DOI: 10.1016/j.jff.2013.09.012

Inhibition of dipeptidyl peptidase IV (DPP-IV) by proline containing casein-derived peptides

Alice B. Nongonierma & Richard J. FitzGerald*

Department of Life Sciences and Food for Health Ireland (FHI), University of Limerick, Limerick, Ireland.

Please cite as:

Nongonierma, A. B. & FitzGerald, R. J. (2013). Inhibition of dipeptidyl peptidase IV (DPP-IV) by proline containing casein-derived peptides. Journal of Functional Foods, 4(5), 1909-1917.

*Corresponding author: dick.fitzgerald@ul.ie
Tel: +353 (0) 61 202598
Fax: + 353 (0) 61 331490

Abstract

Dipeptides with a C terminal Pro inhibit dipeptidyl peptidase IV (DPP-IV), a key enzyme in incretin hormone processing. It was hypothesised that tri- and tetrapeptides with a proline at the C-terminus may also be DPP-IV inhibitors. Therefore, an *in silico* hydrolysis approach was used to release short (4 ≤ amino acids) C terminal Pro peptides from the individual caseins which constitute Pro rich substrates. This was achieved using theoretical digestion of caseins with a prolyl oligopeptidase activity. Fifteen peptides were subsequently selected for *in vitro* DPP-IV inhibitory analysis. Stability of these peptides to gastrointestinal enzymes was also evaluated *in silico* and the predicted breakdown peptides were assessed for their DPP-IV inhibitory and antioxidant potential. New DPP-IV inhibitors were identified, the most potent being Phe-Leu-Gln-Pro (IC_{50} 65.3 ± 3.5 μM). A low *in vitro* antioxidant (2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging) activity was also associated with the peptides studied. The strategy presented highlights the utility of employing an *in silico* approach for the prediction of food-derived peptides with a potential role in glycaemic management for subsequent development of functional foods.

Key words: dipeptidyl peptidase IV inhibitors, antioxidant, bioactive peptides, milk, proline

1. Introduction

Inhibition of dipeptidyl peptidase IV (DPP-IV) has been exploited as a new therapeutic strategy in the treatment of Type 2 diabetes (T2D) (Drucker, 2006b; Nauck & El-Ouaghlidi, 2005). DPP-IV can cleave incretins such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). These hormones enhance insulin secretion from pancreatic beta cells in response to different nutrients (Drucker, 2006a). Different drugs, known as gliptins, are currently being used as DPP-IV inhibitors for the treatment of T2D (Lacroix & Li-Chan, 2014; Scheen, 2012). It has also been demonstrated that various food protein-derived peptides, including animal and plant-derived peptides, have DPP-IV inhibitory properties (Harnedy & FitzGerald, 2013; Li-Chan, Hunag, Jao, Ho, & Hsu, 2012; Velarde-Salcedo *et al.*, 2013). An *in silico* approach demonstrated that various DPP-IV inhibitory peptides may be found within dietary proteins, including milk proteins (Lacroix & Li-Chan, 2012b). Several studies have demonstrated that enzymatic hydrolysates of milk proteins were a good source of DPP-IV inhibitory peptides (Lacroix & Li-Chan, 2013; Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013a; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013; Tulipano, Sibilia, Caroli, & Cocchi, 2011; Uchida, Ohshiba, & Mogami, 2011; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). Recently, interactions between sitagliptin, a DPP-IV inhibitory drug, and milk-derived peptides were studied, showing an additive effect between sitagliptin and the milk-derived peptides for DPP-IV inhibition *in vitro* (Nongonierma & FitzGerald, 2013b). These studies indicate that food-derived peptides may play an important role in glycaemic regulation.

Peptide inhibitors of DPP-IV with various amino acid sequences have been reported in the literature (Lacroix & Li-Chan, 2012b). It has been shown that dipeptides with the sequence Xaa-Pro (with Xaa representing an amino acid) can act as DPP-IV inhibitors. Different dipeptide sequences with a Pro residue at the C terminus have previously been identified as DPP-IV

inhibitors (Hatanaka *et al.*, 2012). The casein-derived peptide, Leu-Pro-Gln-Asn-Ile-Pro-Pro (f70-76), was found to be a DPP-IV inhibitor with an IC_{50} value of 160 µM (Uenishi *et al.*, 2012). In addition, various peptide sequences without Pro residues have also been reported as potent DPP-IV inhibitors (Lacroix & Li-Chan, 2012b; Nongonierma & FitzGerald, 2013a). However, there appears to be a limited amount of data available in the literature describing the role of short (4 ≤ amino acids) casein-derived peptides having C terminal Pro residues on DPP-IV inhibition.

The increased oxidative stress associated with T2D may potentiate the development of secondary complications such as cardiovascular and renal disease (Hayden & Tyagi, 2001). It has been demonstrated that caseins and casein-derived peptides can scavenge free radicals *in vitro* (Irshad, Kanekanian, Peters, & Masud, 2013; Kitts, 2005; Suetsuna, Ukeda, & Ochi, 2000) and increase cellular catalase activity and glutathione levels in human lymphocyte (Jurkat) cells (Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, & O'Brien, 2009). Much interest has focused on the role of dietary antioxidants on health. To date, there appears to be a lack of consensus on the role of these compounds *in vivo* (Chang & Chuang, 2010; Power, Jakeman, & FitzGerald, 2013). However, given the potential of bioactive peptides to display multifunctional activities, we also investigated the *in vitro* antioxidant activity of the synthetic peptides.

The aim of this study was to evaluate the ability of selected short (4 ≤ amino acids) C-terminal Pro containing peptides, predicted *in silico* to be released from casein, a Pro-rich substrate, on incubation with prolyl oligopeptidase, to inhibit DPP-IV activity. In addition, the *in vitro* antioxidant (DPPH scavenging) activity of these peptides was studied. The DPP-IV inhibitory peptides evaluated herein were also subjected to *in silico* analysis to predict their stability to gastrointestinal enzyme digestion. Peptides predicted to be released following *in silico* gastrointestinal digestion were tested *in vitro* for both their DPP-IV inhibitory and antioxidant potential.

2. Materials and methods

2.1. Reagents

Asn-Pro, Leu-Pro, Gln-Pro, Gly-Pro, Tyr-Pro, Ile-Pro, Val-Arg, Ile-Thr-Pro, Lys-His-Pro, His-Gln-Pro, Lys-Tyr-Pro, Val-Glu-Pro, Ile-Gln-Pro, Trp-Ile-Gln-Pro, Asn-Ser-Leu-Pro, Val-Leu-Gly-Pro, Phe-Leu-Gln-Pro and Val-Arg-Gly-Pro were from Thermo Fisher Scientific (Ulm, Germany). Tris(hydroxymethyl)aminomethane (TRIS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}), Gly-Pro-pNA, diprotin A (Ile-Pro-Ile), ethanol and porcine DPP-IV ($\geq$ 10 Units.mg^{-1} protein) were obtained from Sigma Aldrich (Dublin, Ireland). HPLC grade water and hydrochloric acid were from VWR (Dublin, Ireland).

2.2. *In silico* digestion of the caseins with prolyl oligopeptidase

The caseins (α_{s1}-, α_{s2}-, β- and κ-casein) were digested *in silico* with a prolyl oligopeptidase (EC 3.4.21.26) activity to release peptides with a Pro residue at the C terminus. This enzyme potentially cleaves at the Pro C side of Pro-Xaa sequences, with Xaa being an amino acid different from Pro. Less effective cleavage at the Ala C-side of Ala-Xaa has also been reported (Polgar, 1992). Fifteen selected di-, tri- and tetra-peptide candidates (Tyr-Pro, Leu-Pro, Asn-Pro, Ile-Pro, Gly-Pro, Ile-Thr-Pro, His-Gln-Pro, Lys-Tyr-Pro, Lys-His-Pro, Val-Glu-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro, Phe-Leu-Gln-Pro, Asn-Ser-Leu-Pro and Val-Arg-Gly-Pro) were synthesised and subsequently evaluated for their DPP-IV inhibitory and DPPH scavenging properties.

Eight DPP-IV inhibitory peptides identified within the 15 peptides evaluated herein were also subjected to *in silico* digestion with pepsin (pH 1.3 and pH > 2), trypsin and chymotrypsin (high and low specificity) using the peptide cutter programme (ExPASy, 2011) in order to predict their

stability to gastrointestinal digestion. The peptide fragments predicted to be released on incubation with these enzymes were tested for their DPP-IV inhibitory and DPPH scavenging properties.

2.3. DPP-IV inhibition assay

Peptides were dispersed in HPLC grade water at concentrations ranging from 12.5×10^{-3} to 1.25 $mg.mL^{-1}$. The DPP-IV inhibition assay was carried out as described by Nongonierma and FitzGerald (2013a). Briefly, the test samples (25 μL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA, the reaction substrate (50 μL, final concentration 0.2 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0 (25 μL) and the reaction substrate Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (50 μL, final concentration 0.0025 $Units.mL^{-1}$). 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 (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. DPP-IV IC_{50} values were determined by plotting the percentage of inhibition as a function of the concentration of test compound.

Lineweaver-Burk analysis was used to study the mode of inhibition as previously described (Nongonierma & FitzGerald, 2013a). The initial rate of the reaction (pNA released from Gly-Pro-pNA) was measured at different Gly-Pro-pNA concentrations ranging between 0.2 and 0.6 mM in the presence and absence of the DPP-IV peptide inhibitors at their IC_{50} concentration. The affinity constant (Km, without inhibitor), the apparent affinity constant (Kapp, with inhibitor) and the maximum rate of the reaction (Vmax) were determined from the double reciprocal plots.

2.4. DPPH radical scavenging assay

The DPPH assay was used to determine the radical scavenging properties of the peptides which

were dispersed in HPLC grade water at concentrations ranging from 1.25×10^{-2} to 1.25 $mg.mL^{-1}$. The DPPH scavenging assay was carried out essentially according to Nongonierma and FitzGerald (2013a). Briefly, the test samples (50 μL) were pipetted onto a 96 well microplate containing a DPPH (150 μL, 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, 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 without scavenger (DPPH solution with 50 μL water) as described by Liu *et al*. (2005). The DPPH scavenging EC_{50} values (concentration of active compound required to observe 50 % DPPH scavenging) were determined by plotting the percentage of DPPH scavenged as a function of the concentration of test compound.

2.5. 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 of $P < 0.05$.

3. Results

3.1. *In silico* digestion of caseins and casein-derived peptides

Peptides containing Pro residues have been of much interest in the area of bioactive peptides research (Hatanaka *et al.*, 2012; Norris & FitzGerald, 2013). Peptides with a Pro residue at the C terminus may be released by the hydrolytic action of prolyl oligopeptidase (Polgar, 1992). Therefore, *in silico* digestion of the caseins (α_{s1}-, α_{s2}-, β- and κ-casein) was conducted with this enzyme activity. To date, most food protein-derived peptides with DPP-IV inhibitory properties have been shown to be short sequences between 2 and 8 amino acid residues in length (Hatanaka *et al.*, 2012; Lacroix & Li-Chan, 2012b; Nongonierma & FitzGerald, 2013a; Tulipano *et al.*,

2011). In addition, some short milk-derived peptides have been shown to survive gastro-intestinal digestion *in vitro* and *in vivo* (Foltz *et al.*, 2007; Foltz, van Buren, Klaffke, & Duchateau, 2009). For this reason, selected short peptides (≤ 4 amino acid residues) with a Pro residue at the C terminus predicted to be released on incubation of the caseins with prolyl oligopeptidase were considered herein. Fig. 1 depicts the locations in the primary sequence of the individual caseins of these short peptides (underlined and boxed sequences). Of the 20 short peptides predicted to be released, fifteen (Tyr-Pro, Leu-Pro, Asn-Pro, Ile-Pro, Gly-Pro, Ile-Thr-Pro, His-Gln-Pro, Lys-Tyr-Pro, Lys-His-Pro, Val-Glu-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro, Phe-Leu-Gln-Pro, Asn-Ser-Leu-Pro and Val-Arg-Gly-Pro) were selected for the study herein. The location of these peptides on α_{s1}-, α_{s2}-, β- and κ-casein is illustrated in Fig. 1 (boxed sequences). β-Casein contained the highest number (16) of short peptides with a Pro residue at the C terminus which were predicted to be released on incubation with prolyl oligopeptidase. α_{s2}-Casein contained the lowest number (2) of short peptides with a Pro residue at the C terminus predicted to be released on incubation with prolyl oligopeptidase. Larger peptides (between 5 and 8 amino acid length), may also be released on incubation of the caseins with prolyl oligopeptidase. For example, three peptides containing 6-7 amino acids (Ile-Lys-His-Gln-Gly-Leu-Pro, Gln-Leu-Glu-Ile-Val-Pro and Ser-Phe-Ser-Asp-Ile-Pro) were predicted to be released on incubation of α_{s1}-casein with prolyl oligopeptidase. However, these peptides were not evaluated herein because the focus of this study was on short peptides.

3.2. DPP-IV inhibition of casein-derived peptides

Of the 15 peptides with a Pro residue at the C terminus studied herein, 8 (Phe-Leu-Gln-Pro, Ile-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro, Tyr-Pro, Leu-Pro, Val-Arg-Gly-Pro and Asn-Pro) were DPP-IV inhibitors and 7 (Gly-Pro, His-Gln-Pro, Ile-Thr-Pro, Lys-His-Pro, Lys-Tyr-Pro, Asn-Ser-Leu-Pro and Val-Glu-Pro) were inactive. The IC_{50} values of the different DPP-IV inhibitory peptides was determined. The most potent compound tested was Phe-Leu-Gln-Pro with an IC_{50}

187 value of 65.3 ± 3.5 µM and the least potent was Asn-Pro with an IC_{50} value > 20,000 µM (Table
188 1).

189 **3.3. *In silico* gastrointestinal digestion of DPP-IV inhibitory peptides**

190 *In silico* digestion with gastrointestinal enzyme activities was subsequently carried out on the
191 eight casein-derived peptides which inhibited DPP-IV (Table 1). Of the eight DPP-IV inhibitory
192 peptides studied herein, two (Asn-Pro and Ile-Pro) could not theoretically be further cleaved by
193 pepsin, trypsin and chymotrypsin and six (Tyr-Pro, Leu-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro,
194 Phe-Leu-Gln-Pro and Val-Arg-Gly-Pro) were theoretically cleaved. The different cleavage sites
195 on these six peptides for pepsin, trypsin and chymotrypsin are illustrated in Fig. 2. *In silico*
196 gastrointestinal digestion of Tyr-Pro yields Tyr and Pro, while Leu-Pro yields Leu and Pro; Trp-
197 Ile-Gln-Pro yields Trp and Ile-Gln-Pro; Val-Leu-Gly-Pro yields Val, Leu and Gly-Pro; Phe-Leu-
198 Gln-Pro yields Phe, Leu and Gln-Pro; and Val-Arg-Gly-Pro yields Val-Arg and Gly-Pro. The
199 predicted breakdown products were subsequently evaluated experimentally for their DPP-IV
200 inhibitory and DPPH scavenging activity.

201

202 The predicted breakdown peptides (Val-Arg, Gln-Pro, Ile-Gln-Pro and Gly-Pro), of the 6 peptides
203 (Tyr-Pro, Leu-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro, Phe-Leu-Gln-Pro and Val-Arg-Gly-Pro)
204 predicted to be susceptible to gastrointestinal enzyme digestion, were tested for their DPP-IV
205 inhibitory potential. Apart from Gly-Pro, which was predicted to be released by chymotryptic
206 action on Val-Leu-Gly-Pro and the tryptic action of Val-Arg-Gly-Pro, all the other breakdown
207 peptides (Val-Arg, Ile-Gln-Pro and Gln-Pro) were DPP-IV inhibitors. However, these were not
208 potent DPP-IV inhibitors as their IC_{50} values ranged from 826.1 ± 30.1 to > 4,000 µM (Table 1).
209 Trp and Leu, which were predicted to be released from four peptides (Leu-Pro, Trp-Ile-Gln-Pro,
210 Val-Leu-Gly-Pro and Phe-Leu-Gln-Pro), have previously been identified as weak DPP-IV
211 inhibitors with IC_{50} values of 4280 ± 48 and 3419 ± 56 µM, respectively (Nongonierma, Mooney,

212 Shields, & FitzGerald, 2013).

213 The milk protein origin of the peptides studied herein is indicated in Table 1. These peptides were
214 found within different caseins (α_{s1}-, α_{s2}-, β- and κ-casein). Interestingly, six (Ile-Pro, Tyr-Pro,
215 Leu-Pro, Val-Arg, Gln-Pro and Asn-Pro) of the casein-derived DPP-IV inhibitory peptides
216 studied herein can also be found in the whey proteins (Table 1). This has potential implications
217 for the DPP-IV inhibitory properties of whole milk protein and for whey protein hydrolysates as
218 these peptides may be released by enzymatic fragmentation.

219 **3.4. Mode of DPP-IV inhibition of casein-derived peptides**

220 The type of DPP-IV inhibition with the positive control (Ile-Pro-Ile) and the 7 most potent
221 peptides (Phe-Leu-Gln-Pro, Ile-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro, Tyr-Pro, Leu-Pro and
222 Val-Arg) studied herein was determined using the Lineweaver-Burk reciprocal representation.
223 The Lineweaver-Burk double reciprocal plots for the positive control (Ile-Pro-Ile), Phe-Leu-Gln-
224 Pro, Ile-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro and Val-Arg are illustrated in Fig. 3. A
225 significant difference in Vmax ($P < 0.05$) in the presence and absence of DPP-IV inhibitor was
226 observed for Trp-Ile-Gln-Pro and Val-Arg. In contrast, the Kapp (in the presence of inhibitor)
227 values were not significantly different ($P \geq 0.05$) from the Km (without inhibitor) value (Fig. 3D
228 and 3F). These results showed that Trp-Ile-Gln-Pro and Val-Arg behaved as non-competitive
229 inhibitors of DPP-IV. There was no significant difference in Vmax ($P \geq 0.05$) in the presence and
230 absence of DPP-IV inhibitor, for the other peptides studied (Ile-Pro-Ile, Phe-Leu-Gln-Pro, Ile-
231 Pro, Val-Leu-Gly-Pro, Tyr-Pro and Leu-Pro). In contrast, the Kapp values for these peptides were
232 significantly different ($P < 0.05$) from the Km value (Fig. 3A, 3B, 3C and 3E). These results
233 indicate that these peptides behaved as competitive inhibitors of DPP-IV.

234

235 **3.5. Antioxidant activity of the casein-derived peptides**

236 The antioxidant behaviour of the peptides was studied by determination of their ability to

scavenge the DPPH radical. Of the 18 different peptides studied herein, only 6 (Trp-Ile-Gln-Pro, Asn-Pro, His-Gln-Pro, Lys-Tyr-Pro, Tyr-Pro and Gly-Pro) were DPPH scavengers. Twelve peptides (Leu-Pro, Gln-Pro, Ile-Pro, Val-Arg, Ile-Thr-Pro, Lys-His-Pro, Val-Glu-Pro, Ile-Gln-Pro, Asn-Ser-Leu-Pro, Val-Leu-Gly-Pro, Phe-Leu-Gln-Pro and Val-Arg-Gly-Pro) did not possess DPPH scavenging activity. The antioxidant potency was evaluated by determining the EC_{50} value. The EC_{50} value ranged from 2.1 ± 0.1 to > 15 mM for Trp-Ile-Gln-Pro and Gly-Pro, respectively (Table 2). Three of the antioxidant peptides described in Table 2 (Trp-Ile-Gln-Pro, Tyr-Pro and Asn-Pro), were also DPP-IV inhibitors. The 3 other antioxidative peptides (His-Gln-Pro, Lys-Tyr-Pro and Gly-Pro) did not inhibit DPP-IV.

4. Discussion

Several studies have suggested that the presence of a Pro residue within a given peptide may be a good indicator for its DPP-IV inhibitory potential (Hatanaka *et al.*, 2012; Uenishi *et al.*, 2012). It has also been shown that various dipeptides with a Pro residue at the C terminus behave as DPP-IV inhibitors (Hatanaka *et al.*, 2012). Hatanaka *et al.* (2012) studied 16 different Pro containing dipeptides, 14 of which were DPP-IV inhibitors. Similarly, we also found herein that Ile-Pro, Tyr-Pro and Leu-Pro were DPP-IV inhibitors. In agreement with the study of Hatanaka *et al.* (2012), we found that Gly-Pro had no DPP-IV inhibitory activity. The DPP-IV inhibitory activity of two other dipeptides with a Pro at the C terminus (Gln-Pro and Asn-Pro), which to our knowledge have not previously been mentioned in the literature, was reported herein. However, their DPP-IV IC_{50} values were high (> 4000 µM, Table 1). Larger peptides (with 3-4 amino acid residues) having a Pro residue at the C terminus including Phe-Leu-Gln-Pro, Trp-Ile-Gln-Pro, Val-Leu-Gly-Pro and Val-Arg-Gly-Pro were also DPP-IV inhibitors (Table 1). In contrast, His-Gln-Pro, Ile-Thr-Pro, Lys-His-Pro, Lys-Tyr-Pro, Val-Glu-Pro and Asn-Ser-Leu-Pro were not able to inhibit DPP-IV even though they had a Pro residue at the C terminus. Phe-Leu-Gln-Pro was found to be the most potent peptide studied herein, which was predicted *in silico* to be released by

prolyl oligopeptidase digestion of β-casein, having an IC_{50} of 65.3 ± 3.5 µM (Table 1). This peptide was ~ 20 times less potent that the positive control Ile-Pro-Ile (diprotin A). Other milk protein-derived peptides with a potency of the same order as Phe-Leu-Gln-Pro have previously been identified as DPP-IV inhibitory peptides including Ile-Pro-Ile-Gln-Tyr, Ile-Pro-Ala-Val-Phe, Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu, Ile-Pro-Ala, Trp-Val, with IC_{50} values of 35, 45, 46, 49 and 66 µM, respectively (Nongonierma & FitzGerald, 2013a, 2014; Silveira *et al.*, 2013; Tulipano *et al.*, 2011; Uenishi *et al.*, 2012). Another DPP-IV inhibitory peptide, Met-Trp-Pro, the N terminal sequence of the human immunodeficiency virus-1 (HIV-1) transactivator Trp2-Tat (f1-3), containing a Pro at the C terminus has been reported in the literature (Lorey *et al.*, 2003). However, for most DPP-IV inhibitory peptides comprising of more than 2 amino acids including a Pro residue, the Pro residue has been found at position 2 or 3 in the peptide (Hoffmann *et al.*, 1995; Nongonierma & FitzGerald, 2014; Silveira *et al.*, 2013; Uenishi *et al.*, 2012). It has also been demonstrated that these specific sequences are more likely to behave as substrate-type inhibitors of DPP-IV (Nongonierma & FitzGerald, 2014; Rahfeld, Schierborn, Hartrodt, Neubert, & Heins, 1991). Substrate-type inhibition involves inhibitors which may be further cleaved by the biomarker enzyme, releasing less potent inhibitory compounds than the parent peptide (Fujita & Yoshikawa, 1999; Nongonierma & FitzGerald, 2014).

Six short (≤ 4 amino acid residues) peptides (Arg-Pro, Phe-Pro, Val-Pro, His-Pro, Leu-Pro-Pro and Thr-Ser-Thr-Pro) which were predicted to be released from caseins on incubation with prolyl oligopeptidase (Fig. 1), were not evaluated herein. Arg-Pro, Phe-Pro, Val-Pro and His-Pro were previously shown to be DPP-IV inhibitors (Hatanaka *et al.*, 2012). To our knowledge, Leu-Pro-Pro and Thr-Ser-Thr-Pro have not been shown to possess DPP-IV inhibitory activity. Larger peptides (> 5 amino acid residues) may also be released from caseins by prolyl oligopeptidase (Fig. 1). However, these may be unstable to gastrointestinal enzyme digestion and therefore were not included in the study herein.

Different modes of DPP-IV inhibition were highlighted herein with the casein-derived peptides. In agreement with previous results, we also found that the Ile-Pro-Ile and Ile-Pro were competitive inhibitors of DPP-IV (Hatanaka *et al.*, 2012; Rahfeld *et al.*, 1991). Most food-derived DPP-IV inhibitory peptides identified to date have been described as competitive inhibitors (Hatanaka *et al.*, 2012; Nongonierma & FitzGerald, 2013a; Tulipano *et al.*, 2011). Similarly, most peptides studied herein were competitive inhibitors, which indicates their direct binding to the active site of DPP-IV. However, two peptides (Trp-Ile-Gln-Pro and Val-Arg) were found to be non-competitive inhibitors of DPP-IV. It has been shown that peptides derived from the N terminus of the HIV-1 transactivator Tat could bind to a secondary site in DPP-IV, giving a linear mixed- or parabolic mixed-type inhibition (Lorey *et al.*, 2003). Recently, a milk-derived dipeptide Trp-Val (α-La (f26-27)) which behaved as a non-competitive inhibitor of DPP-IV has been identified. Using a molecular docking approach it was shown that Trp-Val was likely to bind DPP-IV at a secondary binding site located in proximity to the active site (Nongonierma *et al.*, 2013). Another mechanism for DPP-IV inhibition has recently been described for larger peptides (> 13 amino acid residues) where the peptides hinder the formation of the active dimeric form of DPP-IV (Velarde-Salcedo *et al.*, 2013).

An increase in oxidative stress is generally found in T2D subjects due to a compromised antioxidative system associated with changes in superoxide dismutase, glutathione peroxidase and catalase activity. This increase in oxidative stress has been linked with the development of cardiovascular and renal disease (Hadi & Al Suwaidi, 2007). It has been suggested that dietary antioxidants may act through the activation of the antioxidant system of the body, involving the nuclear factor like 2 (Nrf2) pathway (Lacroix & Li-Chan, 2014). Therefore, the antioxidant properties of milk-derived peptides could be further exploited to reduce oxidative stress in T2D

subjects. The *in vitro* antioxidant properties of casein-derived peptides have been well documented (Pihlanto, 2006; Power *et al.*, 2013). However, translation of *in vitro* antioxidant capacity of dietary components in humans and their positive role in the prevention of T2D has yet to be demonstrated (Chang & Chuang, 2010; Lacroix & Li-Chan, 2014; Power *et al.*, 2013). The peptides evaluated herein only had a modest DPPH scavenging activity compared to other peptides reported in the literature. EC_{50} values of around 10 µM have been reported for DPPH scavenging by decapeptides extracted from venison (Kim *et al.*, 2009), while a value of 98 µM for the casein-derived peptide Tyr-Pro-Tyr-Pro-Glu-Leu and an EC_{50} value of 23 µM for carnosine (Ala-His) were reported (Suetsuna *et al.*, 2000). EC_{50} values of 242 and 654 µM have previously been reported for Trp-Val and Val-Trp, respectively (Nongonierma & FitzGerald, 2013a). Three peptides (Asn-Pro, Tyr-Pro and Trp-Ile-Gln-Pro) evaluated herein had a dual bioactivity combining antioxidant and DPP-IV inhibitory properties. Trp-Ile-Gln-Pro (IC_{50} 237.3 ± 1.3 µM) was the most potent DPP-IV inhibitor which also behaved as a DPPH (EC_{50} 2.1 ± 0.1 mM) scavenger (Tables 1 and 2).

Some of the fragments which were predicted to be released from the parent peptides after *in silico* digestion with gastrointestinal enzymes were also subsequently experimentally shown to have DPP-IV inhibitory properties (Table 1). In general, peptides or amino acids (Nongonierma *et al.*, 2013) which were predicted to be released by gastrointestinal digestion of the casein-derived peptides had a lower DPP-IV inhibitory potential compared to the parent peptide. The exception was Val-Arg which was > 3 times more potent than the parent peptide (Val-Arg-Gly-Pro). In the case of Phe-Leu-Gln-Pro and Trp-Ile-Gln-Pro, *in silico* digestion with gastrointestinal enzymes resulted in peptides with > 10 fold decrease in the DPP-IV inhibitory potency. While some of the peptides were predicted to be unstable to gastrointestinal enzymes, the second most potent C terminal Pro DPP-IV inhibitor evaluated herein, Ile-Pro (DPP-IV IC_{50} 149.6 ± 6.1 µM), was

predicted to be stable to gastrointestinal digestion. It has been demonstrated elsewhere that Ile-Pro was stable to intestinal *in vitro* digestion, with > 75% intact dipeptide remaining after 60 min of simulated intestinal digestion (Foltz *et al.*, 2009). These results would indicate that Ile-Pro would be bioavailable. However, the bioavailability and efficacy of Ile-Pro needs to be evaluated *in vivo* in order to validate the results described herein. It is interesting to note that Ile-Pro is present in the primary sequence of several milk proteins (Table 1).

The results described herein using a combined *in silico* and experimental strategy further demonstrate that peptides with a Pro residue at the C terminus were not always associated with DPP-IV inhibitory activity. In addition, some of the C-terminal Pro peptides had a relatively low DPP-IV inhibitory potency. There appear to be other physicochemical characteristics which may play a role in the binding of peptides to the active site of DPP-IV, affecting their inhibitory properties. Studies involving reverse peptides (Hatanaka *et al.*, 2012; Nongonierma & FitzGerald, 2013a) have demonstrated that peptide sequence appears to be a primary determinant for its DPP-IV inhibitory potential. However, more research is required to fully establish which physicochemical characteristics of peptides are linked with their DPP-IV inhibitory potential. The results described herein are solely based on *in silico* prediction of peptide release with different enzymatic activities including prolyl oligopeptidase, pepsin, chymotrypsin and trypsin. Validation of the *in silico* prediction with *in vitro* digestion of caseins with the relevant enzymes would help to confirm the results described herein. Ultimately, *in vivo* testing of the hydrolysates generated should be conducted to study the stability and biological activity of these peptides in humans.

Conclusion

The work flow described herein allowed use of *in silico* analysis to predict peptide sequences with DPP-IV inhibitory properties and to predict their stability to gastrointestinal digestion. This

has allowed determination of potent casein-derived DPP-IV inhibitory peptides some of which were predicted to be stable to gastrointestinal enzyme digestion. The application of relevant enzyme activities during hydrolysis may help to specifically release these fragments with the view to developing more potent DPP-IV inhibitory milk protein-derived hydrolysates. The findings described herein are relevant to the development of milk protein hydrolysates for application as functional food ingredients with serum glucose lowering and antioxidative properties in the management of T2D.

Acknowledgements

The work described herein was supported by Enterprise Ireland under Grant Number CC20080001.

Conflicts of interests

The authors declare that they have no conflict of interest.

References

Chang, Y.-C., & Chuang, L.-M. (2010). The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication. *American Journal of Translational Research, 2*, 316-331.

Drucker, D. J. (2006a). The biology of incretin hormones. *Cell Metabolism, 3*, 153-165.

Drucker, D. J. (2006b). Enhancing the action of incretin hormones: A new whey forward? *Endocrinology, 147*, 3171-3172.

ExPASy. (2011). Swiss Institute of Bioinformatics, bioinformatics resource portal. URL: http://web.expasy.org/peptide_cutter/. Acessed on the 28th March 2014.

Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T. M. M. G., & Kloek, J. (2007). Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *The Journal of Nutrition, 137*, 953-958.

Foltz, M., van Buren, L., Klaffke, W., & Duchateau, G. S. M. J. E. (2009). Modeling of the relationship between dipeptide structure and dipeptide stability, permeability, and ACE inhibitory activity. *Journal of Food Science, 74*, H243-H251.

Fujita, H., & Yoshikawa, M. (1999). LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology, 44*, 123-127.

Hadi, H. A. R., & Al Suwaidi, J. (2007). Endothelial dysfunction in diabetes mellitus. *Vascular Health Risk Management, 3*, 8523-8876.

Harnedy, P. A., & FitzGerald, R. J. (2013). *In vitro* assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates. *Journal of Applied Phycology*, 1-11.

399 Hatanaka, T., Inoue, Y., Arima, J., Kumagai, Y., Usuki, H., Kawakami, K., Kimura, M., &
400 Mukaihara, T. (2012). Production of dipeptidyl peptidase IV inhibitory peptides from
401 defatted rice bran. *Food Chemistry, 134*, 797-802.

402 Hayden, M. R., & Tyagi, S. C. (2001). Uric acid: a new look at an old risk marker for
403 cardiovascular disease, metabolic syndrome, and type 2 diabetes mellitus: the urate redox
404 shuttle. *Nutrition and Metabolism, 1*, 1-10.

405 Hoffmann, T., Reinhold, D., Kähne, T., Faust, J., Neubert, K., Frank, R., & Ansorge, S. (1995).
406 Inhibition of dipeptidyl peptidase IV (DP IV) by anti-DP IV antibodies and non-substrate
407 X-X-Pro- oligopeptides ascertained by capillary electrophoresis. *Journal of*
408 *Chromatography A, 716*, 355-362.

409 Irshad, I., Kanekanian, A., Peters, A., & Masud, T. (2013). Antioxidant activity of bioactive
410 peptides derived from bovine casein hydrolysate fractions. *Journal of Food Science and*
411 *Technology*, 1-9.

412 Kim, E.-K., Lee, S.-J., Jeon, B.-T., Moon, S.-H., Kim, B., Park, T.-K., Han, J.-S., & Park, P.-J.
413 (2009). Purification and characterisation of antioxidative peptides from enzymatic
414 hydrolysates of venison protein. *Food Chemistry, 114*, 1365-1370.

415 Kitts, D. D. (2005). Antioxidant properties of casein-phosphopeptides. *Trends in Food Science &*
416 *Technology, 16*, 549-554.

417 Lacroix, I. M., & Li-Chan, E. C. Y. (2013). Inhibition of dipeptidyl peptidase (DPP)-IV and α-
418 glucosidase activities by pepsin-treated whey proteins. *Journal of Agricultural and Food*
419 *Chemistry, 61*, 7500–7506.

420 Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012a). Dipeptidyl peptidase-IV inhibitory activity of

dairy protein hydrolysates. *International Dairy Journal, 25*, 97-102.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012b). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. *Journal of Functional Foods, 4*, 403-422.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2014). Overview of food products and dietary constituents with antidiabetic properties and their putative mechanisms of action: a natural approach to complement pharmacotherapy in the management of diabetes. *Molecular Nutrition & Food Research, 58*, 61-78.

Li-Chan, E. C. Y., Hunag, S.-L., Jao, C.-L., Ho, K.-P., & Hsu, K.-C. (2012). Peptides derived from Atlantic salmon skin gelatin as dipeptidyl-peptidase IV inhibitors. *Journal of Agricultural and Food Chemistry, 60*, 973-978.

Liu, J.-R., Lin, Y.-Y., Chen, M.-J., & Lin, W.-L. (2005). Antioxidative activities of Kefir. *Asian-Australian Journal of Animal Sciences, 18*, 567-573.

Lorey, S., Stöckel-Maschek, A., Faust, J., Brandt, W., Stiebitz, B., Gorrell, M. D., Kähne, T., Mrestani-Klaus, C., Wrenger, S., Reinhold, D., Ansorge, S., & Neubert, K. (2003). Different modes of dipeptidyl peptidase IV (CD26) inhibition by oligopeptides derived from the N-terminus of HIV-1 Tat indicate at least two inhibitor binding sites. *European Journal of Biochemistry, 270*, 2147-2156.

Nauck, M., & El-Ouaghlidi, A. (2005). The therapeutic actions of DPP-IV inhibition are not mediated by glucagon-like peptide-1. *Diabetologia, 48*, 608-611.

Nongonierma, A. B., & FitzGerald, R. J. (2012). Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase. *Peptides, 37*, 263-272.

Nongonierma, A. B., & FitzGerald, R. J. (2013a). Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk-derived dipeptides and hydrolysates. *Peptides, 39*, 157-163.

Nongonierma, A. B., & FitzGerald, R. J. (2013b). Dipeptidyl peptidase IV inhibitory properties of a whey protein hydrolysate: Influence of fractionation, stability to simulated gastrointestinal digestion and food-drug interaction. *International Dairy Journal, 32*, 33–39.

Nongonierma, A. B., & FitzGerald, R. J. (2014). Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis. *Food Chemistry, 145*, 845-852.

Nongonierma, A. B., Mooney, C., Shields, D. C., & FitzGerald, R. J. (2013). Inhibition of dipeptidyl peptidase IV and xanthine oxidase by amino acids and dipeptides. *Food Chemistry, 141*, 644-653.

Norris, R., & FitzGerald, R. J. (2013). Antihypertensive peptides from food proteins. In B. Hernandez-Ledezma & B. Hseih (Eds.), *BIOACTIVE FOOD PEPTIDES IN HEALTH AND DISEASE* (pp. 45-72). Croatia: In Tech.

Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N. M. (2009). Potential bioactive effects of casein hydrolysates on human cultured cells. *International Dairy Journal, 19*, 279-285.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*, 1306-1314.

Polgar, L. (1992). Unusual secondary specificity of prolyl oligopeptidase and the different reactivities of its two forms toward charged substrates. *Biochemistry, 31*, 7729-7735.

Power, O., Jakeman, P., & FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids, 44*, 797-820.

Rahfeld, J., Schierborn, M., Hartrodt, B., Neubert, K., & Heins, J. (1991). Are diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase IV? *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1076*, 314-316.

Scheen, A. J. (2012). DPP-4 inhibitors in the management of type 2 diabetes: A critical review of head-to-head trials. *Diabetes & Metabolism, 38*, 89-101.

Silveira, S. T., Martínez-Maqueda, D., Recio, I., & Hernández-Ledesma, B. (2013). Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. *Food Chemistry, 141*, 1072–1077.

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry, 11*, 128-131.

Tulipano, G., Sibilia, V., Caroli, A. M., & Cocchi, D. (2011). Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides, 32*, 835-838.

Uchida, M., Ohshiba, Y., & Mogami, O. (2011). Novel dipeptidyl peptidase-4-inhibiting peptide derived from β-lactoglobulin. *Journal of Pharmacological Sciences, 117*, 63-66.

Uenishi, H., Kabuki, T., Seto, Y., Serizawa, A., & Nakajima, H. (2012). Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-4)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats. *International*

Dairy Journal, 22, 24-30.

Velarde-Salcedo, A. J., Barrera-Pacheco, A., Lara-González, S., Montero-Morán, G. M., Díaz-Gois, A., González de Mejia, E., & Barba de la Rosa, A. P. (2013). *In vitro* inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of amaranth (*Amaranthus hypochondriacus* L.) proteins. *Food Chemistry, 136*, 758-764.

Table captions

Table 1 Inhibitory concentration inducing 50 % inhibition (IC_{50}) of dipeptidyl peptidase IV (DPP-IV) in the presence of short (≤ 4 amino acid residues) casein-derived C terminal proline containing peptides, type of inhibition and milk protein source of DPP-IV inhibitory peptides. The 15 selected C terminal proline containing peptides predicted to be released from casein following enzymatic digestion with prolyl oligopeptidase are indicated in bold.

Table 2 Inhibitory concentration inducing 50 % scavenging (EC_{50}) for 2,2-diphenyl-1-picrylhydrazyl (DPPH) in the presence of short (≤ 4 amino acid residues) casein-derived C terminal proline containing peptides.

Table 1

Compound	DPP IV IC_{50} (μM)	Type of inhibition	Milk protein source
Ile-Pro-Ile	3.5 ± 0.2[a]	competitive	positive control (diprotin A) κ-CN (f26-28)
Phe-Leu-Gln-Pro	65.3 ± 3.5[b]	competitive	β-CN (f87-90)
Ile-Pro	149.6 ± 6.1[c]	competitive	β-CN (f66-67), β-CN (f74-75), α_{s1}-CN (f 182-183), α_{s2}-CN (f201-202), κ-CN (f26-27), κ-CN (f108-109), κ-CN (f119-120), β-Lg (f78-79), BSA (f297-298), LF (f127-128), LF (f310-311), LF (f469-470)
Trp-Ile-Gln-Pro	237.3 ± 1.3[d]	non-competitive	α_{s2}-CN (f193-196)
Val-Leu-Gly-Pro	580.4 ± 11.3[e]	competitive	β-CN (f197-200)
Tyr-Pro	658.1 ± 8.0[e]	competitive	β-CN (f60-61), β-CN (f114-115), β-CN (f180-181), α_{s1}-CN (f146-147), α_{s1}-CN (f159-160), κ-CN (f35-36), κ-CN (f68-69), LF (f166-167)
Leu-Pro	712.5 ± 11.0[e]	competitive	β-CN (f135-136), β-CN (f137-138), β-CN (f151-152), β-CN (f171-172), α_{s1}-CN (f11-12), α_{s2}-CN (f176-177), κ-CN (f56-57), β-Lg (f143-144), α-La (f23-24), BSA (f112-113), BSA (f179-179), BSA (f301-302), BSA (f515-516), LF (f218-219)

Val-Arg	826.1 ± 30.1[f]	non-competitive	β-CN (f201-202), κ-CN (f67-68), β-Lg (f123-124), BSA (f398-399), LF (f6-7), LF (f37-38)
Val-Arg-Gly-Pro	> 3,000	nd	β-CN (f201-204)
Gln-Pro	> 4,000	nd	β-CN (f89-90), β-CN (f146-147), β-CN (f149-150), α_{s2}-CN (f195-196), κ-CN (f7-8), LF (f13-14)
Ile-Gln-Pro	> 4,000	nd	α_{s2}-CN (f194-196)
Asn-Pro	> 20,000	nd	α_{s1}-CN (f184-185), α_{s2}-CN (f29-30), α_{s2}-CN (f107-108), β-Lg (f152-153), α-La (f66-67)
Gly-Pro	-	na	β-CN (f64-65), β-CN (f199-200), β-CN (f203-204), α_{s2}-CN (f102-103), BSA (f571-572)
His-Gln-Pro	-	na	β-CN (f145-147), β-CN (f148-150)
Ile-Thr-Pro	-	na	α_{s2}-CN (f119-121)
Lys-His-Pro	-	na	α_{s1}-CN (f3-5)
Lys-Tyr-Pro	-	na	β-CN (f113-115)
Asn-Ser-Leu-Pro	-	na	β-CN (f68-71)

Val-Glu-Pro	-	na	β-CN (f116-118)

Values represent mean IC_{50} values ± confidence interval (*P = 0.05*) n=3 and triplicate determination. Values with different superscript letter are significantly different (*P < 0.05*)

The selected C terminal proline containing peptides predicted to be released from casein following enzymatic digestion with prolyl oligopeptidase are indicated in bold

IC_{50} values and type of inhibition for Ile-Pro, Tyr-Pro and Leu-Pro were taken from Nongonierma and FitzGerald (2014)

Type of inhibition determined using Lineweaver-Burk plots as described in Nongonierma & FitzGerald (2012); nd: not determined, na: not applicable; -: no DPP-IV inhibition

α-La: α-lactalbumin; β-Lg : β-lactoglobulin; BSA: bovine serum albumin; CN: casein; LF: lactoferrin.

Table 2

Compound	DPPH EC_{50} (mM)
Trp-Ile-Gln-Pro	2.1 ± 0.1^{b}
Asn-Pro	>5
His-Gln-Pro	>5
Lys-Tyr-Pro	>5
Tyr-Pro	>5
Gly-Pro	>15
Trolox	$(17.2 \pm 5.5)\times10^{-3a}$

Values represent mean EC_{50} values ± confidence interval ($P = 0.05$) n=3 and triplicate determination. Values with different superscript letter are significantly different ($P < 0.05$)

Values for Tyr-Pro were taken from Nongonierma and FitzGerald (2014)

Figure captions

Fig 1. Peptide mapping of the short peptides (≤ 4 amino acid residues) with a proline residue at the C terminus, which can theoretically be released by *in silico* digestion with a prolyl oligopeptidase activity (using one letter amino acid code). The fifteen casein-derived peptides studied herein are boxed and the other sequences are underlined, the proline residues are indicated in bold.

Fig 2. *In silico* digestion of casein-derived C-terminal proline containing dipeptidyl peptidase IV (DPP-IV) inhibitory peptides with gastrointestinal enzymes. Possible cleavage sites on various peptides by pepsin (P), trypsin (T) and chymotrypsin (C) are indicated by an arrow.

Fig 3. Lineweaver-Burk double reciprocal plots for dipeptidyl peptidase IV (DPP-IV) inhibition with casein-derived peptides at their half maximum inhibitory concentration (IC_{50}). (A) Ile-Pro-Ile (B) Phe-Leu-Gln-Pro, (C) Ile-Pro, (D) Trp-Ile-Gln-Pro, (E) Val-Leu-Gly-Pro and (F) Val-Arg. Values are the mean of three determinations (n=3) ± SD. Vi: initial velocity.

α_{s1}-casein, variant B

RP KHP IKHQGLPQEVLNENLLRFFVAP FP EVFGKEKVNELSKDIGSESTEDQAMED
IKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNS
AEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGT
QYTDAPSFSDIP NP IGSENSEKTTMPLW

α_{s2}-casein, variant A

KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSE
ESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRN
AVP ITP TLNREQLSTSEENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQ
KFALPQYLKTVYQHQKAMKP WIQP KTKVIPYVRYL

β-casein, variant A2

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY
P FP GP IP NSLP QNIPPLTQTPVVVPP FLQP EVMGVSKVKEAMAPKHKEMP FP
KYP VEP FTESQSLTLTDVENLHLP LP LLQSWMHQP HQP LPP TVMFPPQSVLSLS
QSKVLP VP QKAVP YP QRDMPIQAFLLYQEP VLGP VRGP FP IIV

κ-casein, variant A

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLP
YP YYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHP HP HLSFMAIPPK
KNQDKTEIPTINTIASGEP TSTP TEAVESTVATLEDSPEVIESPPEINTVQVTSTAV

Fig 1.

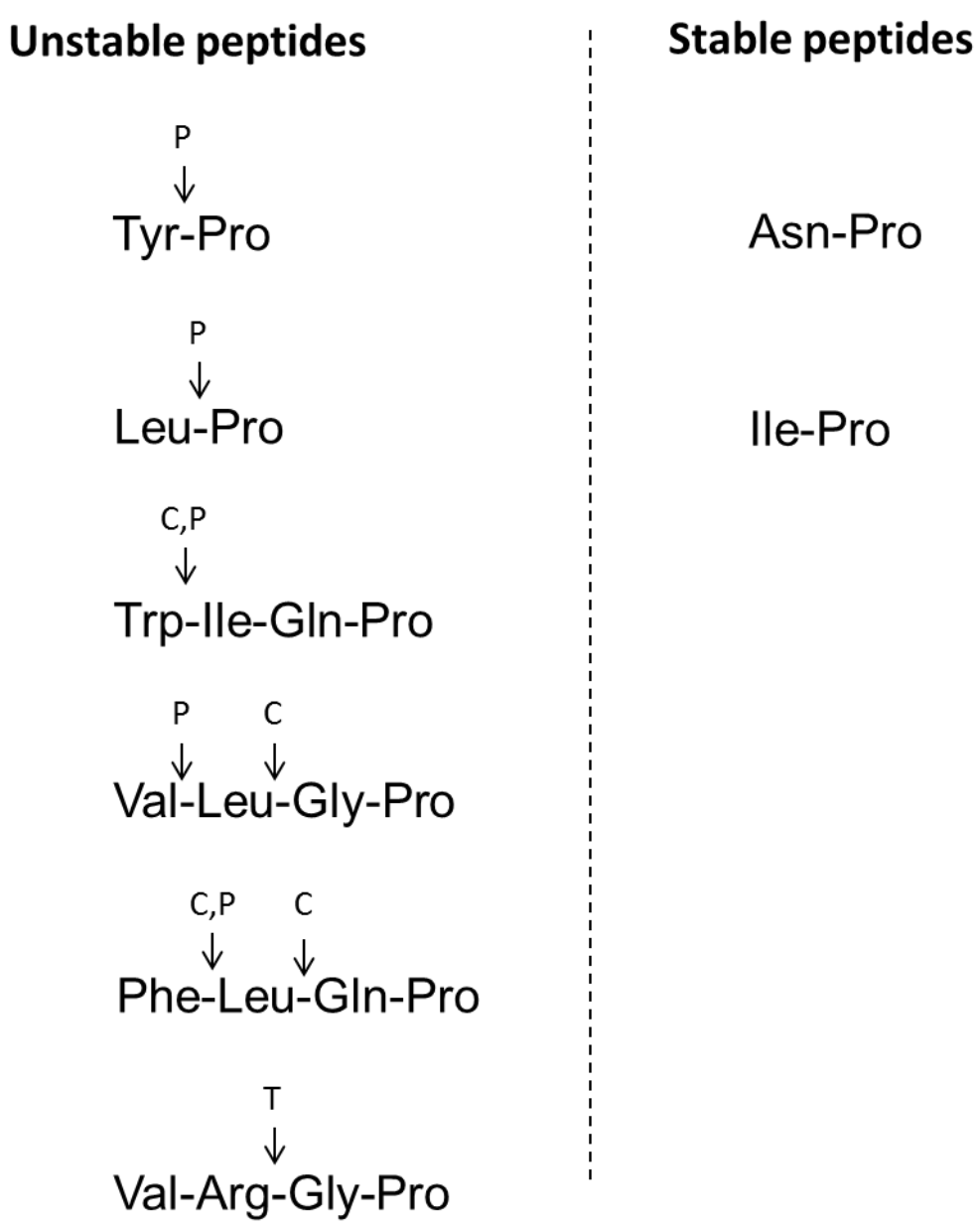
Unstable peptides
Stable peptides
P
Tyr-Pro
P
Leu-Pro
C,P
Trp-Ile-Gln-Pro
P C
Val-Leu-Gly-Pro
C,P C
Phe-Leu-Gln-Pro
T
Val-Arg-Gly-Pro
Asn-Pro
Ile-Pro

Fig 2.

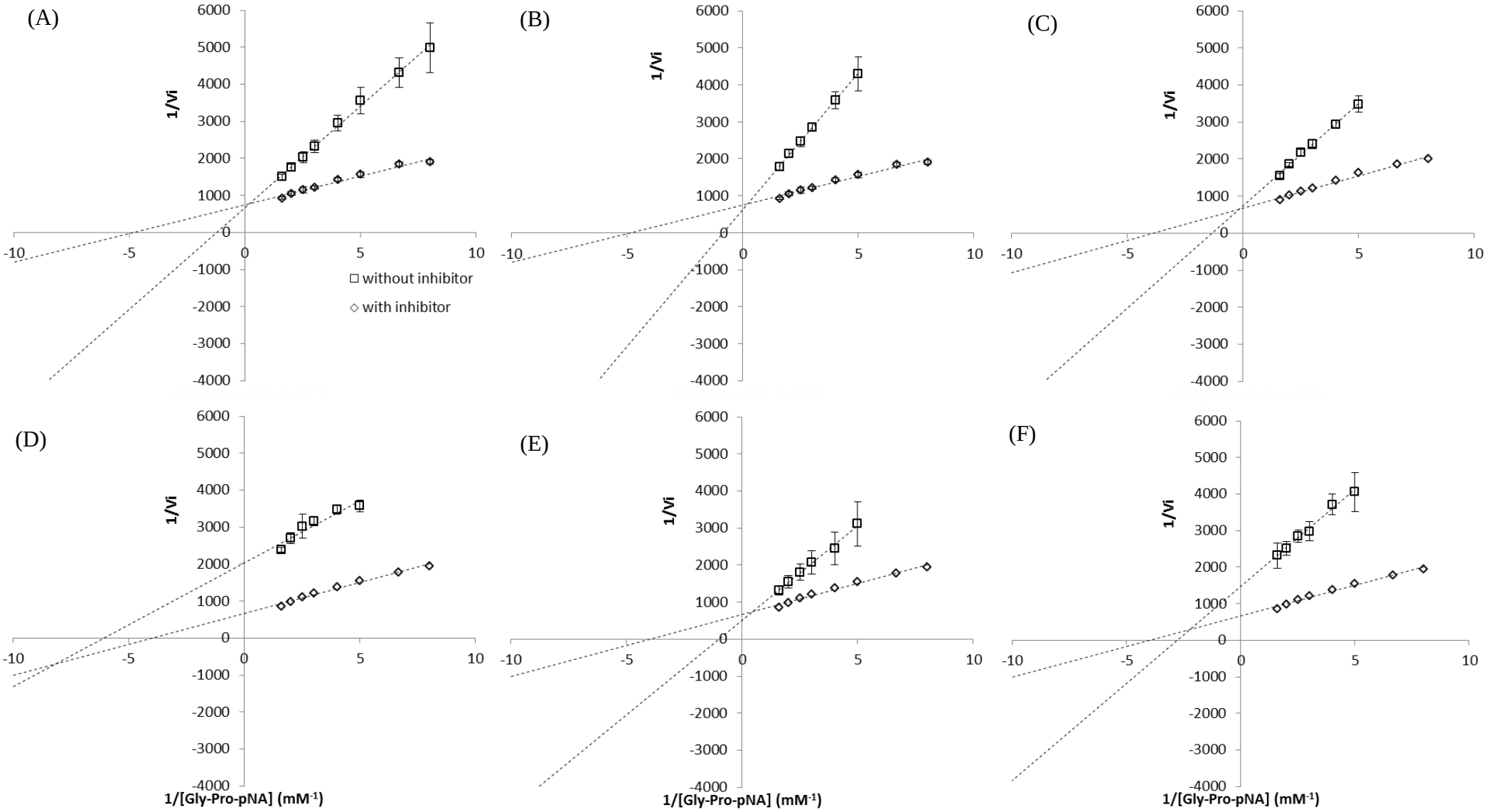
(A)
(B)
(C)
(D)
(E)
(F)
1/Vi
1/[Gly-Pro-pNA] (mM-1)
without inhibitor
with inhibitor

Fig 3.