

## **Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates**

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## Abstract

Selected synthetic dipeptides and milk protein hydrolysates were evaluated for their dipeptidyl peptidase IV (DPP-IV) inhibitory properties, and their superoxide (SO) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities. DPP-IV inhibition was seen with eight out of the twelve dipeptides and 5 of the twelve hydrolysates studied. Trp-Val inhibited DPP-IV, however, inhibition was not observed with the reverse peptide Val-Trp. The most potent hydrolysate inhibitors were generated from casein (CasH2) and lactoferrin (LFH1). Two Trp containing dipeptides, Trp-Val and Val-Trp, and three lactoferrin hydrolysates scavenged DPPH. The dipeptides had higher SO EC<sub>50</sub> values compared to the milk protein hydrolysates (arising from three lactoferrin and one whey protein hydrolysates). Higher molecular mass fractions of the milk protein hydrolysates were associated with the SO scavenging activity. Trp-Val and one lactoferrin hydrolysate (LFH1) were multifunctional displaying both DPP-IV inhibitory and antioxidant (SO and DPPH scavenging) activities. These compounds may have potential as dietary ingredients in the management of type 2 diabetes by virtue of their ability to scavenge reactive oxygen species and to extend the half-life of incretin molecules.

*Key words: dipeptidyl peptidase IV inhibitors, 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, antioxidant, tryptophan, bioactive peptides, lactoferrin*

# 1. Introduction

The worldwide incidence of type 2 diabetes is increasing. It was estimated in 2000 that there were 171 million diabetics while incidences for 2030 are estimated to reach 366 million people [45]. Different strategies have been developed to help in the management of type 2 diabetes; one of which is inhibition of dipeptidyl peptidase IV (DPP-IV). Incretins such as glucose dependent insulinotropic peptide (GIP) and more particularly glucagon-like peptide-1 (GLP-1) can enhance insulin secretion from pancreatic beta cells in the presence of glucose *in vivo* [2, 42]. However, GLP-1 and GIP can be degraded by DPP-IV, resulting in major losses in the bioactive properties of these incretins. DPP-IV is located at the surface of various cells and can also be found in a soluble form in the circulation [2]. An increase in circulating GLP-1 by a factor 4 to 6 has been reported *in vivo* following the utilization of DPP-IV drug inhibitors [27]. DPP-IV inhibitors are reported to protect GLP-1 and GIP from enzymatic degradation and therefore can increase their half-life, resulting in a prolonged action *in vivo* [2]. Peptide-like DPP-IV inhibitors such as vildagliptin and saxagliptin [17] have been shown to normalize blood glucose concentration in type 2 diabetic subjects [27]. However, contradictory results are also reported in the literature with some studies indicating no direct correlation between the insulinotropic properties of DPP-IV inhibitors and plasma GLP-1 levels. This suggests that other incretins and neuropeptides also play a role in the regulation of blood glucose homeostasis in the presence of DPP-IV inhibitors [27].

Peptide inhibitors of DPP-IV having Xaa-Pro, Pro-Xaa or Xaa-Ala (with Xaa an amino acid residue) sequences have been reported in the literature [46]. Furthermore, various studies have highlighted the possibility of using food proteins as natural sources of DPP-IV inhibitory peptides. Milk proteins, particularly, milk protein-derived peptides and amino acids have also been linked with the regulation of postprandial glycaemia and insulin secretion in normoglycaemic and type 2 diabetic subjects [6, 8, 23, 24, 26, 32]. Peptides derived from  $\beta$ -lactoglobulin have been shown to possess DPP-IV inhibitory activity *in vitro* [42, 43]. Furthermore, the DPP-IV inhibitory peptide Leu-Pro-Glu-Arg-Ile-Pro-Pro-Leu from Gouda-type cheese induced a significant reduction of blood glucose in rats following a glucose challenge [44]. Similarly, a DPP-IV inhibitory tryptic digest of  $\beta$ -lactoglobulin induced a decrease in blood glucose level in mice following an oral glucose tolerance test when administered orally at 300 mg/kg body weight [43]. More recently, an *in silico* approach has been utilized to identify potential dietary protein precursors of DPP-IV inhibitory peptides [18].

In type 2 diabetic subjects, the natural antioxidant protective systems including superoxide dismutase, glutathione (GSH) peroxidase and catalase may be compromised due to an overall increase in oxidative stress. It is well recognized that cardiovascular and renal disease can develop as secondary complications in subjects suffering from type 2 diabetes and insulin resistance syndrome [10, 12]. Different studies have demonstrated the anti-oxidative potential of milk proteins and peptides [3, 20, 31, 36, 38, 41]. Whey protein hydrolysates can help decrease oxidative stress by their radical scavenging activity and by their ability to increase the production of antioxidant enzymes *in vivo* [13, 21, 31]. Similarly, caseins and casein-derived peptides have been associated with radical-scavenging properties *in vitro* [1, 9, 15, 41] and with their ability to increase cellular catalase activity and GSH levels in human lymphocyte (Jurkat) cells [30]. More recently we have reported on two dipeptides, Val-Trp and Trp-Val, and different lactoferrin hydrolysates which can inhibit xanthine oxidase (XO) *in vitro* [28].

The aim of this study was to evaluate the potential of selected dipeptides for their antioxidant (SO and DPPH scavenging) activity and for their DPP-IV inhibitory properties. In addition, a range of milk protein hydrolysates was also evaluated for their antioxidant and DPP-IV inhibitory properties.

## 2. Material and methods

### 2.1. Reagents

The synthetic dipeptides Val-Trp, Trp-Val, Ala-Leu, Asp-Lys, Glu-Lys, Gly-Leu, Ser-Leu, Ser-Phe and Val-Ala were obtained from Bachem (Bubendorf, Switzerland) while Phe-Leu, His-Leu, Gly-Gln, Trp and Val were obtained from Sigma-Aldrich (Dublin, Ireland). Sodium phosphate monobasic, sodium phosphate dibasic, ethylenediamine tetraacetic acid (EDTA), Tris(hydroxymethyl)aminomethane (TRIS),  $\beta$ -nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue (NBT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox<sup>TM</sup>), Gly-Pro-pNA, diprotin A (Ile-Pro-Ile), trifluoroacetic acid (TFA), ethanol, porcine DPP-IV ( $\geq 10$  units/mg protein) and bovine superoxide dismutase (SOD  $\geq 3000$  units/mg) were obtained from Sigma Aldrich (Dublin, Ireland). Hydrochloric acid, sodium hydroxide, HPLC grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Whey proteins (WP), caseins (Cas), and lactoferrin (LF) were obtained from commercial suppliers.

### 2.2. Methods

### 2.2.1. Hydrolysis of milk protein substrates

Hydrolysis of the milk protein substrates was carried out with food-grade commercial gastrointestinal preparations essentially as described by Spellman *et al.* [39]. Five WP (WPH1 to WPH5), four Cas (CasH1 to CasH4) and three LF (LFH1 to LFH3) hydrolysates were manufactured. The starting milk protein substrates were suspended at 10 % (w/w) on a protein basis in water and dispersed under agitation at 50°C for 1 h using an overhead stirrer (Heidolph RZR 1, Germany) and hydrolysed at 50°C for 240 min. A control sample without enzyme was removed from the protein dispersion and maintained at 50°C for the duration of the hydrolysis reaction. The enzyme was inactivated by heating the hydrolysate samples at 90°C for 20 min. Hydrolysates were freeze-dried (FreeZone 18L, Labconco, Kansas City, U.S.A.) and stored at -20°C until further analysis.

### 2.2.3. Reversed-phase ultra-performance liquid chromatography of milk protein hydrolysates

The starting substrates and corresponding hydrolysates were analysed by liquid chromatography using an ultra-performance liquid chromatograph (UPLC Acquity - Waters, Dublin, Ireland) as described by Nongonierma and FitzGerald [28].

### 2.2.4. DPPH radical scavenging assay

The DPPH assay was used to determine the proton radical scavenging properties of the dipeptides and milk protein hydrolysates. Test samples (dipeptides and hydrolysates) were dispersed in HPLC grade water at concentrations ranging from  $1.25 \times 10^{-3}$  to  $1.25 \text{ mg.mL}^{-1}$ . The DPPH scavenging assay was carried out essentially according to Liu *et al.* [20]. The test samples (50  $\mu\text{L}$ ) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing a DPPH (final concentration 0.088 mM) solution in 50 % (v/v) ethanol. The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the DPPH radical was monitored at 517 nm. Each sample was analysed in triplicate. Trolox was used as a positive control. Scavenging of the DPPH radical was determined with respect to a control containing no scavenger (DPPH solution added with 50  $\mu\text{L}$  water) as described by Liu *et al.* [20]. The DPPH scavenging  $\text{EC}_{50}$  values (concentration of active compound required to observe 50 % DPPH scavenging) were determined by plotting the percentage of DPPH scavenging as a function of the concentration of test compound.

### 2.2.5. Superoxide radical scavenging assay

The test samples (dipeptides and hydrolysates) were dispersed in HPLC grade water at concentrations ranging from  $5.0 \times 10^{-3}$  to  $1.0 \text{ mg.mL}^{-1}$ . The superoxide (SO) scavenging assay was carried out following the method of Ewing and Janero [4]. Test samples (25  $\mu\text{L}$ ) were pipetted onto a 96 well microplate containing NADH (final concentration 78.4  $\mu\text{M}$ ) and NBT (final concentration 49.6  $\mu\text{M}$ ). All the reagents and samples were diluted in 0.1 mM EDTA in 50 mM phosphate buffer pH 7.4. The negative control contained the same reagents, except that the test sample was replaced by a solution of 0.1 mM EDTA in 50 mM phosphate buffer pH 7.4 (25  $\mu\text{L}$ ). The reaction was initiated by adding 25  $\mu\text{L}$  of PMS (final concentration 3.3  $\mu\text{M}$ ). Superoxide dismutase (SOD) was used as a positive control for radical scavenging. Each sample was analysed in triplicate. The microplate was incubated at 25°C for 60 min in a microplate reader; absorbance of the reduced NBT was monitored at 560 nm. The SO scavenging  $\text{EC}_{50}$  values were determined by plotting the percentage SO scavenged as a function of the concentration of test compound.

#### 2.2.6. DPP-IV inhibition assay

The test samples (dipeptides and hydrolysates) were dispersed in HPLC grade water at concentrations ranging from  $12.5 \times 10^{-3}$  to  $6.3 \text{ mg.mL}^{-1}$ . The DPP-IV inhibition assay was carried out as described by Lacroix and Li-Chan [17]. Test samples (50  $\mu\text{L}$ ) were pipetted onto a 96 well microplate containing Gly-Pro-pNA, the reaction substrate (final concentration 0.2 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0 (50  $\mu\text{L}$ ) and the reaction substrate Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 units/mL). All the reagents and samples were diluted in 100 mM Tris-HCl buffer pH 8.0. Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate was incubated at 37°C for 60 min in a microplate reader, absorbance of the released pNA was monitored at 405 nm. The DPP-IV  $\text{IC}_{50}$  values (concentration of active compound required to observe 50 % DPP-IV inhibition) were determined by plotting the percentage of inhibition as a function of the concentration of test compound. The mode of inhibition of the different compounds was investigated using Lineweaver and Burk analysis by measuring the initial rate of the reaction at different Gly-Pro-pNA concentrations ranging between 0.2 and 0.6 mM without inhibitors and in the presence of peptides or hydrolysates at their  $\text{IC}_{50}$  concentrations.  $K_m$  and  $V_{max}$  values were deduced from the Lineweaver and Burk double reciprocal plots.

#### 2.2.7. Statistical analysis

Means comparison was carried out with a one way ANOVA followed by a Student Newman-

Keuls test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level  $P < 0.05$ . For the Lineweaver and Burk plots, experimental data was fitted by linear regression using SPSS.

### 3. Results

#### 3.1. Superoxide scavenging activity of dipeptides and milk protein hydrolysates

Of the twelve dipeptides studied, scavenging activity for superoxide radicals was seen with nine dipeptides (Table 1). Three dipeptides (Gly-Gln, Asp-Lys and Ser-Phe) did not scavenge SO radicals. A wide range of  $EC_{50}$  values were found depending on dipeptide sequence with the highest values obtained with Val-Ala and His-Leu ( $> 370$  mM) and the lowest values with Ala-Leu. Twelve different milk protein hydrolysates were evaluated for their ability to scavenge superoxide radicals (Table 1). Scavenging activity was found with 4 of the hydrolysates studied. Three of the hydrolysates were generated from LF and one from WP. The  $EC_{50}$  values ranged from  $0.10 \pm 0.01$  to  $0.41 \pm 0.01$  mg.mL<sup>-1</sup>. The  $EC_{50}$  values obtained for the four milk protein hydrolysates were lower (on a weight basis) than that of the dipeptides studied (Table 1). These results indicate that the hydrolysates were more potent, at least on a unit weight basis, than the dipeptides for SO scavenging. This may be due to the fact that a certain peptide chain length is required to observe scavenging activity. This was further confirmed by the  $EC_{50}$  values obtained for ultrafiltered fractions of WPH1 where the 5 kDa permeate and retentate had an  $EC_{50}$  of  $0.37 \pm 0.01$  and  $0.14 \pm 0.01$  mg. mL<sup>-1</sup>, respectively (Table 1).

#### 3.2. DPPH scavenging activity of dipeptides and milk protein hydrolysates

With the exception of Val-Trp and its reverse peptide Trp-Val, the different dipeptides studied showed no activity for scavenging of DPPH radicals (Table 2). Differences were seen for the  $EC_{50}$  of both peptides with Trp-Val ( $242.0 \pm 0.1$   $\mu$ M) being significantly more potent than Val-Trp ( $654.2 \pm 21.0$   $\mu$ M). The amino acid constituents of these dipeptides were evaluated for their DPPH scavenging activity. Scavenging of DPPH with Val was relatively low ( $4.9 \pm 1.8$  % scavenged at  $0.06$  mg.mL<sup>-1</sup>) whereas Trp ( $47.3 \pm 3.8$  % scavenged at  $0.06$  mg.mL<sup>-1</sup>) behaved as a good scavenger for DPPH having an  $EC_{50}$  of  $358.5 \pm 0.3$   $\mu$ M (Table 2). All 12 hydrolysates studied were able to scavenge DPPH radicals. However, this antioxidant activity was not higher than that of the unhydrolysed milk protein (negative control) with the exception of LF (data not shown). All three LF hydrolysates studied displayed a scavenging activity for DPPH radicals. The  $EC_{50}$  values determined for the LF hydrolysates were 5 to 10 times higher than that of Trp, Val-Trp and Trp-Val (Table 2).

### 3.3. DPP-IV inhibition of dipeptides and milk protein hydrolysates

DPP-IV inhibition was seen with 8 of the dipeptides studied (Table 3) of which 5 could be released from milk according to *in silico* analysis using the Peptide Cutter Program [28]. The DPP-IV IC<sub>50</sub> values for the dipeptides ranged from  $65.69 \pm 2.95$  to  $3216.73 \pm 2.12$   $\mu\text{M}$  for Val-Trp and Gly-Leu, respectively (Table 3). The IC<sub>50</sub> value for Diprotin A ( $8.49 \pm 0.15$   $\mu\text{M}$ ) was about 15 times less than that of the most potent dipeptide studied (Trp-Val). It is interesting to note that the reverse peptide Val-Trp did not inhibit DPP-IV. Five of the milk protein hydrolysates studied were able to inhibit DPP-IV. The most potent inhibitors were LFH1 and CasH2 (Table 3), their comparative inhibitory activity was not significantly different ( $P \geq 0.05$ ). The dose-response curves obtained for three dipeptides (Trp-Val, His-Leu and Val-Ala) and three hydrolysates (CasH1, WPH2 and LFH1) are illustrated on Fig. 1. The IC<sub>50</sub> values ( $< 0.66$   $\text{mg}\cdot\text{mL}^{-1}$ ) obtained with the dipeptides were always lower than that of the milk protein hydrolysates studied, with IC<sub>50</sub> values for the hydrolysates being between 1.4 to 40 times higher than that of the dipeptides. The RP-UPLC profiles of hydrolysates manufactured from 3 different starting substrates (Cas, WP and LF) are illustrated in Fig. 2. As expected, major peptide compositional differences were seen between these hydrolysates (CasH1, WPH2 and LFH1) due to different peptide sequences being released from these substrates. However, apart from these differences, all three hydrolysates displayed similar DPP-IV IC<sub>50</sub> values ( $P \geq 0.05$ ). Lineweaver and Burk plots allowed study of the mode of inhibition of the different dipeptides and hydrolysates. The double reciprocal plots for CasH1, WPH2 and LFH1 are illustrated on Fig. 3. For all hydrolysates, there was no significant difference in V<sub>max</sub> ( $P \geq 0.05$ ) in the presence and absence of inhibitor. However, K<sub>m</sub> values were significantly different ( $P < 0.05$ ) in the presence and absence of inhibitor. These results indicate that the different hydrolysates studied behaved as competitive inhibitors of DPP-IV. Similarly, the dipeptides behaved as competitive inhibitors of DPP-IV with the exception of Trp-Val which behaved as a non-competitive type inhibitor (Table 3).

## 4. Discussion

Power *et al.* [33] recently reviewed the antioxidant properties of peptides with a particular focus on milk-derived peptides and hydrolysates. Since reactive oxidative species (ROS) are found both in the intracellular and extracellular compartments within the human body, it is conceivable that different antioxidative peptide structures may function in these compartments. Low molecular mass peptides, such as carnosine (Ala-His) and GSH (Cys-Glu-Gly), have been shown to act as antioxidants within cells whereas larger molecular mass peptides (which may be present within



hydrolysates) can display their antioxidative activity in the plasma [33]. The antioxidant activity of selected dipeptides and milk protein hydrolysates was investigated. For SO scavenging activity, previous results have reported SO EC<sub>50</sub> values for dipeptides of the same order as those found herein. Suetsuna and Chen [40] reported SO EC<sub>50</sub> values ranging from 2.5 to 11.9 mM for lysine containing dipeptides. The results herein highlight differences in scavenging activities of dipeptides depending on dipeptide structure, with Ala-Leu (8.60 ± 0.01 mM) being the most potent and Val-Ala or His-Leu (EC<sub>50</sub> > 370 mM) the least potent. Dipeptides containing a Leu residue appeared to have relatively low EC<sub>50</sub> values (< 25 mM) with the exception of His-Leu (EC<sub>50</sub> > 370 mM, Table 1). A potent Leu containing casein-derived dipeptide, Glu-Leu, has previously been described for its high SO scavenging activity [41]. Aromatic amino acids have been reported to display high antioxidant activity as the radicals formed with phenol and indole amino acids are relatively stable due to their potential to act as hydrogen donors [3, 16, 25, 31, 35, 36]. More specifically, Komagoe *et al.* [16] showed that Tyr and Trp were potent scavengers of SO radicals (EC<sub>50</sub> values of 0.16 mM). However, our results show that within the 12 dipeptides studied, Trp, His and Phe containing dipeptides do not have a high SO scavenging activity. It has been shown that fermented milk such as kefir is able to scavenge SO radicals [20]. In addition, peptic hydrolysates of casein and casein derived-peptides have been identified for their SO scavenging activity [41]. A potent scavenger of SO radicals has been isolated from a peptic digest of casein with the peptide structure Tyr-Pro-Tyr-Pro-Glu-Leu [41]. Removal of the amino acids at the N terminal position (Tyr, Tyr-Pro or Tyr-Pro-Tyr residues) of this peptide resulted in a reduction of the SO scavenging activity. However, bioactivity was restored with the dipeptide Glu-Leu [41]. In comparison with dipeptides, milk protein hydrolysates exhibited higher SO scavenging activities on a weight basis, showing lower EC<sub>50</sub> values than the dipeptides (Table 1). More potent SO scavenger peptides than the ones studied may be present within these milk protein hydrolysates. Upon fractionation of a peptic casein hydrolysate, three fractions with a wide range of EC<sub>50</sub> values from less than 0.1 to greater than 10 mg.mL<sup>-1</sup> have been identified [41]. This suggests that potent SO scavengers can be found within milk protein hydrolysates. In addition enrichment of bioactive peptides following fractionation could result in an increased potency for SO scavenging.

Various milk derived peptides have previously been reported for their DPPH radical scavenging activity [31, 34]. Within the 12 dipeptides studied herein only Val-Trp and Trp-Val were able to scavenge DPPH radicals. Different EC<sub>50</sub> values were found for these dipeptides with Trp-Val being twice as potent as the reverse peptide Val-Trp. EC<sub>50</sub> values for DPPH scavenging by decapeptides extracted from venison protein were around 10 µM [14] and values of 98 µM for

the casein derived peptide Tyr-Pro-Tyr-Pro-Glu-Leu and of 23.3  $\mu\text{M}$  for carnosine (Ala-His) [41] have been reported. Higher DPPH  $\text{EC}_{50}$  values were found with the dipeptides Val-Trp and Trp-Val herein. However, these activities were 20-40 times less potent than Trolox. To the best of our knowledge, this is the first time that these dipeptides have been described for their DPPH radical scavenging activity. It has previously been reported that the antioxidant activity of dipeptides depends on the C or N terminal position of certain amino acid residues with stronger antioxidant activity associated with Trp-Lys compared to Lys-Trp [40]. Similarly, in this study, the dipeptide with Trp at the N terminus (Trp-Val) was the most potent DPPH scavenger. Previous studies reported differences in other bioactivities for these two dipeptides. Val-Trp, a non-competitive angiotensin converting enzyme (ACE) inhibitor, had an ACE inhibition  $\text{IC}_{50}$  value 200 times less than Trp-Val, a competitive inhibitor of ACE [29]. Free Trp also showed scavenging properties for DPPH, however, it was less potent than the dipeptide Trp-Val (Table 2). The lower antioxidant activity observed for amino acids compared to peptides has been explained by their lower radical scavenging activity [3]. *In silico* analysis showed that theoretically, Val-Trp can be released from LF following digestion with gastrointestinal activities [28]. In agreement with these findings, the three LF hydrolysates evaluated showed scavenging activity for DPPH radicals.

Eight out of the twelve dipeptides studied herein were shown to be DPP-IV inhibitors (Table 3). To the best of our knowledge, some of the dipeptides described herein have not previously been reported as DPP-IV inhibitors. These include Trp-Val, Ala-Leu, Glu-Lys, Gly-Leu, Ser-Leu, Phe-Leu and His-Leu. The dipeptide Val-Ala has been identified in previous studies as an inhibitor for DPP-IV. This dipeptide occurs in different milk proteins including  $\alpha$ -,  $\beta$ - and  $\kappa$ -casein,  $\beta$ -lactoglobulin and LF [18]. The  $\text{IC}_{50}$  values found for the dipeptides studied herein are of the same order as values reported in the literature for other DPP-IV inhibitory peptides. For example, an  $\text{IC}_{50}$  of 46  $\mu\text{M}$  was found for Leu-Pro-Glu-Arg-Ile-Pro-Pro-Leu and other  $\beta$ -casein peptides had an  $\text{IC}_{50}$  value between 110 and 1500  $\mu\text{M}$  [44]. The most potent DPP-IV inhibitory peptide studied herein was Trp-Val (Table 3). Surprisingly, the reverse peptide Val-Trp did not inhibit DPP-IV. Similar findings as those for Val-Trp and its reverse peptide have been mentioned in another study, with Ile-Pro ( $\text{IC}_{50}$   $0.41 \pm 0.07$   $\mu\text{M}$ ) inhibiting DPP-IV whereas its reverse peptide Pro-Ile did not [11]. This result was explained by the fact the N terminal residue of the peptide plays an important role in the inhibition of DPP-IV [11]. Different milk protein hydrolysates have been reported for their DPP-IV inhibitory potential including hydrolysates of  $\beta$ -lactoglobulin [17, 43]. Of 22 milk protein hydrolysates screened for their DPP-IV inhibitory potential, Lacroix and Li-Chan [17] found that casein hydrolysates were generally more potent than WP hydrolysates with

the exception of a peptic whey protein hydrolysate. Out of 5 hydrolysates with DPP-IV inhibition herein, 2 were generated from Cas and WP and one from LF. The most potent hydrolysates in this study were LFH1 and CasH2 (Table 3). The higher potency of Cas hydrolysates has been linked to the fact that  $\beta$ -casein is the milk protein substrate displaying the highest number of DPP-IV inhibitory peptide sequences (which is defined as the number of peptide sequences with DPP-IV inhibitory activity divided by the length of the protein) [18]. The DPP-IV  $IC_{50}$  values observed for the milk protein hydrolysates described herein are of the same order as values reported in the literature.  $IC_{50}$  values ranging from 0.075 to  $6.4 \pm 2.3 \text{ mg}\cdot\text{mL}^{-1}$  have previously been reported for various food protein-derived hydrolysates [11, 17, 19]. It is interesting to note that the milk protein hydrolysates were less potent on a weight basis than the dipeptides (Table 3). This may be explained by the fact that the bioactive peptides within the milk protein hydrolysates may be present at relatively low concentrations [17]. As expected, the RP-UPLC (Fig. 2) profiles of the hydrolysates studied differed, which indicates that the peptides involved in the bioactive properties determined within this study were different. All hydrolysates studied herein were competitive inhibitors of DPP-IV. Competitive inhibition suggested a direct interaction between the hydrolysates and DPP-IV. It has been suggested that some milk-derived peptides may also behave as DPP-IV substrates, resulting in a direct interaction of the peptide with the active site of the enzyme [17]. Similarly, most of the dipeptides were competitive DPP-IV inhibitors with the exception of Trp-Val which was non-competitive. Non-competitive inhibition of DPP-IV with peptides derived from the human immunodeficiency virus-1 (HIV) transactivator Tat has been reported [22]. The non-competitive inhibition was due to peptides binding to a secondary site different from the active site of DPP-IV.

Trp-Val and the LF hydrolysate were both able to scavenge DPPH and SO radicals, and were also able to inhibit DPP-IV. To the best of our knowledge, this is the first time that an LF hydrolysate has been reported for its DPP-IV inhibitory activity. Trp-Val and LF hydrolysates have recently been described for their XO inhibitory properties [28]. This result is of interest as Trp-Val and LFH1 may act as multifunctional components which could modulate different targets beneficial, for example, in the management of type 2 diabetes. Intestinal transport of intact peptides and even proteins is reported to occur *in vivo* [7]. Hydrophobic short peptide sequences from milk such as Leu-Trp, Phe-Tyr and Ile-Tyr, Ile-Pro-Pro could reach the circulation without being degraded in the gastrointestinal tract [5]. Furthermore, dipeptides with hydrophobic characteristics such as Trp-Val may be bioavailable especially since hydrophobic peptides have been associated with a high trans-epithelial permeation through a caco-2 cell monolayer [37]. Further studies are needed to validate that the *in vitro* bioactive properties described herein pertain *in vivo*.

## Conclusion

Protein hydrolysates and dipeptides which can be released by the action of food-grade gastrointestinal enzyme preparations on milk proteins behave as scavengers of DPPH and superoxide radicals. In addition, eight dipeptides were shown to inhibit DPP-IV of which five could in theory be released by the action of gastro-intestinal enzyme activities on milk proteins. An LF-derived hydrolysate (LFH1) and Trp-Val may act as multifunctional agents in the management of type 2 diabetes. Both components reduce oxidative species (SO and DPPH scavenging properties) and inhibit DPP-IV activity *in vitro* in a dose-dependent manner. Identification of the peptides within the milk protein hydrolysates presented herein would help to better understand the origin of the DPP-IV inhibition properties of these food-grade hydrolysates. Future *in vivo* studies on these components are required to validate the *in vitro* data and to confirm the bioavailability and bioactivity of these milk protein hydrolysates and dipeptides in humans.

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# Conflicts of interests

The authors declare that they have no conflict of interest.

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## Table captions

**Table 1** Inhibitory concentration inducing 50 % scavenging ( $EC_{50}$ ) for superoxide (SO) radicals with milk derived dipeptides, lactoferrin (LF) and whey protein (WP) hydrolysates.

**Table 2** Inhibitory concentration inducing 50 % scavenging ( $EC_{50}$ ) for 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals with Trp, Trp-Val, Val-Trp and lactoferrin (LF) hydrolysates

**Table 3** Inhibitory concentration inducing 50 % inhibition ( $IC_{50}$ ) for dipeptidyl peptidase IV (DPP-IV) in the presence of dipeptides and milk protein hydrolysates. LFH: lactoferrin hydrolysate, CasH: casein hydrolysate and WPH: whey protein hydrolysate.

**Table 1**

| <b>Compound</b>      | <b>SO EC<sub>50</sub> (mM)<sup>*</sup></b> | <b>SO EC<sub>50</sub> (mg.mL<sup>-1</sup>)<sup>*</sup></b> |
|----------------------|--|--|
| Glu-Lys              | 45.16 ± 0.02 <sup>d</sup>                  | 9.18 ± 0.01 <sup>g</sup>                                   |
| Gly-Leu              | 20.73 ± 0.01 <sup>c</sup>                  | 3.90 ± 0.01 <sup>f</sup>                                   |
| Ala-Leu              | 8.60 ± 0.01 <sup>a</sup>                   | 1.74 ± 0.01 <sup>e</sup>                                   |
| Val-Ala              | > 370 <sup>g</sup>                         | 72.71 ± 0.01 <sup>i</sup>                                  |
| Trp-Val              | 131.02 ± 0.01 <sup>e</sup>                 | 39.75 ± 0.01 <sup>h</sup>                                  |
| Val-Trp              | 114.52 ± 0.01 <sup>e</sup>                 | 34.75 ± 0.01 <sup>h</sup>                                  |
| Ser-Phe              | 163.54 ± 0.01 <sup>f</sup>                 | 41.26 ± 0.01 <sup>h</sup>                                  |
| Phe-Leu              | 13.81 ± 0.01 <sup>b</sup>                  | 3.84 ± 0.01 <sup>f</sup>                                   |
| His-Leu              | > 370 <sup>g</sup>                         | > 100 <sup>j</sup>   |
| Ser-Leu              | 24.92 ± 0.01 <sup>c</sup>                  | 5.44 ± 0.01 <sup>f</sup>                                   |
| LFH1                 | na   | 0.10 ± 0.01 <sup>a</sup>                                   |
| LFH2                 | na   | 0.41 ± 0.01 <sup>d</sup>                                   |
| LFH3                 | na   | 0.19 ± 0.01 <sup>b</sup>                                   |
| WPH1                 | na   | 0.28 ± 0.01 <sup>c</sup>                                   |
| WPH1-5 kDa Retentate | na   | 0.14 ± 0.01 <sup>a</sup>                                   |
| WPH1-5kDa Permeate   | na   | 0.37 ± 0.01 <sup>c</sup>                                   |
| SO <sup>†</sup>      |  | 100.78 ± 1.29  |

\*Values represent mean EC<sub>50</sub> values ± confidence interval ( $P = 0.05$ ) for quadruplicate determination. Within the same column, figures with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>†</sup>EC<sub>50</sub> expressed in units/mL

na: not applicable

**Table 2**

| Compound | DPPH EC <sub>50</sub> (μM)* | DPPH EC <sub>50</sub> (mg.mL <sup>-1</sup> )* |
|----------|-----------------------------|---|
| Trp-Val  | 242.0 ± 0.1 <sup>b</sup>    | 0.07 ± 0.01 <sup>b</sup>                      |
| Val-Trp  | 654.2 ± 21.0 <sup>d</sup>   | 0.20 ± 0.01 <sup>c</sup>                      |
| Trp      | 358.5 ± 0.3 <sup>c</sup>    | 0.07 ± 0.01 <sup>b</sup>                      |
| LFH1     | na                          | 1.15 ± 0.40 <sup>d</sup>                      |
| LFH2     | na                          | 1.33 ± 0.01 <sup>d</sup>                      |
| LFH3     | na                          | 1.37 ± 0.01 <sup>d</sup>                      |
| Trolox   | 16.5 ± 2.1 <sup>a</sup>     | 0.004 ± 0.001 <sup>a</sup>                    |

\*Values represent mean EC<sub>50</sub> values ± confidence interval ( $P = 0.05$ ) for quadruplicate determination. Within the same column, figures with different superscript letters are significantly different ( $P < 0.05$ ).

na: not applicable

**Table 3**

| <b>Compound</b> | <b>DPP-IV IC<sub>50</sub> (μM)*</b> | <b>DPP-IV IC<sub>50</sub> (mg.mL<sup>-1</sup>)*</b> | <b>Type of inhibition<sup>#</sup></b> |
|-----------------|-------------------------------------|---|---------------------------------------|
| Glu-Lys         | 3216.73 ± 2.12 <sup>h</sup>         | 0.654 ± 0.001 <sup>f</sup>                          | competitive                           |
| Gly-Leu         | 2615.03 ± 612.80 <sup>g,h</sup>     | 0.492 ± 0.115 <sup>f</sup>                          | competitive                           |
| Ala-Leu         | 882.13 ± 68.66 <sup>f</sup>         | 0.178 ± 0.014 <sup>e</sup>                          | competitive                           |
| Val-Ala         | 168.24 ± 7.96 <sup>d</sup>          | 0.032 ± 0.001 <sup>b</sup>                          | competitive                           |
| Trp-Val         | 65.69 ± 2.95 <sup>b</sup>           | 0.020 ± 0.001 <sup>b</sup>                          | non-competitive                       |
| Phe-Leu         | 399.58 ± 10.81 <sup>e</sup>         | 0.111 ± 0.003 <sup>d</sup>                          | competitive                           |
| His-Leu         | 143.19 ± 0.35 <sup>c</sup>          | 0.038 ± 0.001 <sup>c</sup>                          | competitive                           |
| Ser-Leu         | 2517.08 ± 36.33 <sup>g</sup>        | 0.549 ± 0.008 <sup>g</sup>                          | competitive                           |
| LFH1            | na                                  | 1.088 ± 0.106 <sup>h,i</sup>                        | competitive                           |
| CasH1           | na                                  | 1.105 ± 0.072 <sup>h</sup>                          | competitive                           |
| CasH2           | na                                  | 0.882 ± 0.057 <sup>i</sup>                          | competitive                           |
| WPH1            | na                                  | 1.430 ± 0.272 <sup>h</sup>                          | competitive                           |
| WPH2            | na                                  | 0.999 ± 0.077 <sup>h</sup>                          | competitive                           |
| Diprotin A      | 4.23 ± 0.08 <sup>a</sup>            | 0.0015 ± 0.0001 <sup>a</sup>                        | competitive                           |

\*Values represent mean IC<sub>50</sub> values ± confidence interval ( $P = 0.05$ ) for quadruplicate determination. Within the same column, figures with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>#</sup>Type of inhibition determined using Lineweaver and Burk plots for the dipeptides [28]  
na: not applicable

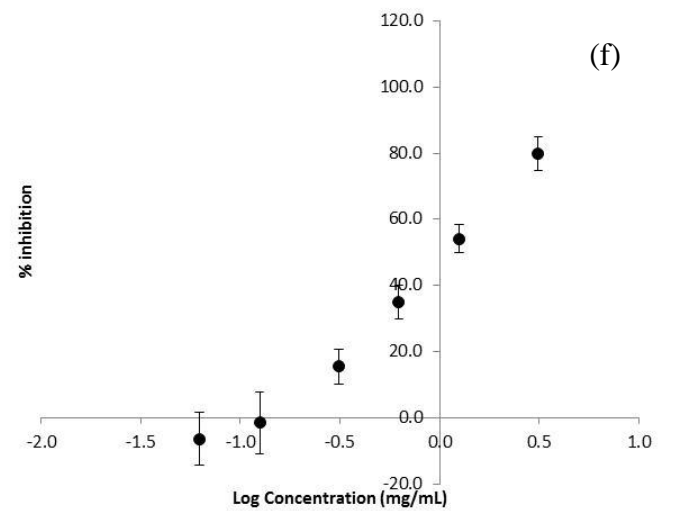
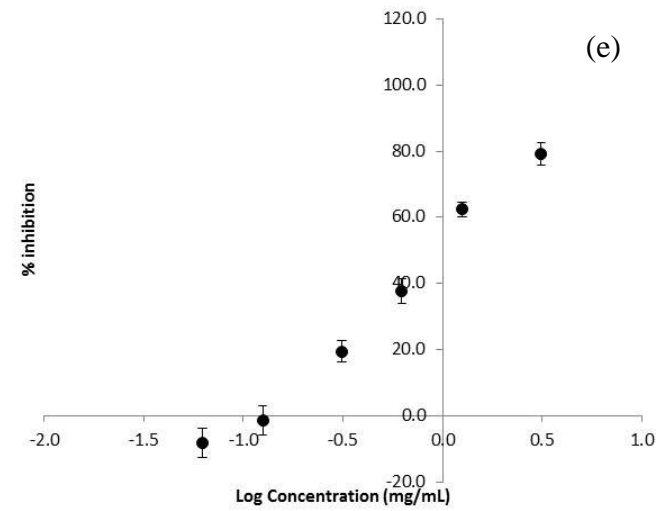
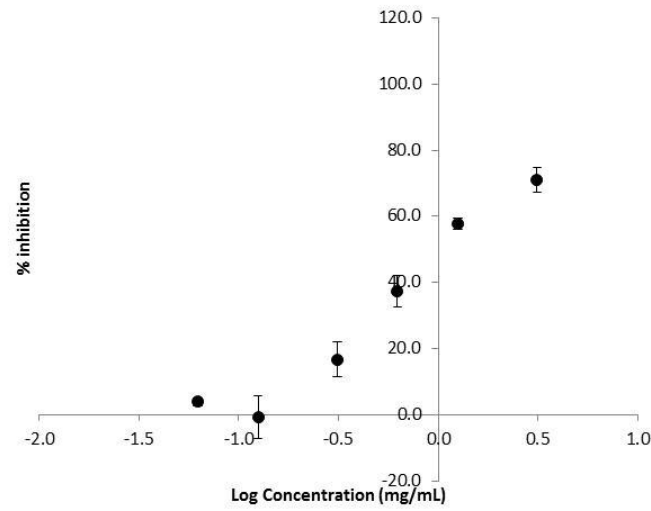
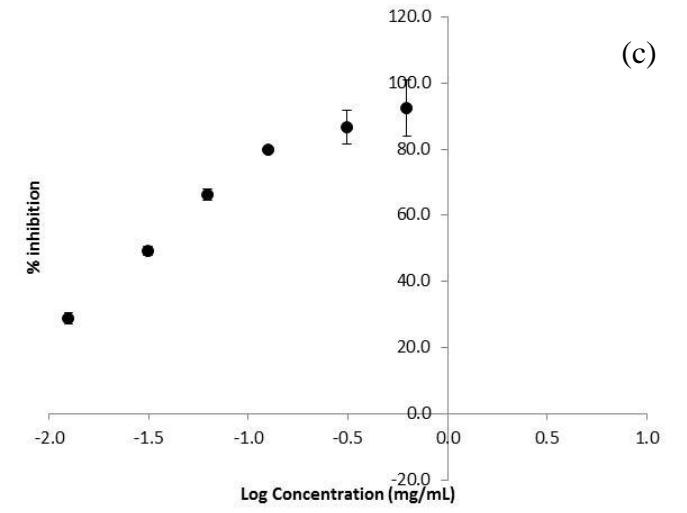
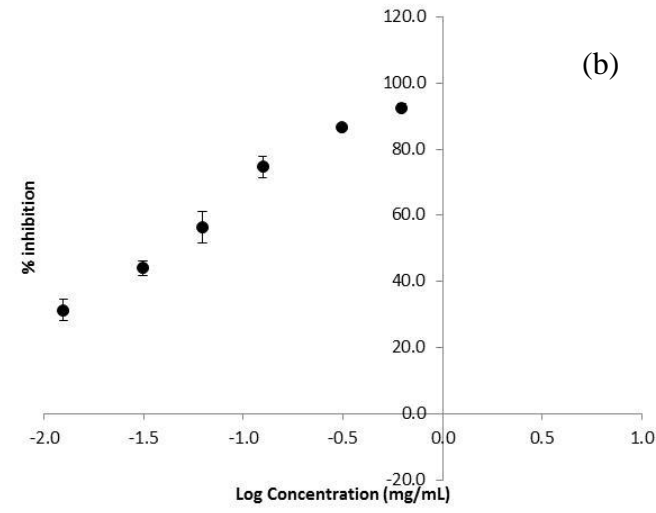
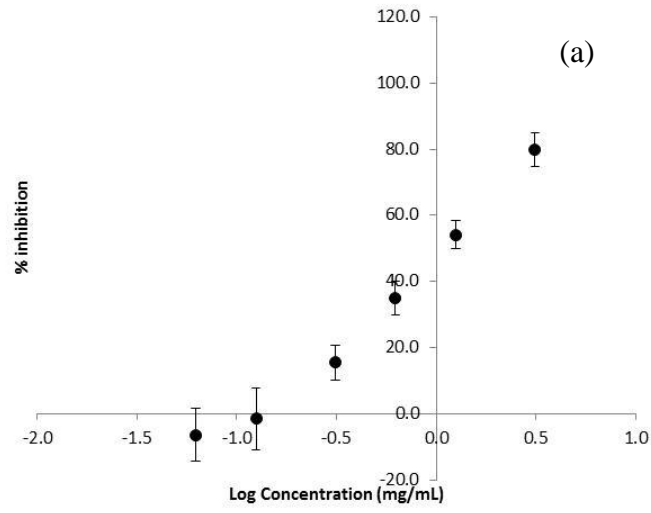


## Figure captions

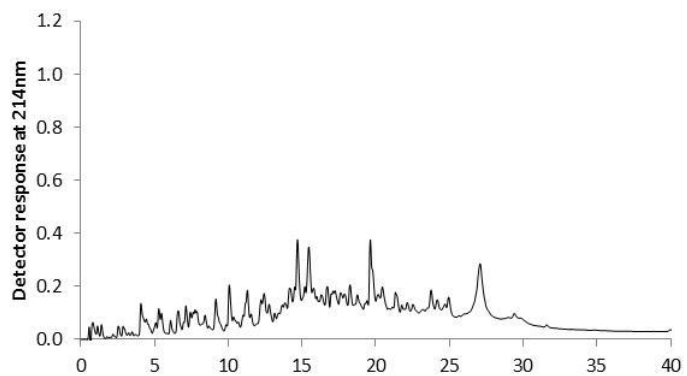
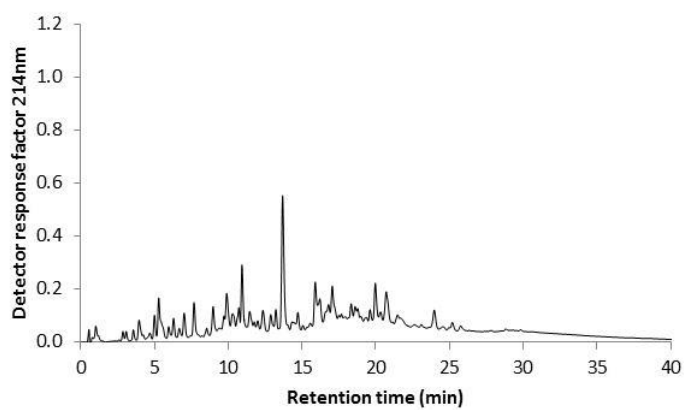
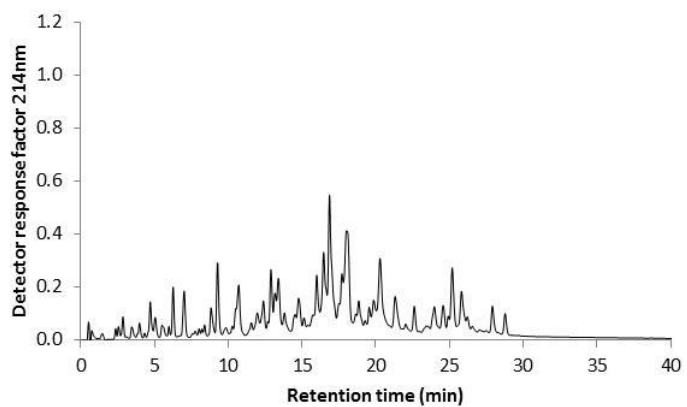
Fig. 1 Dose response curve for dipeptidyl peptidase IV (DDP-IV) inhibition with dipeptides (a ) Trp-Val (b) His-Leu, (c) Val-Ala and milk protein hydrolysates (d) CasH1, (e) WPH2 and (f) LFH1. CasH: casein hydrolysate, WPH: whey protein hydrolysate and LFH: lactoferrin hydrolysate. Values are the average of three determination (n=3)  $\pm$  SD.

Fig. 2 Reversed-phase ultra-performance liquid chromatography of milk protein hydrolysates with dipeptidyl peptidase IV (DDP-IV) inhibitory properties. (a) CasH1, (b) WPH2 and (c) LFH1. CasH: casein hydrolysate, WPH: whey protein hydrolysate and LFH: lactoferrin hydrolysate.

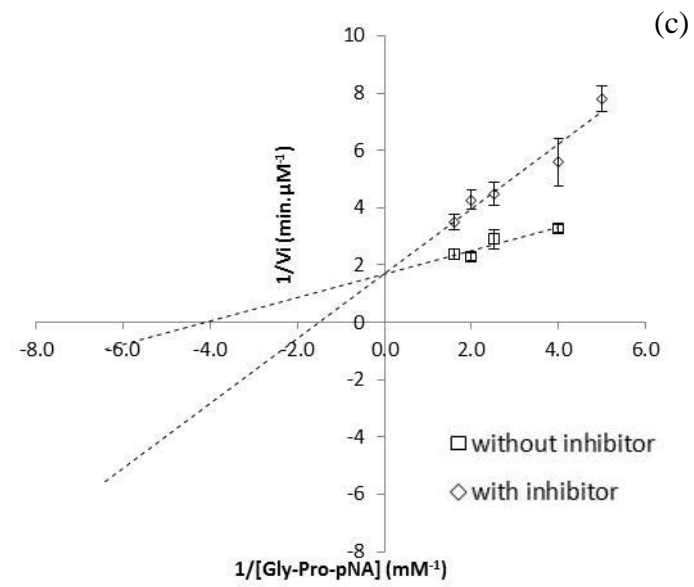
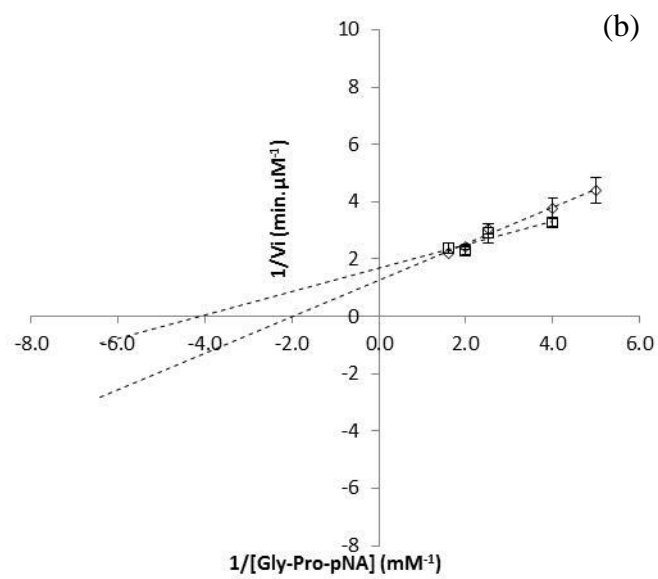
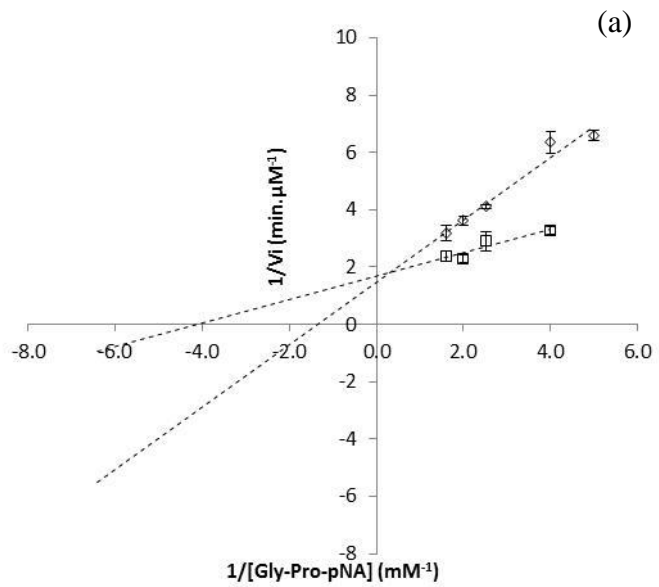
Fig. 3 Lineweaver and Burk plots for dipeptidyl peptidase IV (DDP-IV) inhibition with milk protein hydrolysates (a) CasH1, (b) WPH2 and (c) LFH1. CasH: casein hydrolysate, WPH: whey protein hydrolysate and LFH: lactoferrin hydrolysate. Each data point is the average of 6 values  $\pm$  SD.



**Fig 1.**



**Fig. 2.**



**Fig 3.**