**Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and
angiotensin I-converting enzyme inhibitory activities?**

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Abstract

Inhibition of dipeptidyl-peptidase IV (DPP-IV) and angiotensin I-converting enzyme (ACE) are useful strategies for managing, respectively, diabetes and hypertension, two conditions often occurring together. In this study, debittered and non-debittered whey protein hydrolysates (WPHs) were assessed for their *in vitro* inhibitory activity against ACE and DPP-IV and characterized for their constituent peptides. All WPHs and several fractions obtained from them had ACE and DPP-IV inhibitory activities, with ACE being generally more strongly inhibited than DPP-IV. Among the identified peptides tested, GYGGVSLPEW derived from α -lactalbumin and LKPTPEGDL from β -lactoglobulin were, respectively, the most effective at inhibiting ACE ($IC_{50} = 2 \mu M$) and DPP-IV ($IC_{50} = 42 \mu M$). Although some identified peptides were able to inhibit both enzymes, the majority did not show a dual inhibitory effect. This research provides new insight on the active peptides responsible for the ACE and DPP-IV inhibitory activities of whey protein hydrolysates.

Key words: Angiotensin I-converting enzyme; dipeptidyl-peptidase IV; whey protein hydrolysates; bioactive peptides; dual inhibitors

1. Introduction

Diabetes and high blood pressure are among the leading risk factors for atherosclerosis and its complications, including strokes and heart attacks. The two conditions are believed to share common pathways and are frequently observed together in the same patients (Campbell et al., 2011; Cheung & Li, 2012). In the American population, hypertension occurs in 50 to 80% of individuals with type 2 diabetes (Landsberg & Molitch, 2004). Moreover, in a prospective cohort study by Gress, Nieto, Shahar, Wofford, and Brancati (2000), subjects with hypertension have been found to be 2.5 times more likely to develop type 2 diabetes than individuals with normal blood pressure.

Over the last two decades, protein hydrolysates and protein-derived peptides have been studied for their potential to help manage or complement pharmacotherapy in the treatment of chronic diseases (Li-Chan, 2015; Mine, Li-Chan, & Jian, 2010).

Dairy proteins are often considered one of the most important precursors of biologically active peptides, a number of proteins from both the whey and casein fractions of milk having been reported to contain within their sequences peptides with an array of activities (Kamau et al., 2010; Nagpal et al., 2011).

Currently, most of the research in the area of biologically active peptides from milk proteins has focused on peptides with inhibitory activity against the angiotensin I-converting enzyme (ACE) (Ricci, Artacho, & Olalla, 2010), a dipeptidyl

carboxypeptidase that promotes sodium retention and vasoconstriction (Brown & Vaughan, 1998). A number of milk protein hydrolysates produced using various enzymatic treatments as well as whey- and casein-derived peptides have been reported in the literature to inhibit ACE activity *in vitro* and exhibit antihypertensive activity *in vivo* (FitzGerald, Murray, & Walsh, 2004; Ricci, Artacho, & Olalla, 2010; Wang et al., 2012). ACE-inhibiting protein hydrolysates and protein-derived peptides, however, often present a bitter taste that may limit their application as functional food ingredients (Cheung & Li-Chan, 2010). Recently, our research group reported that the treatment of a bitter whey protein hydrolysate (WPH) with aminopeptidase or carboxypeptidase produced hydrolysates with reduced bitterness, while still exhibiting ACE inhibitory activity and ability to decrease systolic blood pressure in spontaneously hypertensive rats (Cheung, Aluko, Cliff, & Li-Chan, 2015). Although these findings suggested that exopeptidase treatment may be an effective approach to generate ACE-inhibitory hydrolysates with acceptable taste, the specific peptides responsible for the observed inhibition of ACE activity were not identified.

In addition to containing within their sequences peptides with ACE inhibitory activity, dairy proteins, particularly those found in whey, have recently been shown to be precursors of fragments able to inhibit the enzyme dipeptidyl-peptidase IV (DPP-IV) (Konrad et al., 2014; Lacroix & Li-Chan, 2012a; Lacroix & Li-Chan, 2013; Lacroix & Li-Chan, 2014; Nongonierma & FitzGerald, 2013; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Uchida, Ohshiba, & Mogami, 2011;

Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). The inhibition of DPP-IV, an ubiquitous enzyme involved in the inactivation of the incretin hormones glucose-dependent insulintropic polypeptide and glucagon-like peptide-1, is considered an effective approach to manage hyperglycemia in type 2 diabetes (Filippatos, Athyros, & Elisaf, 2014). The finding that proteins can be precursors of peptides with DPP-IV inhibitory activity *in vitro* has triggered a great interest in their potential to complement pharmacotherapy in the management of type 2 diabetes. However, although several protein hydrolysates have been shown to be able to inhibit DPP-IV activity, only a limited number of studies have actually isolated and identified DPP-IV inhibitory peptides from these hydrolysates (Lacroix & Li-Chan, 2014; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012).

Several specific milk protein-derived peptides have been reported to have more than one bioactivity. The β -casein-derived hepta-peptide YPFPGPI, for example, has been reported to have opioid, immunomodulating and ACE inhibitory activities (Gobbetti, Vini, & Rizzello, 2004). On the other hand, literature on dairy protein-derived peptides displaying both ACE and DPP-IV inhibitory activities is sparse. To date, only a few short peptides found within the sequence of milk proteins, including the tryptophan-containing peptides WL, WY and LW (Nongonierma & FitzGerald, 2015), have been reported to have a dual inhibitory effect on the two enzymes. These peptides, however, were not generated empirically by hydrolysis of milk proteins, but were rather chemically synthesized and tested for their effect on ACE

and DPP-IV activities. A recent study by Konrad et al. (2014) showed that hydrolysates of whey protein concentrate and β -lactoglobulin produced by enzymatic treatment with a serine protease isolated from Asian pumpkin had an inhibitory effect on both ACE and DPP-IV. However, since the authors did not identify the hydrolysates' constituent peptides, it is unknown whether the peptides responsible for the inhibition of ACE were also the ones causing the inhibition of the DPP-IV enzyme.

The objectives of the present study were to assess whether ACE-inhibiting WPHs debittered by exopeptidase treatment are also able to cause the inhibition of the DPP-IV enzyme *in vitro* and if so, to determine whether the same whey protein-derived peptides are responsible for the inhibition of both enzymes.

2. Materials and Methods

2.1 Materials

Whey protein isolate (WPI 895, Fonterra, New Zealand; 92.4% protein) donated by Caldic (Delta, BC, Canada) was used to produce the whey protein hydrolysates (WPHs) investigated in this study. The enzymes Thermoase PC10F, Peptidase R, and ProteAX were donated by Amano Enzyme U.S.A, LTD. (Elgin, IL, USA), while Accelerzyme® CGP was donated by DSM Food Specialties B.V (Delft, The

Netherlands). The commercially available whey protein hydrolysate Hilmar™ 8390, referred to as “H” in this study, was donated by Hilmar Ingredients (Hilmar, CA, USA). Dipeptidyl-peptidase IV (DPP-IV, EC 3.4.14.5, from porcine kidney, ≥10 units per mg protein), angiotensin I-converting enzyme (ACE, EC 3.4.15.1, from rabbit lung, ≥2.0 units per mg protein), N-hippuryl-His-Leu hydrate (≥98% by HPLC), Ile-Pro-Ile (diprotin A), *N*-[(S)-3-mercapto-2-methylpropionyl]-L-proline (captopril), teprotide (Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, ≥95% by TLC) and HPLC peptide standard mixture were purchased from Sigma-Aldrich (Oakville, ON, Canada). Antifreeze protein type 1 was donated by A/F Protein Canada (St Johns, NF, Canada). Gly-Pro-*p*-nitroanilide (H-Gly-Pro-*p*-NA•HCl) was from Bachem Americas (Torrance, CA, USA). Synthesized peptides (≥ 95% purity) were prepared and purified by GL Biochem (Shanghai) Ltd (Shanghai, China). All other chemicals used were of analytical grade.

2.2 Preparation of whey protein isolate hydrolysates

The hydrolysates (referred to as T, T-AC, T-PR and T-PX) were prepared in duplicate as described in Cheung, Aluko, Cliff, and Li-Chan (2015). Briefly, WPI (3% WPI, w/v, in deionized distilled (dd) H₂O, at the unadjusted pH of 6.9) was hydrolyzed with Thermoase PC10F (3 g/100 g WPI) for 180 min at 65°C. The mixture was then heated at 90°C for 15 min to inactivate the enzyme and centrifuged (10 min at 13,200 × *g*) using a DuPont Sorvall Centrifuge RC 5B (Mandel Scientific Co. Ltd.,

Guelph, ON, Canada). The supernatant was collected and freeze-dried (Labconco Corporation, Kansas City, MO, USA). This hydrolysate is referred to as “T”.

The hydrolysate produced after 180 min of hydrolysis with Thermoase PC10F (T) was further treated with one of three exopeptidase enzyme preparations. Briefly, a solution of T (10% hydrolysate, w/v, in ddH₂O) was hydrolyzed for 7 h with Accelerzyme® CGP (“AC”; 37°C, pH 4), Peptidase R (“PR”; 52°C, pH 6) or ProteAX (“PX”; 60°C, pH 6) at 4g/100g hydrolysate. The resulting exopeptidase-treated hydrolysates, respectively referred to as “T-AC”, “T-PR” and “T-PX”, were heated at 90°C for 15 min to inactivate the enzymes and freeze-dried.

2.3 Isolation of the ACE and DPP-IV inhibitory peptides from the hydrolysates

2.3.1 Size-exclusion chromatography

The hydrolysates H, T, T-AC, T-PR and T-PX were fractionated by size-exclusion chromatography. The samples were dissolved in 20 mM Tris-HCl buffer pH 8.0 to a concentration of 20 mg/mL, 500 µL was loaded into a Superdex™ Peptide 10/300 GL column (10 x 310 mm, 24 mL, GE Healthcare Uppsala, Sweden) connected to a fast protein liquid chromatography (FPLC) system (ÄKTAPurifier 100/10; GE Healthcare Life Science, Baie d’Urfé, QC, Canada), and eluted with Tris-HCl buffer (20 mM, pH 8.0) at a flow rate of 0.2 mL/min. The eluates were collected, pooled into fractions based on the peaks observed on the elution profiles monitored at 215

nm and 280 nm (similar elution profiles were observed at these two wavelengths), freeze-dried and assessed for their ACE and DPP-IV inhibitory activities. The separations were repeated multiple times to obtain enough samples for the subsequent purification and analysis. The antifreeze protein (MW 3240 Da) as well as the peptides DRVYIHPF (MW 1046.2 Da), YGGFM (MW 573.7 Da), YGGFL (MW 555.6 Da), VYV (MW 379.5 Da) and GY (MW 238.2 Da) from the HPLC peptide mixture standard were used to evaluate the molecular weight of the fractions.

2.3.2 Reversed-phase high performance liquid chromatography (RP-HPLC)

The peptide fraction (T-AC-4) obtained from the size-exclusion chromatography of T-AC that displayed the highest ACE and DPP-IV inhibitory activities was further fractionated by RP-HPLC as described in Lacroix and Li-Chan (2014). The collected fractions were assessed for their effect on ACE and DPP-IV activities.

2.4 Identification of peptide sequences by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

The hydrolysates T and T-AC and the purified fractions displaying the highest inhibition of ACE and DPP-IV were analyzed at the Fred Hutchinson Cancer Research Center (Seattle, WA, USA) to identify their constituent peptides by LC-ESI-MS/MS.

Samples (0.5-2 μg) were analyzed using a Thermo Scientific Easy-nLC II nano HPLC system coupled to a hybrid Orbitrap Elite ETD mass spectrometer (Thermo Scientific, Waltham, MA, USA) using an instrument configuration as described in Yi, Lee, Aebersold, and Goodlett (2003). In the case of T, T-AC and T-AC-4, the samples were first desalted using C18 ZipTips (Millipore, Billerica, MA, USA). In-line desalting was performed using a reversed-phase trap column (100 $\mu\text{m} \times 20 \text{ mm}$) packed with Magic C₁₈AQ (5- μm 200Å resin; Michrom Bioresources, Auburn, CA, USA) followed by peptide separations on a Magic C₁₈AQ reversed-phase column (75 $\mu\text{m} \times 250 \text{ mm}$) directly mounted on the electrospray ion source. Chromatographic separation was realized using a linear gradient of acetonitrile (2-40% in 60 min) and 0.1% formic acid in H₂O at a flow rate of 400 nL/min (300 nL/minute for direct inject runs). The heated capillary temperature was set to 300°C and a spray voltage of 2500 V was applied to the electrospray tip. The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between MS survey scans in the Orbitrap (automatic gain control (AGC) target value 1,000,000, resolution 240,000, and injection time 250 ms) with MS/MS spectra acquisition in the linear ion-trap (AGC target value of 10,000 and injection time of 100 ms). The 20 most intense ions from the Fourier-transform full scan were selected for fragmentation in the linear ion trap by collision-induced dissociation with a normalized collision energy of 35%. Selected ions were dynamically excluded for 15 s with a list size of 500 and exclusion mass by a mass width of $\pm 10 \text{ ppm}$. Each sample was run at least twice, one run having +2 and +3 charge states selected for

MS/MS (m/z range of 300 to 1800) and the second run having +1, +2 and +3 charge states for MS/MS (m/z range of 300 to 5000).

Collected data were analyzed using Proteome Discoverer 1.4 (Thermo Scientific, San Jose, CA, USA) and the amino acid sequence of each peptide was identified by comparison with peptide sequences from *Bos taurus* in the UniProt database.

Searches were performed with no enzyme; the precursor ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.6 Da. Variable modifications included oxidation on methionine and proline (+15.995 Da). The tools Sequest HT and Fixed Value PSM Validator were used for database searching and scoring.

2.5 Determination of ACE inhibitory activity

The effect of the whey protein hydrolysates and synthesized peptides on the activity of ACE was determined using the substrate N-hippuryl-His-Leu (HHL) according to the method described in Cheung and Li-Chan (2010) with some modifications. The hydrolysates were dissolved in 0.05 M Tris-HCl buffer at pH 8.3 containing 0.3 M NaCl (hereinafter referred to as “assay buffer”) while the synthesized peptides were first solubilized in a mixture of acetonitrile and water, then further diluted with the assay buffer to the desired concentrations. Samples (30 µL) were pre-incubated with 30 µL of ACE enzyme (0.083 U/mL in assay buffer) for 15 min at 37°C, thereupon 150 µL of HHL (6.5 mM in assay buffer) was added. The enzymatic reaction was carried out at 37°C for 60 min after which 250 µL of 1 N HCl was added

to inactivate the enzyme, followed by 1 mL of ethyl acetate to extract the hippuryl acid (HA). The mixtures were vortexed for 30 s and centrifuged at $2000 \times g$ for 5 min. After centrifugation, the ethyl acetate layer (0.7 mL) was removed and heated at 120°C for 30 min to evaporate the solvent. The HA residues were re-dissolved with 1.3 mL of ddH₂O and the absorbance was measured at 228 nm. The positive and negative controls, corresponding to the activity of ACE in the absence of inhibitor and the absence of ACE activity, respectively, were prepared by using assay buffer in lieu of the sample and in lieu of the sample and enzyme solution, respectively. For the synthesized peptides, a mixture of Tris-HCl and acetonitrile was used to prepare the positive controls.

The concentrations of samples required to cause a 50% inhibition of the enzyme activity (IC₅₀) were calculated from the logarithmic regression equations obtained from plotting the percent ACE inhibition against the sample concentrations. The IC₅₀ values of captopril and the angiotensin converting enzyme inhibitory peptide teprotide (Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were also determined for comparison.

2.6 Determination of DPP-IV inhibitory activity

The effect of the whey protein hydrolysates and synthesized peptides on the activity of DPP-IV was determined using the chromogenic substrate Gly-Pro-*p*-NA as

described by Lacroix and Li-Chan (2014). The tri-peptide Ile-Pro-Ile (diprotin A) was used as a reference inhibitor.

2.7 Statistical analysis

One-way analysis of variance using the general linear model and pairwise comparison with Tukey's method were performed using Minitab Statistical Software (Version 17, Minitab Inc., State College, PA, USA). All assays were conducted at least in triplicate and significant difference was established at $P < 0.05$.

3. Results

3.1 ACE and DPP-IV inhibitory activities of whey protein hydrolysates

The whey protein hydrolysates prepared by treatment of WPI with Thermoase PC10F alone (T) or with Thermoase PC10F followed by one of the exopeptidases, namely Accelerzyme® CPG (T-AC), Peptidase R (T-PR) or ProteAX (T-PX), as well as a commercial whey protein hydrolysate Hilmar™ 8390 (H), were tested for their effect on ACE and DPP-IV activities.

Although all five of these hydrolysates were able to inhibit the two enzymes to some extent, they appeared to cause a stronger inhibition of ACE than of DPP-IV (**Figure 1**). The greatest inhibition of ACE activity was observed with T, whereas the

carboxypeptidase treated T-AC was the most effective at inhibiting the DPP-IV enzyme. Treatment of T with ProteAX, an enzyme preparation exhibiting aminopeptidase as well as endoproteinase activity, resulted in the hydrolysate T-PX with the lowest ACE and DPP-IV inhibitory activities. While both the aminopeptidase and carboxypeptidase treatments of T led to a reduction in ACE inhibitory activity, only the aminopeptidase-treated hydrolysates (T-PR and T-PX) showed a significantly reduced ability to inhibit DPP-IV (**Figure 1**).

3.2 Fractionation of whey protein hydrolysates with ACE and DPP-IV inhibitory activities

3.2.1 Size-exclusion chromatography of H, T, T-AC, T-PR and T-PX

The four whey protein hydrolysates produced in this study (T, T-AC, T-PR and T-PX) and the commercial whey protein hydrolysate (H) were fractionated by size-exclusion chromatography (**Figure 2**). Treatments of T with exopeptidase (T-AC-7, T-PR-5 and T-PX-7 in **Figures 2C, 2D** and **2E**, respectively) led to the formation of short peptides and free amino acids, as shown by the presence of fractions eluting at about 30 mL, corresponding to MW < 238.2 Da. Interestingly, the elution profile of the commercial hydrolysate H was also characterized by a prominent peak (H-5) at around 30 mL (**Figure 2A**).

The resulting size exclusion fractions were tested for their effect on both ACE and DPP-IV activities. With 82% ACE inhibition, T-3 (MW 750-1230 Da) was the most potent size-exclusion fraction obtained from all five hydrolysates. Among the 31 fractions collected, H-3, H-4, T-3, T-AC-4, T-AC-5, T-PR-3, T-PX-2, T-PX-3, T-PX-4 and T-PX-6 were found to be significantly more effective at inhibiting the ACE enzymes than the un-fractionated hydrolysates from which they were obtained. The peptide fractions eluting around 30 mL and having the lowest molecular weight (H-5, T-AC-7, T-PR-5, and T-PX-7) all displayed low or no ACE inhibitory activity.

On the other hand, the fraction T-AC-4 (MW 645-1065 Da), with 47% inhibition, was found to be the most effective at inhibiting DPP-IV activity. While several fractions were able to inhibit the ACE enzyme more strongly than the crude hydrolysates they originated from, only fractions T-AC-4, T-PX-2 and T-PX-4, were more effective at inhibiting DPP-IV than their respective non-fractionated hydrolysates. Similarly to what was observed for the ACE enzyme, peptide fractions eluting around 30 mL had little or no effect on DPP-IV activity (**Figure 2**).

Displaying the highest DPP-IV inhibitory activity and being among the most effective at inhibiting ACE, T-AC-4 was selected to undergo further purification by RP-HPLC.

3.2.2 Reversed-phase high performance liquid chromatography of T-AC-4

As shown in **Figure 3**, among the seven fractions collected from the RP-HPLC fractionation of T-AC-4, the most non-polar fraction T-AC-4-g, eluting at ~ 27–29 min, caused the strongest inhibition of the DPP-IV enzyme (74% inhibition). The other fractions had some inhibitory activity, but their potency was far lower. Conversely, T-AC-4-g had no effect on the ACE enzyme. Instead, the fractions T-AC-4-b and T-AC-4-f were the most effective ACE inhibitors, causing 77 and 84% inhibition respectively. The enzyme was also inhibited by the other four fractions (T-AC-4-a, T-AC-4-c, T-AC-4-d and T-AC-4-e), but to a lesser extent.

3.3 ACE and DPP-IV inhibitory activities (IC_{50} values) of fractions isolated from T-AC

The potency of the most active size-exclusion and RP-HPLC fractions isolated from T-AC were determined and compared to the un-fractionated hydrolysates T and T-AC (**Table 1**). The fraction T-AC-4 obtained by size exclusion chromatography had significantly lower IC_{50} values against both ACE and DPP-IV than the un-fractionated hydrolysate T-AC as well as the starting hydrolysate T. Further purification of T-AC-4 by RP-HPLC produced fractions with even lower IC_{50} values; those displaying the highest ACE inhibitory activity, however, were not the same that caused the strongest inhibition of DPP-IV.

With an IC_{50} value of 25 $\mu\text{g/mL}$, the RP-HPLC fraction T-AC-4-f was the most effective at inhibiting the ACE enzyme. The potency of this fraction was roughly two times greater than that of the size exclusion fraction T-AC-4 and four times greater

than the hydrolysate T. The more polar RP-HPLC fraction T-AC-4-b also showed high ACE inhibitory activity, with an IC_{50} value of 36 $\mu\text{g/mL}$. In contrast, the hydrolysate T-AC, with an IC_{50} value of 195 $\mu\text{g/mL}$, was much less potent than the fractions isolated from it.

On the other hand, the T-AC-4-g fraction caused the strongest inhibition of DPP-IV activity ($IC_{50} = 50 \mu\text{g/mL}$). This RP-HPLC fraction was about 3 times more potent against DPP-IV than T-AC-4 and about 5 times more effective at inhibiting the enzyme than the whole hydrolysates T-AC and T.

3.4 Identification by LC-ESI-MS/MS of the peptides present in the hydrolysates T and T-AC and purified fractions T-AC-4, T-AC-4-b, T-AC-4-f and T-AC-4-g

Causing the strongest inhibition of the DPP-IV or ACE enzyme, the RP-HPLC fractions T-AC-4-b, T-AC-4-f and T-AC-4-g were analyzed for their constituent peptides by LC-ESI-MS/MS. The peptides present in the hydrolysates T and T-AC as well as the fraction T-AC-4 from size-exclusion chromatography were also determined and compared to those identified in the potent RP-HPLC fractions.

The sequences of the 378 peptides identified, whose length varied from 4 to 24 amino acid residues, are presented in the **Supplementary Table S1**. These peptides originated from β -lactoglobulin and α -lactalbumin, which are the two major protein constituents in the WPI used to produce the hydrolysates investigated in this study.

A few peptides derived from minor protein constituents of the WPI, such as lactoferrin and bovine serum albumin, were also identified in the hydrolysates and purified peptide fractions (data not shown).

A total of 201 peptide sequences were identified in T, 162 derived from β -lactoglobulin and 39 from α -lactalbumin, while 101 peptides derived from β -lactoglobulin and 33 from α -lactalbumin were identified in the T-AC hydrolysate. Forty-five of these sequences were common to both hydrolysates. The fraction T-AC-4 was found to be composed of 80 peptides derived from β -lactoglobulin and 27 from α -lactalbumin. Of these, 75 were also identified in T-AC. On the other hand, the three RP-HPLC fractions were found to contain 115 β -lactoglobulin- and 48 α -lactalbumin-derived peptides. Among these 163 sequences, 63 were also found in T-AC-4. While all peptide sequences identified in T-AC-4 should have also been found in T-AC and all peptides in T-AC-4-b, T-AC-4-f and T-AC-4-g should have been identified in T-AC-4, this was not the case. This discrepancy is likely due to the fact that crude whey protein hydrolysates and their size-exclusion fractions are composed of a complex mixture of peptides, making the identification of each individual peptide sequence by mass spectrometry a very challenging task. It is probable that some peptides, particularly those present in small amount, might not have been found in T, T-AC or T-AC-4 by LC-ESI-MS/MS analysis, but were identified in the more purified RP-HPLC fractions from T-AC-4.

3.5 ACE and DPP-IV inhibitory activities of identified peptides

Among the peptides identified in T, T-AC, T-AC-4, T-AC-4-b, T-AC-4-f and/or T-AC-4-g, 15 peptides derived from α -lactalbumin and 33 peptides from β -lactoglobulin were chemically synthesized and their effect on the activity of the ACE and DPP-IV enzymes was assessed (**Figure 4, Table 2**). These peptides were selected based on similarities between their amino acid sequences and α -lactalbumin- and β -lactoglobulin-derived peptides reported to have ACE and/or DPP-IV inhibitory activity, or because of the presence in their sequence of structural features (i.e. particular amino acid residues at certain positions) often observed in ACE and/or DPP-IV inhibitory peptides.

The peptides were found to have various levels of effectiveness on the ACE and DPP-IV enzymes (**Figure 4**). Among the 48 peptides tested, 20 had no detectable effect on ACE activity, whereas 8 were able to cause at least 50% inhibition of the enzyme at a final assay concentration of 143 μ M (**Figure 4A**). The β -lactoglobulin-derived peptides LDIQKVAGTW and LKALPMH, with IC_{50} values of 21 and 11 μ M, respectively, and α -lactalbumin derived peptides GYGGVSLPEW and WLAHKAL, with IC_{50} values of 2 and 29 μ M, respectively, were the most effective at inhibiting the ACE enzyme. The potency of these peptides was, however, lower than that of the peptidomimetic ACE inhibitors teprotide and captopril (IC_{50} = 0.48 and 0.0054 μ M, respectively) (**Table 2**).

With the exception of the tetra-peptide RTPE and deca-peptide LDDDLTDDIM, which had no detectable effect on DPP-IV, all other peptides were able to inhibit the enzyme, albeit mostly only to a moderate extent (**Figure 4B**). Displaying IC₅₀ values of 42 and 57 µM, the β-lactoglobulin-derived peptides LKPTPEGDLE and LKPTPEGDLEIL were the most effective at inhibiting DPP-IV. All peptides tested were less potent towards the enzyme than the reference inhibitor diprotin A (IC₅₀ = 4.7 µM) (**Table 2**).

4. Discussion

While the treatment of WPH with the aminopeptidases Peptidase R and ProteAX or the carboxypeptidase Accelerzyme® CPG generated hydrolysates with reduced ACE inhibitory activity, only the hydrolysates produced by treatment with the aminopeptidases were found to be less effective at inhibiting the DPP-IV enzyme (**Figure 1**). Peptides with ACE inhibitory activity have been reported to preferentially have hydrophobic/aromatic amino acids at one or both termini, and/or bulky and basic charged residues at the C-terminal (Nongonierma & FitzGerald, 2015). Since all three exopeptidases used were reported to have specificity for hydrophobic amino acids (Cheung, Aluko, Cliff, & Li-Chan, 2015), the removal of these residues from the peptides present in the starting hydrolysate appear to have led to the production of new peptides with reduced inhibitory activity. Unlike ACE which is a carboxypeptidase, DPP-IV releases di-peptides from the N-terminal of its substrates. The finding that only the aminopeptidase-treated

T3 showed reduced inhibitory activity suggests that residues at the C-terminal might have little effect on the ability of a peptide to inhibit DPP-IV.

Most fractions isolated from the WPHs tested in this study were able to inhibit the activity of ACE and DPP-IV; however, the extent of the inhibition differed greatly. In general, hydrolysates and fractions with the strongest ACE inhibitory activity were not the most effective at inhibiting the DPP-IV enzyme. Similar findings were recently reported in a study by Konrad et al. (2014) in which the authors determined the ACE and DPP-IV inhibitory activities of RP-HPLC fractions obtained from hydrolysates of a whey protein concentrate and β -lactoglobulin. Among their isolated fractions, a number were able to inhibit both enzymes, but their effectiveness against them varied. Since the authors did not identify the constituent peptides in these fractions, it is unknown whether the same peptides were responsible for the inhibition of the DPP-IV enzyme as well as the ACE enzyme.

Although a few of the peptides tested in the present study had inhibitory activity against both ACE and DPP-IV (**Figure 4**), the susceptibility of the two enzymes to inhibition by the whey protein-derived peptides differed greatly. In fact, the most potent ACE inhibitory peptide GYGGVSLPEW had very weak effect on DPP-IV activity. Similarly, the fragments with the strongest DPP-IV inhibitory activity (LKPTPEGDLLEIL and LKPTPEGDLLE) had low or no ACE inhibitory activity (**Table 2**). All peptides bearing a tryptophan residue at the N- or C-terminal or a histidine residue at the C-terminal showed strong inhibitory activity against ACE. The shorter

β -lactoglobulin fragment ALPMH ($IC_{50} = 521 \mu M$) (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000) and α -lactalbumin-derived peptides KGYGGVSLPEW and WLAHK ($IC_{50} = 0.7$ and $77 \mu M$, respectively) (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Tavares et al., 2011) have also been reported to have inhibitory effect against ACE. On the other hand, the most potent DPP-IV inhibitory peptides have a proline residue at their P_1' position, a structural feature found in a number of peptides reported to have inhibitory activity against the DPP-IV enzyme (Lacroix & Li-Chan, 2012b). Even though the amino acid sequence, rather than individual characteristics such as length or net charge, seems to be the predominant factor determining the effect of peptides on the activity of ACE and DPP-IV, the specific sequences required to cause the inhibition of ACE appeared to be different than those needed to inhibit DPP-IV activity.

5. Conclusion

Findings from the present research show that hydrolysates presenting inhibitory activities against both ACE and DPP-IV can be obtained from the enzymatic treatment of whey proteins, including hydrolysates debittered by exopeptidase treatments. By fractionating the hydrolysates and identifying the sequences of peptides in the most active fractions, the possibility of dual inhibitory activity of the constituent peptides in the hydrolysates was tested. Although a few of the identified peptides were able to inhibit both enzymes, most in fact did not display a dual

inhibitory activity. These results suggest that the peptides that are responsible for the observed inhibition by the hydrolysate of the ACE enzyme are most likely not the same ones that cause the inhibition of DPP-IV.

The present findings suggest that whey protein hydrolysates could have the potential to help improve both blood pressure and blood glucose regulation by means of their ability to inhibit the ACE and DPP-IV enzymes. While the experimental hydrolysates investigated in this study had been previously shown to have antihypertensive activity in spontaneously hypertensive rats (Cheung, Aluko, Cliff, & Li-Chan, 2015), their *in vivo* effect on ACE and DPP-IV activities as well as blood glucose regulation are unknown and, therefore, should be further investigated. Since the inhibitory peptides have to first be absorbed through the gastrointestinal wall before reaching the target enzymes, more research is also needed to evaluate the fate of the whey protein-derived peptides during digestion and their cellular permeability.

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Table 1. ACE and DPP-IV inhibitory activities (IC₅₀ values) of whey protein isolate hydrolysates obtained by enzymatic treatment with Thermoase PC10F (T) or with Thermoase PC10F and Accelerzyme® CPG (T-AC), and the most active size-exclusion chromatography (T-AC-4) and RP-HPLC (T-AC-4-b, T-AC-4-f and T-AC-4-g) fractions isolated from T-AC.

Hydrolysate/fraction	IC ₅₀ (µg/mL) ^a	
	ACE	DPP-IV
T	102 c	250 c
T-AC	195 d	268 d
T-AC-4	59 b	151 b
T-AC-4-b	36 ab	ND
T-AC-4-f	25 a	ND
T-AC-4-g	ND	50 a

^aIC₅₀ values are reported as the mean from at least three determinations and expressed as final assay concentrations. Within the same column, values with different lower case letters are significantly different ($P < 0.05$). ND, IC₅₀ values were not determined as the samples had no or low inhibitory activity.

Table 2. ACE and DPP-IV inhibitory activities (IC₅₀ values) of synthesized peptides

Origin of peptide	Peptide sequence	IC ₅₀ (μM) ^a	
		ACE	DPP-IV
β-Lactoglobulin	LDIQKVAGTW	21 ab	ND
	IQKVAGTW	51 c	329 c
	LKPTPEGDLEIL ^b	ND	57 a
	LKPTPEGDLE	ND	42 a
	VLDTDY	128 e	471 d
	LDTDY	121 de	ND
	LKALPMH	11 ab	193 b
	LSFNPTQ	106 d	ND
α-Lactalbumin	LKGYGGVSLPE	ND	486 d
	GYGGVSLPEW	2 a	ND
	WLAHKAL ^b	29 bc	286 c
Diprotin A ^c	IPI ^b	ND	4.7
Teprotide ^d	GlpWPRPQIPP	0.48	ND
Captopril ^d	NA	0.0054	ND

^a IC₅₀ values are reported as the mean from at least three determinations and expressed as final assay concentrations. Within the same column, values with different lower case letters are significantly different ($P < 0.05$).

^b IC₅₀ values of these peptides against DPP-IV are from Lacroix & Li-Chan (2014).

^c Diprotin A was used as reference DPP-IV inhibitor.

^d Teprotide and captopril were used as reference ACE inhibitors.

NA, not applicable; ND, IC₅₀ values were not determined as the peptides/peptidomimetics had no or low inhibitory activity.

Figure 1. ACE and DPP-IV inhibitory activities of whey protein hydrolysates. **H**, commercial whey protein hydrolysate Hilmar™ 8390; **T**, whey protein isolate (WPI) hydrolyzed with Thermoase PC10F; **T-AC**, WPI successively hydrolyzed with Thermoase PC10F and Accelerzyme® CPG; **T-PR**, WPI isolate successively hydrolyzed with Thermoase PC10F and Peptidase R; **T-PX**, WPI successively hydrolyzed with Thermoase PC10F and ProteAX. The percent inhibition values of ACE and DPP-IV were determined using 0.143 mg/mL and 0.125 mg/mL of samples, respectively (final assay concentrations). Each bar represents the mean and standard deviation of at least three determinations. Bars with different lower or upper case letters are significantly different ($P < 0.05$).

Figure 2. Elution profiles obtained by size-exclusion chromatography of **H** (A), **T** (B), **T-AC** (C), **T-PR** (D) and **T-PX** (E), and the ACE and DPP-IV inhibitory activities of the unfractionated hydrolysates and resulting fractions. The percent inhibition values of ACE and DPP-IV are reported as the mean from at least three determinations and were obtained using 0.143 mg/mL and 0.125 mg/mL of samples, respectively (final assay concentrations). Within the same row, values with different lower case letters are significantly different ($P < 0.05$). Molecular weight markers were eluted at the following volumes: antifreeze protein (3240 Da) = 8.90 mL; DRVYIHPF (1046.2 Da) = 15.57 mL; YGGFM (MW 573.7 Da) = 15.94 mL; YGGFL (MW 555.6 Da) = 16.31 mL; VYV (MW 379.5 Da) = 17.36 mL; GY (MW 238.2 Da) = 19.67 mL.

Figure 3. Elution profile obtained by reversed-phase HPLC of **T-AC-4**, and the ACE and DPP-IV inhibitory activities of the resulting fractions. The percent inhibition values of ACE and DPP-IV are reported as the mean from at least three determinations and were obtained using 0.143 mg/mL and 0.125 mg/mL of samples, respectively (final assay concentrations). Within the same row, values with different lower case letters are significantly different ($P < 0.05$).

Figure 4. Percent inhibition of ACE (A) and DPP-IV (B) by synthesized peptides. The percent inhibition values of ACE and DPP-IV were determined using 143 μ M and 125 μ M of peptides, respectively (final assay concentrations). Each bar represents the mean and standard deviation of at least three determinations.

Figure 1

Figure 2

Enzyme	% Inhibition						
	T-AC-4-a	T-AC-4-b	T-AC-4-c	T-AC-4-d	T-AC-4-e	T-AC-4-f	T-AC-4-g
ACE	38 d	77 b	17 e	48 c	13 e	84 a	-5 f
DPP-IV	23 c	22 c	11 e	16 d	27 b	19 cd	74 a

Table S1. β -Lactoglobulin- and α -lactalbumin-derived peptide sequences identified in the whey protein hydrolysate T, the exopeptidase treated hydrolysate T-AC, and/or the SE-FPLC and RP-HPLC fractions isolated from T-AC.

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
β -lactoglobulin	LIVTQTMKGLDIQKVAGTWYS				
	LIVTQTMKGLDIQKVAGTW				
	LIVTQTMKGLDIQKVAGT				
	LIVTQTMKGLDIQKVA				
	LIVTQTMKGLDIQK				
	LIVTQTMKGLDIQ				
	LIVTQTMKGLD				
	LIVTQTMKGL				
	LIVTQTMKG				
	LIVTQTMK				
	LIVTQTM				
	LIVTQT				
	IVTQTMKGLDIQKVAGTWYS				
	IVTQTMKGLDIQKVAGTW				
	IVTQTMKGLDIQ				
	IVTQTMKGLD				
	IVTQTMKGL				
	IVTQTMKG				
	IVTQTMK				
	IVTQT				
	TMKGLDIQKVAGTW				
	MKGLDIQKVAGTWYS				
	MKGLDIQKVAGTW				
	MKGLDIQKVAGT				
	MKGLDIQKVAG				
	MKGLDIQKVA				
	MKGLDIQK				
	MKGLDIQ				
	GLDIQKVAGTW				
	GLDIQKVAGT				
	LDIQKVAGTWYSL				
	LDIQKVAGTWYS				
	LDIQKVAGTWY				
	LDIQKVAGTW				
	LDIQKVAGT				
	LDIQKVAG				
	LDIQKVA				
	LDIQK				
	IQKVAGTWYS				
	IQKVAGTW				
	IQKVAGT				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	VAGTWYSLAM				
	VAGTWYSL				
	VAGTWY				
	VAGTW				
	TWYSL				
	LAMAASDISLLDAQSAPLRVY				
	LAMAASDISLLDAQSAPLR				
	LAMAASDISLLDAQSAPL				
	LAMAASDISL				
	LAMAASDIS				
	AMAASDISL				
	MAASDISLLDAQSAPLR				
	MAASDISLLDAQSAPL				
	MAASDISLLDAQSAP				
	MAASDISLL				
	MAASDISL				
	MAASDIS				
	AASDISLLDAQSAPLRVYVEE				
	AASDISLLDAQSAPLRVY				
	AASDISLLDAQSAPLRV				
	AASDISLLDAQSAPLR				
	AASDISLLDAQSAPL				
	AASDISLLDAQSAP				
	AASDI				
	ASDISLLDAQSAPLR				
	SDISLLDAQSAPL				
	DISLLDAQSAPL				
	DISLLDAQSAP				
	ISLLDAQSAPLR				
	ISLLDAQSAPL				
	LLDAQSAPLRVYVEEL				
	LLDAQSAPLRVYVEE				
	LLDAQSAPLRVY				
	LLDAQSAPLRV				
	LLDAQSAPLR				
	LLDAQSAPL				
	LLDAQSAP				
	LDAQSAPLRVYVEELKPTPEGDL				
	LDAQSAPLRVYVEELKPTPEGDL				
	LDAQSAPLRVYVEEL				
	LDAQSAPLRVYVEE				
	LDAQSAPLRVY				
	LDAQSAPLRV				
	LDAQSAPLR				
	LDAQSAPL				
	LDAQSAP				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	LDAQSA				
	DAQSAPL				
	SAPLRVYVEE				
	LRVYVEEL				
	LRVYVEE				
	VYVEELKPTPEGDLEIL				
	VYVEELKPTPEGDLEI				
	VYVEELKPTPEGDLE				
	VYVEELKPTPEGD				
	VYVEELKPTPEGD				
	VYVEELKPTPEG				
	VYVEELKPTPE				
	VYVEELK				
	VYVEEL				
	VYVEE				
	VEELKPTPEGDLEIL				
	VEELKPTPEGDLE				
	EELKPTPEGDLEILLQ				
	ELKPTPEGDLEIL				
	ELKPTPE				
	LKPTPEGDLEILLQKWENGE				
	LKPTPEGDLEILLQKW				
	LKPTPEGDLEILLQK				
	LKPTPEGDLEILLQ				
	LKPTPEGDLEILL				
	LKPTPEGDLEIL				
	LKPTPEGDLEI				
	LKPTPEGDLE				
	LKPTPEGD				
	LKPTPEGD				
	LKPTPEG				
	LKPTPE				
	KPTPEG				
	KPTPEGD				
	KPTPE				
	ILLQKW				
	LQKWENGECQKK				
	LLQKWENGE				
	LLQKWENG				
	LQKWENGE				
	AQKKIIAEK				
	KIIAEKTKIPA				
	KIIAEK				
	IIAEKTKIPAVFKIDALNENKV				
	IIAEKTKIPAVFKIDALNENK				
	IIAEKTKIPAVFKIDA				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	IIAEKTKIPAVFKID				
	IIAEKTKIPAVFKI				
	IIAEKTKIPAVFK				
	IIAEKTKIPAVF				
	IIAEKTKIPAV				
	IIAEKTKIPA				
	IIAEKTKIP				
	IIAEKT				
	IIAEK				
	IAEKTIPAVFK				
	IAEKTIPAV				
	IAEKTIPA				
	IAEKTIP				
	AEKTIPA				
	AEKTIP				
	EKTIP				
	KTKIPA				
	TKIPAVFKID				
	IPAVFKIDALNENKV				
	IPAVFKID				
	AVFKIDALNENK				
	VFKIDALNENKVL				
	VFKIDALNENKV				
	VFKIDALNENK				
	VFKIDALNEN				
	VFKIDALNE				
	VFKIDALN				
	VFKIDAL				
	VFKIDA				
	FKIDALNENKV				
	FKIDALNENK				
	FKIDALN				
	FKIDAL				
	IDALNENKVLVLDTDYKKYL				
	IDALNENKVLVLDTDYKKY				
	IDALNENKVLVLDTDYKK				
	IDALNENKVLVLDTDYK				
	IDALNENKVLVLDTDY				
	IDALNENKVLVLDTD				
	IDALNENKVLVLD				
	IDALNENKVLV				
	IDALNENKVL				
	IDALNENKV				
	IDALNENK				
	IDALNEN				
	IDALNE				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	DALNENKVLVLDTDYKKYL				
	DALNENKV				
	DALNENK				
	ALNENKVLVLDTDY				
	ALNENKVLVLDTD				
	ALNENK				
	LNENKVLVLDTDYKKYLL				
	LNENKVLVLDTDYKKYL				
	LNENKVLVLDTDYKKY				
	LNENKVLVLDTDYKK				
	LNENKVLVLDTDYK				
	LNENKVLVLDTDY				
	LNENKVLVLDTD				
	LNENKVLVLD				
	LNENKVL				
	LNENKV				
	LNENK				
	NKVLVLDTDY				
	VLVLDTDYKKYL				
	VLVLDTDYKK				
	VLVLDTDYK				
	VLVLDTDY				
	VLVLDTD				
	LVLDTDYKKYLL				
	LVLDTDYKKYL				
	LVLDTDYKKY				
	LVLDTDYKK				
	LVLDTDYK				
	LVLDTDY				
	LVLDTD				
	VLDTDYKKYL				
	VLDTDYKKY				
	VLDTDYKK				
	VLDTDYK				
	VLDTDY				
	LDTDYKKYL				
	LDTDYKKY				
	LDTDYKK				
	LDTDYK				
	LDTDY				
	DTDYKKY				
	DTDYKK				
	LLFCMENSAEPEQS				
	LFCMENSAEPEQSL				
	LFCMENSAEPEQS				
	FCMENSAEPEQS				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	CMENSAEPEQS				
	MENSAEPEQSL				
	MENSAEPEQS				
	SAEPEQSL				
	LVRTPEVDDEALEKFDKA				
	LVRTPEVDDEALEK				
	LVRTPEVDDEAL				
	LVRTPEVDDEA				
	LVRTPEVDDE				
	LVRTPEVDD				
	LVRTPEVD				
	LVRTPEV				
	LVRTPE				
	VRTPEVDDEALEKFDKA				
	VRTPEVDDEALEKF				
	VRTPEVDDEALEK				
	VRTPEVDDEAL				
	VRTPEVDDEA				
	VRTPEVDDE				
	VRTPEVDD				
	VRTPEVD				
	VRTPE				
	RTPEVDDEALEK				
	RTPE				
	TPEVDDEA				
	VDDEALEK				
	DDEALEK				
	DEALEKFDKA				
	DEALEK				
	LEKFDKALKALP				
	FDKALKALPMHIR				
	FDKALKALPMH				
	FDKALKALPM				
	FDKALKALP				
	KALKALP				
	ALKALPMHIR				
	ALKALP				
	LKALPMHIRLS				
	LKALPMHIR				
	LKALPMHI				
	LKALPMH				
	LKALPM				
	KALPMH				
	LPMHIR				
	IRLSFNPTQLEEQCHI				
	IRLSFNPTQLEEQ				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	IRLSFNPTQ				
	RLSFNPTQ				
	LSFNPTQLEEQCHI				
	LSFNPTQLEEQ				
	LSFNPTQLEE				
	LSFNPTQL				
	LSFNPTQ				
	LSFNPT				
	SFNPTQLEEQCHI				
	SFNPTQLEEQCH				
	SFNPTQLEEQ				
	FNPTQLEEQCHI				
	FNPTQLEEQCH				
	FNPTQLEEQ				
	FNPTQLEE				
	FNPTQ				
	NPTQ				
	PTQLEEQ				
	LEEQCHI				
α -lactalbumin	FRELKDLKGYGG				
	LKGYGGVSLPEWVCTT				
	LKDLKGYGGVSLPEW				
	LKDLK				
	DLKGYGG				
	LKGYGGVSLPEWV				
	LKGYGGVSLPEW				
	LKGYGGVSLPE				
	LKGYGGVSLP				
	LKGYGGVSL				
	LKGYGGVS				
	LKGYGG				
	KGYGGVSLPEW				
	KGYGGVSLPE				
	GYGGVSLPEW				
	GYGGVSLPE				
	YGGVSLPEW				
	YGGVSLPE				
	GGVSLPEW				
	GGVSLPE				
	GVSLPEW				
	VSLPEWVCTT				
	TTFHTSGYDTQA				
	TTFHTSGYDTQ				
	TTFHTSGYD				
	TFHTSGYDTQAI				
	TFHTSGYDTQA				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	TFHTSGYDTQ				
	TFHTSGYDT				
	TFHTSGYD				
	TFHTSG				
	FHTSGYDTQAIVQNNDSTEYG				
	FHTSGYDTQAIVQ				
	FHTSGYDTQAI				
	FHTSGYDTQA				
	FHTSGYDTQ				
	FHTSGYDT				
	FHTSGYD				
	FHTSGY				
	HTSGYDTQ				
	TSGYDTQAI				
	TSGYDTQA				
	TSGYDTQ				
	SGYDTQAI				
	AIVQNNDSTEY				
	AIVQNNDSTE				
	IVQNNDSTEYGLFQ				
	IVQNNDSTEYGLF				
	IVQNNDSTEYGL				
	IVQNNDSTEYG				
	IVQNNDSTEY				
	IVQNNDSTE				
	IVQNND				
	LFQINN				
	LFQINN				
	FQINN				
	FQINN				
	IWCKDDQNP				
	IWCKDDQNP				
	WCKDDQNP				
	KDDQNP				
	DDQNP				
	DDQNP				
	DQNP				
	DQNP				
	FLDDDLTDD				
	FLDDDLTDD				
	FLDDDLT				
	FLDDDLT				
	LDDDLTDD				
	LDDDLTDD				
	LDDDLT				
	DLTDD				

Protein of origin	Peptide sequence ^a	Hydrolysate		SE-FPLC fraction	RP-HPLC fractions
		T	T-AC		
	LTDDIM				
	ILDKVGINYWL				
	ILDKVGINYW				
	ILDKVGINY				
	ILDKVGIN				
	ILDKVG				
	ILDK				
	LDKVGINYW				
	LDKVGINY				
	LDKVGIN				
	DKVGIN				
	KVGIN				
	INYWL				
	WLAHKAL				
	LDQWLCEKL				

^a Peptides in bold blue font were chemically synthesized and their effect on ACE and DPP-IV activities was tested.