contain a higher proportion of large peptides with lower ACE inhibitory activity. Another reason could be that $\beta$-lactoglobulin, which is still partially present after in vitro gastrointestinal digestion, binds low-molecular-weight ACE inhibitory peptides, preventing them from crossing the ultrafiltration membrane.\(^{22}\)

Ultrafiltration is a simple and reliable technique to separate peptides according to molecular weight, while removing proteins. Moreover, it presents an effective sample preparation and clean-up technique for RP-HPLC.\(^{23}\) Bioactive peptides usually contain 2–20 amino acid residues per molecule,\(^{8}\) and the lower their molecular weight, the higher is their chance to cross the intestinal barrier and exert a biological effect.\(^{24}\) The MWCO in our study was 3000 Da, which corresponds to oligopeptides of about 25 amino acids. Comparing the HPLC chromatograms of the physiological digest and the permeate (Figs 1–3), despite the different gradient programmes, reveals that large proteins such as $\beta$-lactoglobulin are no longer present in the permeate, while the proportion of low-molecular-weight compounds has risen considerably. The pea permeate especially has a high initial peak, pointing to a considerable concentration of amino acids and small hydrophilic peptides.

When fractionating ACE inhibitory peptides from whey hydrolysate, Pihlanto-Leppälä \textit{et al.}\(^{13}\) found higher ACE inhibitory activity in the $<1$ kDa fraction than in the other fractions. However, Mullally \textit{et al.}\(^{12}\) reported that the permeates of tryptic digests of whey protein hydrolysates and $\beta$-lactoglobulin obtained after ultrafiltration through 3 kDa as opposed to 1 kDa membranes are more potent inhibitors of ACE. In a three-step recycling ultrafiltration membrane reactor the gelatine hydrolysate fraction gained after the third hydrolysis with an ultrafiltration membrane of 1 kDa MWCO showed a higher ACE inhibitory activity than after the first and second hydrolysis steps with ultrafiltration membranes of 10 and 5 kDa respectively.\(^{25}\) Hence our results confirm the observation that ultrafiltration may separate high ACE inhibitory activity.

**Fractionation by RP-HPLC**

A further purification of the permeates was performed by RP-HPLC. Fig 3 shows the RP-HPLC profiles of the pea and whey permeates and indicates the five different fractions that were collected. The first fraction remained a gel even after extensive lyophilisation. As observed for the physiological digests, the whey permeate showed sharp individual peaks, while most of the peaks merged in the case of the pea permeate.

An overview of the different ACE inhibitory activities obtained by ultrafiltration/centrifugation of the digests and RP-HPLC fractionation of the permeates is depicted in Table 1. For both pea and whey proteins the ACE inhibitory activity in the first fraction was lower than in the permeate and even lower than in the digest. Most likely, this can be attributed to the presence of amino acids and short hydrophilic peptides with low ACE inhibitory activity. In fractions II, III and IV of the pea permeate a higher ACE inhibitory activity was observed. The lowest IC$_{50}$ value of 0.016 mg protein ml$^{-1}$ was found in fraction

![Figure 3. HPLC profiles of pea and whey permeate and various fractions (I, II, III, IV and V) collected.](image-url)
IV, which was more than four times lower than the IC₅₀ value of the pea digest. For whey the fractionation resulted in an even greater enrichment of ACE inhibitory activity. Fractions III, IV and V had higher ACE inhibitory activities compared with the permeate. The lowest IC₅₀ value, 0.003 mg protein ml⁻¹ found in fraction III, was more than 13 times lower than the IC₅₀ value of the whey digest. The fractions with the highest ACE inhibitory activity corresponded with a gradient of about 24–28% acetonitrile. Hence these fractions contained more hydrophobic peptides, which are more likely to exert an ACE inhibitory effect according to the structure–activity relationship of ACE inhibitory peptides. The treatment was again more effective for whey than for pea. This suggests that very potent ACE inhibitory peptides were present alongside low active peptides in whey hydrolysate, while all peptides had more or less the same ACE inhibitory activity in pea digest.

The yield of the three most active fractions was estimated for pea and whey proteins (Table 2). For fractions II, III and IV from pea a yield of about 5% was observed. For fractions III, IV and V from whey the yield was approximately 2%. These values are comparable to the yield of 3.3% of the di- and tripeptide fraction of a royal jelly protein gastrointestinal digest.

The ACE inhibitory hydrolysates and fractions have the advantage of resisting in vivo gastrointestinal digestion after oral administration. This increases their potential to exert an antihypertensive effect.

We chose not to isolate one or a few peptides with high ACE inhibitory activity, but to enrich for activity in two purification steps. In protein hydrolysates the ACE inhibition measured is due to various peptides, while the nutritional value of the protein is preserved. Moreover, it is suggested that peptide mixtures may exert a synergistic ACE inhibitory effect. The presence of other peptides and amino acids in the ACE inhibitory food protein hydrolysates may have important consequences on the susceptibility to peptidase degradation and intestinal transport after oral administration. Active transport of amino acids initiates a solvent drag through the tight junctions in the intestine, by which ACE inhibitory oligopeptides may be absorbed.

**CONCLUSIONS**

In this study the ACE inhibitory activity of pea and whey in vitro gastrointestinal digests was considerably enriched upon purification by ultrafiltration/centrifugation and RP-HPLC. These ACE inhibitory fractions have the potential to lower the blood pressure upon administration to hypertensive patients.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


**Table 2.** Estimated yields (g protein) of physiological digest, permeate and three most active fractions from 40 g l⁻¹ pea and whey protein (n = 3)

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<th>Pea</th>
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<tr>
<td>Digest</td>
<td>32.0</td>
<td>38.1</td>
</tr>
<tr>
<td>Permeate</td>
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<td>5.1</td>
</tr>
<tr>
<td>Fractions</td>
<td>1.9</td>
<td>0.8</td>
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</table>
Fractionation of ACE inhibitory activity from pea and whey protein digests