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1 **Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV**

2 **(DPP-IV) hydrolysis**

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21 **Abstract**

22 *In silico* digestion of milk protein-derived peptides with gastrointestinal enzyme activities was
23 used to predict the release of peptides with a Pro residue at position 2 from the N terminus. These
24 peptides are known to act as preferred dipeptidyl peptidase IV (DPP-IV) substrates. Five casein-
25 derived synthetic peptides (Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu, Tyr-Pro-Tyr-Tyr, Leu-
26 Pro-Tyr-Pro-Tyr and Ile-Pro-Ile) and a casein (CasH), whey (WPH) and lactoferrin hydrolysate
27 (LFH) generated with gastrointestinal enzymes were incubated with DPP-IV at 37°C for 18 or 24
28 h. Peptide breakdown was evident following incubation with DPP-IV. Different modes of DPP-
29 IV inhibition were observed depending on the test compound. Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-
30 Tyr and Leu-Pro-Tyr-Pro-Tyr were substrate-, Leu-Pro-Leu-Pro-Leu and CasH were prodrug-
31 while WPH and LFH were true DPP-IV inhibitors. These results are relevant for the bioactivity
32 and bioavailability of functional foods targeting DPP-IV inhibition with potential blood glucose
33 regulatory properties in humans.

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36 **Key words:** dipeptidyl peptidase IV inhibitors, substrate-type inhibition, prodrug-type
37 inhibition, antioxidant, bioactive peptides, milk

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39 **1. Introduction**

40 Inhibition of dipeptidyl peptidase IV (DPP-IV) has been proposed as a new avenue for the
41 treatment of Type 2 diabetes (T2D). DPP-IV is an ubiquitous enzyme which can be found in
42 different locations of the body including the surface of various cells and in the circulation. It
43 hydrolyses incretin hormones such as glucose dependent insulinotropic peptide (GIP) and
44 glucagon-like polypeptide-1 (GLP-1). Those incretins can enhance insulin secretion from
45 pancreatic beta cells in the presence of nutrients i*n vivo* (Bjelke et al., 2006). The degradation of
46 GLP-1 and GIP by DPP-IV results in a loss in the bioactive properties of these hormones. DPP-
47 IV drug inhibitors are utilized to prevent incretin degradation *in vivo*, thereby increasing their
48 half-life (Bjelke et al., 2006).

49 Various studies have highlighted the possibility of using food-derived proteins and peptides as
50 a natural source of DPP-IV inhibitors (Hatanaka et al., 2012; Huang, Jao, Ho & Hsu, 2012;
51 Lacroix & Li-Chan, 2012b). These sources notably include milk proteins (Lacroix & Li-Chan,
52 2012a; Silveira, Martínez-Maqueda, Recio & Hernández-Ledesma, 2013; Tulipano, Sibilia,
53 Caroli & Cocchi, 2011; Uchida, Ohshiba & Mogami, 2011; Uenishi, Kabuki, Seto, Serizawa &
54 Nakajima, 2012). Casein and whey protein hydrolysates have been identified as DPP-IV
55 inhibitors (Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013; Tulipano et al., 2011;
56 Uenishi et al., 2012). Hydrolysates generated from β-lactoglobulin (Lacroix & Li-Chan, 2013;
57 Silveira et al., 2013; Uchida et al., 2011), α-lactalbumin (Lacroix & Li-Chan, 2013), bovine
58 serum albumin (Lacroix & Li-Chan, 2013) and lactoferrin (Lacroix & Li-Chan, 2013;
59 Nongonierma & FitzGerald, 2013) have also been identified as potent DPP-IV inhibitors. Various
60 DPP-IV inhibitory peptide sequences have been reported in the literature (Lacroix & Li-Chan,
61 2012b). The potent DPP-IV inhibitory peptides, diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu),
62 originating from microbial cultures of *Bacillus cereus*, display half maximal inhibitory
63 concentration (IC_{50}) values in the µM range (Rahfeld, Schierborn, Hartrodt, Neubert & Heins,
64 1991). Recently, different dipeptides from rice protein with a Pro residue at the C terminus have

65 been identified as DPP-IV inhibitors (Hatanaka et al., 2012). However, various dipeptide
66 sequences without Pro residues have also been identified as potent DPP-IV inhibitors
67 (Nongonierma & FitzGerald, 2013). Several food-derived DPP-IV inhibitory peptide sequences
68 longer than 2 amino acid residues have also been reported in recent studies (Silveira et al., 2013;
69 Tulipano et al., 2011; Uchida et al., 2011; Uenishi et al., 2012).

70 It is recognised that peptides which act as inhibitors of key enzymes in metabolic pathways
71 may have different susceptibilities to further cleavage on binding to these enzymes. For instance,
72 it has been proposed that inhibition of angiotensin I converting enzyme (ACE), a key enzyme in
73 blood pressure control, by peptide inhibitors could be classified into three main categories based
74 on their stability toward ACE. Peptides displaying an inhibitor-type behaviour are not cleaved
75 following incubation with ACE. Peptides with a prodrug-type behaviour are cleaved by ACE,
76 resulting in the release of a "true" inhibitory peptide with a lower IC_{50} value compared to the
77 parent peptide. The third category of peptides, which show a substrate-type behaviour, are
78 cleaved following incubation with ACE, resulting in an increase in the IC_{50} value compared to the
79 parent peptide (Fujita & Yoshikawa, 1999). It has been demonstrated that many DPP-IV
80 inhibitory peptides behaved as substrates for this enzyme. Rahfeld et al. (1991) reported for the
81 first time that diprotin A and B were substrates for DPP-IV. This result could have been
82 anticipated as both peptides have the structural features of DPP-IV preferred substrates, where a
83 Pro residue is located at the penultimate position (Kühn-Wache, Bär, Hoffmann, Wolf, Rahfeld &
84 Demuth, 2011; Vanhoof, Goossens, De Meester, Hendriks & Scharpé, 1995). Milk proteins and
85 particularly caseins are relatively rich in Pro residues, therefore, it could be anticipated that
86 various peptide sequences showing structural characteristics of DPP-IV substrates may be
87 released upon enzymatic hydrolysis of milk proteins. Lacroix and Li-Chan (2012a) hypothesised
88 that DPP-IV inhibition by various milk protein hydrolysates may actually involve milk protein-
89 derived peptides behaving as DPP-IV substrates.

90 A link between the secondary complications of T2D such as cardiovascular disease

91 (atherosclerosis, stroke and coronary heart disease) and oxidative stress has been proposed. It has
92 been suggested that natural antioxidants may be used as adjuncts to therapeutic approaches to
93 help in preventing cardiovascular complications induced by T2D (Xu, Tappia, Neki & Dhalla,
94 2013). Milk proteins and peptides have been identified for their antioxidant properties. The
95 utilization of anti-diabetic milk peptides/hydrolysates with additional antioxidant properties in the
96 management of T2D has therefore been proposed (Nongonierma & FitzGerald, 2013).

97 Earlier studies have demonstrated the cleavage of milk protein-derived peptides by DPP-IV.
98 However, to our knowledge, no studies have shown that milk protein-derived peptides display a
99 prodrug- or substrate-type of inhibition towards DPP-IV. The aim of this study was to predict the
100 release of DPP-IV substrate–like peptide sequences (peptides having a penultimate Pro residue)
101 by gastrointestinal enzymes using an *in silico* digestion of the major individual milk proteins.
102 Several casein-derived sequences were then synthesized and incubated with DPP-IV in order to
103 assess their stability to further hydrolysis by DPP-IV. The parent peptides and their breakdown
104 products were then tested for their DPP-IV inhibitory and antioxidant potential. A similar
105 approach was followed with milk protein hydrolysates generated with gastrointestinal enzymes.

106 **2. Materials and methods**

107 *2.1. Reagents*

108 The synthetic peptides Leu-Pro, Tyr-Pro, Ile-Pro, Tyr-Tyr, Leu-Pro-Leu, Tyr-Pro-Tyr, Ile-Gln-
109 Tyr, Tyr-Pro-Tyr-Tyr, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr were
110 from Thermo Fisher Scientific (Ulm, Germany). Ile, Tyr, Leu, trifluoroacetic acid (TFA),
111 tris(hydroxymethyl)aminomethane (TRIS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-
112 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}), Gly-Pro-pNA, diprotin A (Ile-Pro-
113 Ile), ethanol, porcine DPP-IV ($\geq$ 10 units.mg^{-1} protein), high performance liquid chromatography
114 (HPLC) grade water and acetonitrile (ACN) were obtained from Sigma Aldrich (Dublin, Ireland).
115 Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were from VWR (Dublin, Ireland).

116 *2.2. In silico digestion of individual milk proteins with gastrointestinal enzymes and peptide*

117 *digestion with DPP-IV*

118 *In silico* digestion of individual milk proteins was carried out with the peptide cutter program 119 (ExPASy, 2011) using gastrointestinal enzymes (pepsin, trypsin and chymotrypsin). The different 120 peptides released were then analysed for their potential to act as DPP-IV preferred substrates, i.e., 121 peptides with a Pro residue at the penultimate position. Three casein-derived peptide sequences 122 (Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr) which contained 123 previously identified DPP-IV inhibitory peptides within their sequence were selected from the *in* 124 *silico* digestion with gastrointestinal enzymes. Ile-Pro-Ile-Gln-Tyr contained the potent DPP-IV 125 inhibitor Ile-Pro-Ile which has also been identified as a DPP-IV substrate (Rahfeld et al., 1991). 126 In addition, its incubation with DPP-IV was predicted to release Ile-Pro, which has been 127 identified as a DPP-IV inhibitor (Hatanaka et al., 2012). Digestion of Leu-Pro-Leu-Pro-Leu and 128 Leu-Pro-Tyr-Pro-Tyr with DPP-IV would theoretically release the previously identified DPP-IV 129 inhibitors Leu-Pro and Tyr-Pro (Hatanaka et al., 2012). Tyr-Pro-Tyr-Tyr (Casoxin B) was used 130 for its structural similarities with Leu-Pro-Tyr-Pro-Tyr. The five peptide sequences were digested 131 *in silico* with DPP-IV and the resulting peptide products were identified.

132 *2.3. Digestion of milk protein hydrolysates and peptides with DPP-IV*

133 Peptides with a Pro residue at the penultimate position (position 2), including Tyr-Pro-Tyr, 134 Leu-Pro-Leu, Leu-Pro-Leu-Pro-Leu, Tyr-Pro-Tyr-Tyr, Leu-Pro-Tyr-Pro-Tyr, Ile-Pro-Ile-Gln-Tyr 135 and Ile-Pro-Ile, were subjected to DPP-IV digestion. The peptides Ile-Gln-Tyr and Pro-Ile, which 136 did not present structural features of DPP-IV substrates, were used as negative controls. Peptides 137 were resuspended in 100 mM Tris-HCl buffer pH 8.0 at 10 mg.mL^{-1} and incubated at 37°C for 18 138 h with DPP-IV at two different enzyme to substrate ratios (E:S), 1 and 10 U DPP-IV: 1 g peptide. 139 The control consisted of the peptide resuspended in 100 mM Tris-HCl buffer pH 8.0 at 10 140 mg.mL^{-1} incubated at 37°C for 18 h without DPP-IV. The enzyme was heat inactivated at 90°C 141 for 20 min, which resulted in complete inactivation of the enzyme as no DPP-IV activity was 142 detected when the heat treated DPP-IV was subsequently incubated with Gly-Pro-pNA. In order

143 to check the retention of activity at the end of the reaction, DPP-IV was also incubated for 18 h at
144 37°C in 100 mM Tris-HCl buffer pH 8.0 without substrate. At the end of the incubation period,
145 this sample was incubated with Gly-Pro-pNA and DPP-IV activity was determined.

146 Milk protein hydrolysates derived from caseins, whey proteins and lactoferrin as described in
147 Nongonierma and FitzGerald (2013) were used in this study. The hydrolysates were resuspended
148 in 100 mM Tris-HCl buffer pH 8.0 at 10 $mg.mL^{-1}$ (final concentration) and incubated at 37°C for
149 24 h with DPP-IV at an E:S of 0.2 and 2 U of $DPP\text{-}IV.g^{-1}$of hydrolysate. The control consisted of
150 the hydrolysates resuspended in 100 mM Tris-HCl buffer pH 8.0 and incubated at 37°C for 24 h
151 without DPP-IV. Hydrolysis with DPP-IV was carried out in duplicate (n=2).

152 *2.4. DPP-IV inhibition assay*

153 Diprotin A was resuspended in HPLC grade water at concentrations ranging from 12.5×10^{-3}
154 to 12.5 $\mu g.mL^{-1}$ (final concentration), the other peptides and milk protein hydrolysates were
155 dispersed at concentrations ranging from 12.5×10^{-3} to 1.25 $mg.mL^{-1}$ (final concentration). The
156 DPP-IV inhibition assay was carried out as described by Nongonierma and FitzGerald (2013).
157 Briefly, the test samples (25 μL) were pipetted onto a 96 well microplate (Sarstedt, Dublin,
158 Ireland) containing Gly-Pro-pNA, the reaction substrate (50 μL, final concentration 0.2 mM).
159 The negative control contained 100 mM Tris-HCl buffer pH 8.0 (25 μL) and the reaction
160 substrate Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (50 μL, final
161 concentration 0.0025 $U.mL^{-1}$). All the reagents and samples were diluted in 100 mM Tris-HCl
162 buffer pH 8.0. Diprotin A was used as a positive control. Each sample was analysed in triplicate.
163 The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT,
164 Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. The DPP-IV
165 IC_{50} values (concentration of active compound required to observe 50 % DPP-IV inhibition) were
166 determined by plotting the percentage inhibition as a function of the concentration of test
167 compound.

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

175 *2.5. DPPH radical scavenging assay*

176 The DPPH assay was used to determine the radical scavenging properties of the peptides
177 which were dispersed in HPLC grade water at concentrations ranging from 1.25×10^{-2} to 2.5
178 $mg.mL^{-1}$. The DPPH scavenging assay was carried out essentially according to Nongonierma and
179 FitzGerald (2013). Briefly, the test samples (50 µL) were pipetted onto a 96 well microplate
180 containing 150 µL of a DPPH (final concentration 0.088 mM) solution in 50 % (v/v) ethanol. The
181 microplate was incubated at 37°C for 60 min in a microplate reader, absorbance of the DPPH
182 radical was monitored at 517 nm. Each sample was analysed in triplicate. Trolox was used as a
183 positive control. Scavenging of the DPPH radical was determined with respect to a control
184 containing no scavenger (DPPH solution added with 50 µL water). The DPPH scavenging EC_{50}
185 values (concentration of active compound required to observe 50 % DPPH scavenging) were
186 determined by plotting the percentage DPPH scavenging as a function of the concentration of test
187 compound.

188 *2.6. Reverse-phase ultra-performance liquid chromatography (RP-UPLC) of peptides and*
189 *hydrolysates*

190 Profiles of different samples, including peptides and milk protein hydrolysates, before and
191 after incubation with DPP-IV at 37°C were determined by reverse-phase (RP) using an ultra-
192 performance liquid chromatograph (UPLC Acquity - Waters, Dublin, Ireland) equipped with a
193 2.1 x 50 mm, 1.7 µm Acquity UPLC C18 BEH column mounted with a 0.2 µm inline filter

194 (Waters) as described by Nongonierma and FitzGerald (2012). All peptides, including the DPP-
195 IV substrate-like peptides and their predicted breakdown products were injected in triplicate
196 (n=3) onto the UPLC column at different concentrations (25, 50, 100, 150, 200 and 250 $\mu g.mL^{-1}$).
197 Retention time of the standards was used to identify amino acids and peptides present in the
198 samples obtained before and after incubation with DPP-IV. The calibration curves (peak area as a
199 function of peptide concentration, $R^2 > 0.95$) for all peptides were generated and used to quantify
200 the amount of each amino acid and peptide present in the samples before and after incubation
201 with DPP-IV.

202 *2.7. Statistical analysis*

203 Means comparison was carried out using a one way ANOVA followed by a Student Newman-
204 Keuls test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$.

205 **3. Results**

206 *3.1. In silico gastrointestinal digestion of DPP-IV inhibitory peptides*

207 Of the different milk protein-derived peptides identified with DPP-IV preferred substrate
208 features, three peptide sequences Ile-Pro-Ile-Gln-Tyr (κ-casein variant A, f26-30), Leu-Pro-Tyr-
209 Pro-Tyr (κ-casein variant A, f56-60) and Leu-Pro-Leu-Pro-Leu (β-casein variant A2, f135-139)
210 were chosen for this study. These were selected on the basis that they contained previously
211 identified DPP-IV inhibitory peptides which may be released during DPP-IV digestion. In
212 addition, Tyr-Pro-Tyr-Tyr (κ-casein variant A, f58-61) which has previously been identified as
213 casoxin B (Chiba, Tani & Yoshikawa, 1989) and diprotin A (Ile-Pro-Ile, κ-casein variant A, f26-
214 28) were also included in this study. Ile-Pro-Ile, a well-known DPP-IV inhibitor, was used as a
215 positive control as it has previously been identified as a DPP-IV substrate (Rahfeld et al., 1991).

216 *In silico* digestion of these peptide sequences by DPP-IV allowed prediction of the potential
217 breakdown products. The possible DPP-IV cleavage sites on the peptides are illustrated in Fig. 1.
218 This analysis predicted that digestion of Ile-Pro-Ile-Gln-Tyr with DPP-IV would yield Ile-Pro and
219 Ile-Gln-Tyr; Leu-Pro-Leu-Pro-Leu was predicted to yield Leu-Pro, Leu-Pro-Leu, and Leu; Leu-

220 Pro-Tyr-Pro-Tyr was predicted to yield Leu-Pro, Tyr-Pro-Tyr, Tyr-Pro and Tyr; Tyr-Pro-Tyr-Tyr
221 was predicted to yield Tyr-Pro and Tyr-Tyr while Ile-Pro-Ile was predicted to yield Ile-Pro and
222 Ile. The five parent peptides and their predicted breakdown products were investigated for their
223 DPP-IV inhibitory properties and their further susceptibility to cleavage by DPP-IV *in vitro*.

224 *3.2. DPP-IV inhibition and antioxidant activity of the milk protein-derived peptides*

225 Of the twelve peptides tested, ten (Leu-Pro, Tyr-Pro, Ile-Pro, Ile-Pro-Ile, Leu-Pro-Leu, Tyr-
226 Pro-Tyr, Tyr-Pro-Tyr-Tyr, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr)
227 were able to inhibit DPP-IV and two peptides were found to be inactive (Tyr-Tyr and Ile-Gln-
228 Tyr). The IC_{50} for the ten DPP-IV inhibitory peptides identified was determined (Table 1). The
229 lowest IC_{50} value was observed for Ile-Pro-Ile (3.4 ± 0.1 μM), which was ten times lower than
230 that of Ile-Pro-Ile-Gln-Tyr (35.2 ± 1.8 μM). The least potent compound studied herein was Leu-
231 Pro (712.5 ± 11.0 μM), which was ~ 200 times less potent than Ile-Pro-Ile.

232 The mode of DPP-IV inhibition for the peptides was determined using the Lineweaver and
233 Burk representation. The Lineweaver and Burk double reciprocal plots for Ile-Pro-Ile, Ile-Pro-Ile-
234 Gln-Tyr, Leu-Pro-Leu-Pro-Leu, Leu-Pro-Tyr-Pro-Tyr, Tyr-Pro-Tyr and Leu-Pro are illustrated in
235 supplementary Fig. S1. For all peptides studied, there was no significant difference for Vmax
236 determined with or without inhibitor ($P \geq 0.05$), whereas Km was significantly different ($P <$
237 0.05) from Kapp. These results suggested that all DPP-IV inhibitory peptides studied herein were
238 competitive inhibitors of DPP-IV and therefore could directly bind to its active site.

239 The antioxidant activity of the twelve peptides was evaluated by determining their ability to
240 scavenge the DPPH radical. Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr, Tyr-
241 Pro-Tyr and Tyr-Tyr were able to scavenge DPPH (Table 1). The other peptides did not show any
242 scavenging activity toward DPPH radicals. The antioxidant potency of the peptides was evaluated
243 by determining their EC_{50} value. The EC_{50} values were relatively high suggesting that these
244 peptides did not have good antioxidant properties at least *in vitro*.

245 *3.3. Degradation of milk protein-derived peptides and milk protein hydrolysates following*

246 *incubation with DPP-IV*

247 Peptides with DPP-IV preferred substrate features (Ile-Pro-Ile, Leu-Pro-Tyr-Pro-Tyr, Leu-Pro-
248 Leu-Pro-Leu, Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr, Leu-Pro-Leu and Tyr-Pro-Tyr) were
249 incubated with DPP-IV at a low and high E:S. After 18 h incubation at 37°C with DPP-IV, all
250 peptides were cleaved by the enzyme with the exception of Ile-Gln-Tyr and Pro-Ile (the negative
251 controls) which in theory could not be cleaved by DPP-IV. It was found at the end of the
252 incubation that DPP-IV was still active as 69.3 ± 2.9 % DPP-IV activity was found. The RP-
253 UPLC profiles for Ile-Pro-Ile, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-
254 Tyr incubated at the low and high E:S are illustrated on Fig. 2. The RP-UPLC profiles show that
255 these peptides were cleaved to release their predicted breakdown products as per *in silico*
256 digestion with DPP-IV (section 3.1.). As expected peptide breakdown was more pronounced at
257 the high compared to the low E:S. The same results were found with Tyr-Pro-Tyr-Tyr, Tyr-Pro-
258 Tyr and Leu-Pro-Leu (data not shown).

259 Quantification of the peptides in each sample was carried out using the response factors from
260 the calibration curves determined for each individual peptide and amino-acid. The concentration
261 of amino acids and peptides present in each sample is shown in Table 2. As expected, breakdown
262 of the parent peptide was higher at the high compared to the low E:S. The mass balance of
263 peptides generated during DPP-IV hydrolysis agreed with the concentration of parent peptide
264 cleaved. Some components were not detected in the hydrolysed samples, this was due to the
265 amount of amino acids or peptides being below the detection threshold. Differences were seen in
266 the extent of peptide breakdown depending on the sequence of the parent peptide. At the high
267 E:S, Tyr-Pro-Tyr-Tyr and Tyr-Pro-Tyr were completely cleaved and could not be detected after
268 18 h incubation. In contrast, modest peptide breakdown was seen with Ile-Pro-Ile with less than
269 10 % (w/w) of this peptide being cleaved at high E:S.

270 Three milk protein hydrolysates studied herein which have previously been shown to be DPP-
271 IV inhibitors (Nongonierma & FitzGerald, 2013) were incubated with DPP-IV. The RP-UPLC

272 profiles for the high E:S digests are illustrated on supplementary Fig. S2. Slight differences were 273 seen on the RP-UPLC profiles of the casein hydrolysate (CasH) before and after incubation with 274 DPP-IV at 2, 20, 21 and 28 min retention times, where peptide peaks did not match between the 275 control and the hydrolysate incubated with DPP-IV (supplementary Fig. S2 a). For the whey 276 protein hydrolysate (WPH), one peak eluting at 15 min was absent from the hydrolysate after 277 incubation with DPP-IV (supplementary Fig. S2 b). In the case of the lactoferrin hydrolysate 278 (LFH), slight differences were seen at 3 and 9 min between the control and after incubation with 279 DPP-IV (supplementary Fig. S2 c).

280 Overall, incubation of the individual peptides with DPP-IV induced major changes in the 281 peptide composition, whereas for the milk protein hydrolysate, only minor modifications were 282 seen.

283 *3.4. DPP-IV inhibitory properties of milk protein-derived peptides and milk protein hydrolysates* 284 *following incubation with DPP-IV*

285 The IC_{50} values for the peptides pre and post DPP-IV hydrolysis was evaluated to study the 286 impact of peptide breakdown on the bioactive properties (Table 3). The IC_{50} value of the control 287 samples were similar to the values previously reported for these peptides (Table 1), suggesting 288 that the bioactive properties of these peptides were not altered following incubation at 37°C for 289 18 h. In addition, the RP-UPLC of the peptides in the control and freshly prepared peptides did 290 not differ (data not shown), suggesting that the peptides were not degraded during incubation in 291 the controls. For most peptides, there was no significant difference ($P \geq 0.05$) in terms of IC_{50} 292 value between the control and the low E:S sample, with the exception of Leu-Pro-Leu-Pro-Leu. 293 Leu-Pro-Leu-Pro-Leu had a significantly ($P < 0.05$) lower IC_{50} value at the low E:S compared to 294 the control. For all peptides, incubation with DPP-IV at the high E:S resulted in a significant 295 increase ($P < 0.05$) in the IC_{50} value compared to the control and the low E:S sample. However, 296 this was not seen for Leu-Pro-Leu-Pro-Leu where the IC_{50} value significantly decreased ($P <$ 297 0.05) following incubation with DPP-IV and no significant difference ($P \geq 0.05$) was seen

298 between the IC_{50} values at the low and high E:S.

299 The IC_{50} values for the milk protein hydrolysates incubated with DPP-IV at a high and low
300 E:S were determined (Fig. 3). There was no significant difference ($P \geq 0.05$) between the IC_{50}
301 value of the three hydrolysate controls. Following incubation with DPP-IV, no significant
302 differences in terms of IC_{50} values were found for WPH and LFH between the control and the
303 high and low E:S digests. In contrast, for CasH, samples incubated with DPP-IV displayed a
304 significantly lower ($P < 0.05$) IC_{50} value compared to CasH control. There was no significant
305 difference ($P \geq 0.05$) between CasH incubated at high or low E:S.

306 **4. Discussion**

307 This study has demonstrated that milk protein-derived peptides, with a Pro at position 2,
308 predicted to be released by gastrointestinal enzymes could act as DPP-IV inhibitors, involving a
309 substrate or prodrug mode of inhibition. With both substrate or prodrug modes of inhibition, the
310 starting peptides are susceptible to further DPP-IV cleavage, resulting in the release of more or
311 less potent peptide inhibitors as compared to the parent peptide. All peptides studied herein were
312 competitive inhibitors, suggesting a direct interaction of the peptides with the active site of DPP-
313 IV.

314 To our knowledge, of the different peptides studied, only Ile-Pro-Ile, Ile-Pro, Tyr-Pro and Leu-
315 Pro have previously been identified for their DPP-IV inhibitory properties (Hatanaka et al.,
316 2012). In agreement with the results of Hatanaka et al. (2012), Ile-Pro also displayed a lower IC_{50}
317 value compared to Leu-Pro (149.6 ± 6.1 and 712.5 ± 11.0 µM, respectively, Table 1). In addition,
318 Ile-Pro was about 6 times more potent than Leu-Pro, which is of the same order as the values
319 reported in Table 1. In contrast, we found that Tyr-Pro was a more potent DPP-IV inhibitor than
320 Leu-Pro, whereas Hatanaka et al. (2012) reported the opposite trend.

321 Relatively potent DPP-IV inhibitors displaying a substrate-type inhibition have been reported
322 in the literature. The well-known inhibitors diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu)
323 isolated from microbial cultures have previously been shown to behave as DPP-IV substrates

324 (Rahfeld et al., 1991). Other DPP-IV inhibitory peptides isolated from casein with a Pro at the
325 penultimate position, have recently been identified, these include Leu-Pro-Gln-Asn-Ile-Pro-Pro-
326 Leu, Leu-Pro-Gln and Val-Pro-Ile-Thr-Pro-Thr-Leu with IC_{50} values of 46, 82 and 110 µM,
327 respectively (Uenishi et al., 2012). Similarly, Ile-Pro-Ala (Tulipano et al., 2011) and Ile-Pro-Ala-
328 Val-Phe (Silveira et al., 2013) were isolated from β-lactoglobulin, with DPP-IV IC_{50} values of 49
329 and 45 µM, respectively. Results obtained from DPP-IV inhibitory potency correlated with the
330 composition of the samples, with the overall IC_{50} value being governed by the amount of the most
331 potent peptide within the sample (Table 2 and 3). Similar results have previously been reported
332 by Hatanaka et al. (2012) with Diprotin A (Ile-Pro-Ile) having an IC_{50} value twice as low as its
333 breakdown product Ile-Pro (0.21 ± 0.01 mM and 0.41 ± 0.07 mM, respectively). For illustration,
334 in the case of Tyr-Pro-Tyr and Tyr-Pro-Tyr-Tyr incubated at a high DPP-IV E:S, all the parent
335 peptide was degraded. As a consequence, these samples only contained Tyr-Pro as DPP-IV
336 inhibitory peptide. Interestingly, the IC_{50} value for Tyr-Pro-Tyr and Tyr-Pro-Tyr-Tyr incubated at
337 high E:S of DPP-IV (523.4 ± 31.9 and 783.7 ± 50.6 µM, respectively, Table 3) was of the same
338 order as that of Tyr-Pro (658.1 ± 8.0 µM, Table 1).

339 To our knowledge, no study has identified milk protein-derived peptides with a prodrug-type
340 behavior for DPP-IV inhibition. One peptide, Leu-Pro-Leu-Pro-Leu, had a different behaviour
341 from the other peptides studied herein, showing a decrease in the IC_{50} value following incubation
342 at low and high E:S as compared to the control (Table 3). This peptide therefore displayed a
343 prodrug-type inhibition according to the classification established by Fujita & Yoshikawa (1999).
344 In the sample containing Leu-Pro-Leu-Pro-Leu incubated with low and high E:S, significant
345 amounts of Leu-Pro-Leu were found (Table 2). This peptide is about 30 % more potent than Leu-
346 Pro-Leu-Pro-Leu and three times more potent than Leu-Pro, which explains the overall decrease
347 in IC_{50} value seen following incubation of Leu-Pro-Leu-Pro-Leu with DPP-IV.

348 Relatively high amounts of Leu-Pro-Leu and Tyr-Pro-Tyr, which could be substrates for DPP-
349 IV were also found with the parent peptides Leu-Pro-Leu-Pro-Leu and Tyr-Pro-Tyr-Pro-Tyr,

350 respectively, incubated with DPP-IV at high E:S. The susceptibility of Leu-Pro-Leu and Tyr-Pro-
351 Tyr to DPP-IV cleavage varied when these sequences were encrypted in a larger peptide (Leu-
352 Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr, respectively). At the high E:S, around 35 % of Tyr-
353 Pro-Tyr was cleaved from Leu-Pro-Tyr-Pro-Tyr, whereas 100 % of Tyr-Pro-Tyr was cleaved
354 with DPP-IV. For Leu-Pro-Leu incubated with DPP-IV, more than 75 % was cleaved whereas 30
355 % Leu-Pro-Leu was cleaved when Leu-Pro-Leu-Pro-Leu was incubated with DPP-IV at the high
356 E:S. These differences may arise from the inhibition exerted by the parent peptide (Leu-Pro-Leu-
357 Pro-Leu or Leu-Pro-Tyr-Pro-Tyr) on DPP-IV, which may have blocked further cleavage of Tyr-
358 Pro-Tyr and Leu-Pro-Leu. This is further supported by the fact that the more potent DPP-IV
359 inhibitory peptides (i.e. Ile-Pro-Ile, Ile-Pro-Ile-Gln-Tyr and Leu-Pro-Tyr-Pro-Tyr, Table 1)
360 studied herein were generally cleaved to a lesser extent by DPP-IV (Table 2 and Fig. 2).
361 For the whey protein-derived hydrolysates (WPH and LFH), preincubation with DPP-IV did
362 not affect the DPP-IV inhibitory properties of the samples (Fig. 3). This was in agreement with
363 the fact that the peptide profile for these samples was essentially unaffected by incubation with
364 DPP-IV (supplementary Fig. S2). However, with CasH, more obvious modifications were seen in
365 the peptide profile, which resulted in a significant decrease in the DPP-IV inhibitory properties.
366 Differences observed between whey proteins and caseins may come from the fact that caseins
367 contain higher amounts of Pro residues on a weight basis compared to whey. In addition, a higher
368 number of peptides with a Pro at the penultimate position were identified with the *in silico*
369 approach used herein for the caseins (α_{s1}-, α_{s2}-, β- and κ-casein) as compared to the whey proteins
370 (β-lactoglobulin and α-lactalbumin). The significant decrease in the IC_{50} value observed with
371 CasH following incubation with DPP-IV may be explained by the fact that it may contain
372 prodrug-type DPP-IV inhibitory peptides. Peptide peaks eluting at the same retention time as
373 Leu-Pro-Leu-Pro-Leu (17.9 min) and Leu-Pro-Leu (12.3 min) were seen on the RP-UPLC profile
374 of CasH. However, there was no difference in peak areas between the control and the high E:S
375 digest of CasH, suggesting that these peptides may not be responsible for the decreased IC_{50}

376 value. However, several other peptides which have not been studied herein may be responsible
377 for this effect. Mass spectrometric identification of the peptides within CasH may help to better
378 understand the effect of DPP-IV pre-digestion on the overall DPP-IV inhibitory properties of the
379 casein hydrolysate.

380 To assess if the peptides studied herein also had the potential to reduce oxidative stress in
381 T2D, their antioxidant activity was studied. The peptides evaluated herein only had a modest
382 DPPH scavenging activity. The EC_{50} value for DPPH scavenging by the casein-derived peptide
383 Tyr-Pro-Tyr-Pro-Glu-Leu was 98 μM while it was 23.3 μM for carnosine (Ala-His) (Suetsuna,
384 Ukeda & Ochi, 2000). EC_{50} values of 242 and 654 μM have been reported for Trp-Val and Val-
385 Trp, respectively (Nongonierma & FitzGerald, 2013).

386 A wide range of biologically active peptides, including immunomodulatory, neuro- and vaso-
387 active peptides, may be cleaved *in vivo* by DPP-IV resulting, in some cases, in the alteration of
388 their biological activity (Vanhoof et al., 1995). In agreement with our results, relatively long
389 incubation times with DPP-IV were required to achieve significant cleavage of the substrate. For
390 instance, with [(Xaa-Pro)n]−[drug] conjugates, it has been shown that up to 92 % of the
391 conjugates were cleaved by DPP-IV following a 24 h incubation period (García-Aparicio et al.,
392 2006). Cleavage of the milk protein-derived peptide, β-casomorphin (f1-5) (Tyr-Pro-Phe-Pro-
393 Gly), by DPP-IV from renal brush border membrane has been demonstrated (Miyamoto,
394 Ganapathy, Barlas, Neubert, Barth & Leibach, 1987; Tiruppathi, Miyamoto, Ganapathy, Roesel,
395 Whitford & Leibach, 1990). In addition, it was also shown that cleavage of Pro containing
396 peptides and gliadin, a Pro rich protein, also occurred with DPP-IV from intestinal brush border
397 membrane of rat (Tiruppathi, Miyamoto, Ganapathy & Leibach, 1993). It was also demonstrated
398 that Leu-Pro-Gly-Gly was degraded in Leu-Pro and Gly-Gly by DPP-IV located in the intestinal
399 brush border membrane of rat (Morita, Chung, Freeman, Erickson, Sleisenger & Kim, 1983).
400 Based on the available scientific evidence, it is anticipated that the results obtained *in vitro* with
401 the milk protein-derived peptides studied herein may also translate *in vivo*. The different milk

402 protein-derived peptides studied herein may be further degraded by DPP-IV in the
403 gastrointestinal tract to release amino acids and smaller peptides as predicted by the *in silico*
404 digestion with DPP-IV (Fig. 1).

405 Degradation of Pro containing peptides by DPP-IV located in the intestinal brush border
406 membrane *in vivo* has been shown to directly affect absorption of peptides. Morita et al. (1983)
407 have shown that ileal absorption rate of the constitutive amino acids of Leu-Pro-Gly-Gly (i.e.
408 Leu, Pro and Gly) and Leu-Pro and Gly-Gly was faster than that of the tetrapeptide. Relatively
409 high levels of DPP-IV (1.03 $g.mL^{-1}$) have been identified in the serum of humans (Cuchacovich,
410 Gatica, Pizzo & Gonzalez-Gronow, 2001). If they can cross the gut barrier, it is anticipated that
411 the short peptides studied herein (Leu-Pro-Leu and Tyr-Pro-Tyr) may be degraded into Leu-Pro
412 and Tyr-Pro. Leu-Pro and Tyr-Pro could in turn inhibit DPP-IV in the circulation. The peptide
413 sequences studied herein are of relevance to multi-site targeting for DPP-IV inhibition. They may
414 display their DPP-IV inhibitory potential directly in the gastrointestinal tract where they may be
415 degraded by intestinal brush border DPP-IV (Tiruppathi et al., 1993). This in turn will release
416 shorter peptides which are better candidates for intestinal permeation possibly allowing these to
417 reach the circulation where they may further display their DPP-IV inhibitory properties. Although
418 the IC_{50} value of the larger peptides was generally found to be lower than that of the shorter
419 peptides studied herein, the IC_{50} values for shorter peptides indicate that these are still moderately
420 potent inhibitors of DPP-IV (Table 1).

421

422 **Conclusion**

423 Milk protein-derived peptides which have been predicted to be released by gastrointestinal
424 enzyme activities have been shown to behave as substrate- or prodrug-type inhibitors of DPP-IV.
425 Besides their DPP-IV inhibitory properties, which make them interesting candidates for
426 protecting incretins against DPP-IV cleavage in the gastrointestinal tract, the instability of these
427 peptides may be further exploited. Cleavage of these peptide sequences by DPP-IV was shown to

428 release peptides which were in some cases more or less potent DPP-IV inhibitors. It is anticipated
429 that such peptides, owing to their small size and relatively high hydrophobicity may be good
430 candidates for intestinal absorption, thereby, allowing them to reach the circulation where they
431 may display their bioactive properties. Validation of these results *in vivo* is still required,
432 however, based on numerous studies conducted *in vivo* with peptides showing DPP-IV substrate-
433 like features, it is anticipated that similar trends may be found *in vivo*. To our understanding, the
434 results presented herein are relevant to the management of type 2 diabetes with functional foods
435 involving multi-target sites for DPP-IV inhibition in humans.
436

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440

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Table 1 Peptide concentration inducing 50 % inhibition (IC_{50}) for dipeptidyl peptidase IV (DPP-IV) and concentration of peptide required to observe 50 % 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging (EC_{50}) in the presence of milk protein-derived peptides.

Peptide sequence	DPP IV IC_{50} (μM)*	DPPH EC_{50} (mM)*
IPI (diprotin A)	3.4 ± 0.1^{a}	nd
IPIQY	35.2 ± 1.8^{b}	4.18 ± 0.61
LPYPY	108.3 ± 2.8^{c}	>5
IP	149.6 ± 6.1^{d}	-
YPYY	194.4 ± 13.0^{e}	> 10
LPL	241.4 ± 11.4^{f}	-
YPY	243.7 ± 2.8^{f}	-
LPLPL	325.0 ± 15.2^{g}	-
YP	658.1 ± 8.0^{h}	>5
LP	712.5 ± 11.0^{h}	-
YY	-	>5
IQY	-	-
Trolox (× 10^3)	nd	17.2 ± 5.5

*Values represent mean IC_{50} values ± confidence interval ($P = 0.05$), n=3. Values with different superscript letters are significantly different ($P < 0.05$).
nd: not determined, -: no activity detected.

Table 2 Concentration of milk protein-derived peptides pre- (control) and post-hydrolysis with dipeptidyl peptidase IV (DPP-IV) following incubation at 37°C for 18 h at a low (1 U: 1 g peptide) and high (10 U: 1 g peptide) enzyme to substrate ratio (E: S).

Parent peptide	Predicted sequences post DPP-IV hydrolysis	Peptide or amino acid concentration (µM)		
		Control	Low E:S	High E:S
IPI (diprotin A)	IPI	0.868 ± 0.001	0.854 ±0.002	0.801 ± 0.047
	IP	nd	nd	0.072 ± 0.002
	I	nd	0.020±0.017	0.076 ± 0.043
LPYPY	LPYPY	0.408 ± 0.001	0.360 ± 0.001	0.172 ± 0.021
	LP	nd	0.036 ± 0.001	0.182 ± 0.064
	YPY	nd	0.034 ± 0.001	0.192 ± 0.001
	YP	nd	nd	0.042 ± 0.001
	Y	nd	nd	0.052 ± 0.001
LPLPL	LPLPL	0.468 ± 0.012	0.288 ± 0.001	0.099 ± 0.001
	LPL	nd	0.156 ± 0.002	0.256 ± 0.001
	LP	nd	0.100 ± 0.001	0.487 ± 0.003
	L	nd	nd	nd
IPIQY	IPIQY	0.450 ± 0.001	0.450 ± 0.001	0.264 ± 0.001
	IP	nd	0.026 ± 0.001	0.158 ± 0.001
	IQY	nd	0.023 ± 0.001	0.162 ± 0.001
YPYY	YPYY	0.441 ± 0.001	0.420 ± 0.001	nd
	YP	nd	0.074 ± 0.001	0.385 ± 0.001
	YY	nd	0.078 ± 0.001	0.397 ± 0.001
LPL	LPL	0.817 ± 0.004	0.801 ± 0.003	0.106 ± 0.055
	LP	nd	0.076 ± 0.002	0.471 ± 0.002
	L	nd	nd	nd
YPY	YPY	0.501 ± 0.001	0.446 ± 0.047	nd
	YP	nd	0.037 ± 0.024	0.445 ± 0.001
	Y	nd	0.048 ± 0.001	0.430 ± 0.001
PI	PI	1.171 ± 0.004	1.208 ± 0.002	1.239 ± 0.001
IQY	IQY	0.506 ± 0.001	0.469 ± 0.001	0.492 ± 0.001

nd: not detected

Table 3 Concentration of milk protein-derived peptides inducing 50 % inhibition (IC_{50}) of dipeptidyl peptidase IV (DPP-IV) pre- (control) and post-hydrolysis of the milk protein-derived peptides with DPP-IV following incubation at 37°C for 18 h at a low (1 U: 1 g peptide) and high (10 U: 1 g peptide) enzyme to substrate ratio (E: S).

Compound	**DPP-IV IC_{50}***		
	Control	**Low E:S**	**High E:S**
IPI (diprotin A)	2.9 ± 0.2[a]	3.3 ± 0.1[a]	4.4 ± 0.3[b]
IPIQY	26.7 ± 0.6[c]	31.1 ± 0.3[c]	40.4 ± 0.4[d]
LPYPY	90.8 ± 2.8[e]	89.7 ± 7.7[e]	124.1 ± 23.3[f]
LPL	186.8 ± 3.1[g]	195.8 ± 3.5[g,h]	542.2 ± 9.1[k]
YPYY	207.9 ± 9.2[g,h]	238.3 ± 48.1[h,i]	523.4 ± 31.9[k]
YPY	282.0 ± 26.8[j]	387.0 ± 40.2[j]	783.7 ± 50.6[l]
LPLPL	358.4 ± 15.6[j]	271.6 ± 18.6[i]	246.7 ± 66.5[h,i]

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