Dipeptidyl peptidase IV inhibitory properties of a whey protein hydrolysate: influence of fractionation, stability to simulated gastrointestinal digestion and food-drug interaction

Alice B. Nongonierma and Richard J. FitzGerald

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

Please cite as:

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

The *in vitro* dipeptidyl peptidase IV (DPP-IV) inhibitory activity of a whey protein hydrolysate (WPH) generated with a food-grade pancreatic enzyme preparation was studied. The 50 % inhibitory concentration (IC$_{50}$) value in the presence of WPH was 1.34 ± 0.11 mg.mL$^{-1}$.

Ultrafiltration (UF) fractionation of WPH allowed enrichment in DPP-IV inhibitory peptides. The permeates generated by UF through 5 and 2 kDa membranes along with the hydrophilic fraction isolated by solid-phase extraction were significantly more potent ($P < 0.05$) DPP-IV inhibitors than WPH. These samples respectively had IC$_{50}$ values of 0.95 ± 0.16, 0.48 ± 0.01 and 1.11 ± 0.09 mg.mL$^{-1}$. Simulated gastrointestinal digestion (SGID) of WPH resulted in an increased DPP-IV inhibitory potency (IC$_{50}$ value of 1.02 ± 0.05 mg.mL$^{-1}$). Competitive inhibition of DPP-IV was observed with WPH and all its fractions, indicating a direct interaction of the bioactive peptides therein with the active site of DPP-IV. Combinations of sitagliptin, a conventional drug-inhibitor of DPP-IV, and whey-derived peptides resulted in an additive effect on DPP-IV inhibition.

*Key words: dipeptidyl peptidase IV inhibitors, type 2 diabetes, bioactive peptides, milk protein hydrolysates, food-drug interaction*
1. Introduction

Worldwide estimates indicate that 366 million people will be affected by diabetes in 2030 (WHO, 2006). Different strategies are being used to prevent/treat type 2 diabetes (T2D), they include treatment with various anti-diabetic medications. Changes in the diet and lifestyle, and regular participation in physical exercise have been recommended as a means to help in the management of T2D (Bantle et al., 2008; Hawley & Gibala, 2012; Mensink et al., 2003). In the presence of glucose, incretins, including glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), can influence insulin secretion from pancreatic beta cells (Drucker, 2006; Tulipano, Sibilia, Caroli, & Cocchi, 2011). Enzymatic degradation of these hormones by DPP-IV results in a significant reduction in their in vivo levels. Therefore, another avenue being explored in the management of T2D is the utilisation of DPP-IV inhibitors (Lacroix & Li-Chan, 2012a; Nauck & El-Ouaghlidi, 2005; Scheen, 2010). Various DPP-IV inhibitory drugs, also termed gliptins, are currently being used as fasting and post-prandial serum glucose lowering agents. These include sitagliptin, the first DPP-IV drug inhibitor available on the market. Classical insulin secretagogues (i.e sulfonylureas) have been associated with adverse effects such as hypoglycemia and weight gain. However, in diabetic subjects, gliptins can stimulate insulin secretion in the presence of glucose, this reduces the risk of hypoglycaemia and weight gain (Herman, Stein, Thornberry, & Wagner, 2007; Scheen, 2010).

Dietary intake of milk proteins and milk protein hydrolysates has been shown to affect several biomarkers of diabetes including postprandial glycaemia and insulin secretion in humans (Manders et al., 2006b; Morifuji et al., 2010; Power, Hallihan, & Jakeman, 2009; Tremblay & Gilbert, 2009). The potential of milk-derived peptides to act as DPP-IV inhibitors has been demonstrated (Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013; Tulipano et al., 2011; Uchida, Ohshiba, & Mogami, 2011; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). A DPP-IV inhibitory peptide present in a tryptic digest of β-lactoglobulin was shown to decrease
blood glucose level in mice following an oral glucose tolerance test (Uchida et al., 2011). Similarly, a casein-derived peptide (Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu) isolated from Gouda-type cheese caused a reduction of blood glucose level in rats following a glucose challenge (Uenishi et al., 2012). To date, most studies have investigated the potential role of milk peptides for their insulinotropic and blood glucose regulation properties. However, a limited amount of work appears to have been carried out studying the DPP-IV inhibitory properties of milk protein hydrolysates. DPP-IV inhibitors may act directly in the gastrointestinal tract, allowing an improved blood glucose homeostasis (Tulipano et al., 2011). Targeting DPP-IV inhibition using food-derived peptides could ultimately result in an increased half-life of the incretins.

In drug development, it is common practice to evaluate drug-drug interactions in order to determine side/adverse effects. However, very little data is available in the literature describing food-drug interactions. Food-drug interactions may have implications in the pharmacokinetics of a drug or the nutritional status of food components. Food may affect bioavailability, distribution, metabolism and elimination of drug molecules. It has been suggested that bioactive food-derived compounds, including polyphenols and bioactive peptides, may interact with drugs (Boullata, 2010; Genser, 2008). Some data is available on the effect of food-drug interactions on metabolic enzymes. It has been shown that grape fruit juice furanocoumarin can selectively inhibit intestinal cytochrome P450 (CYP) 34A enzymes (Paine et al., 2006; Paine et al., 2008). These enzymes are drug metabolising enzymes, the inhibition of which may result in an increased exposure to a drug which in turn may lead to adverse effects in humans (Genser, 2008). The outcomes of different pharmacokinetic studies suggest minor risks of drug-drug interactions in the specific case of sitagliptin (Herman et al., 2007; Scheen, 2010). It has been reported that gliptins have a good bioavailability which is not affected by food intake. In healthy volunteers, it was shown that administration of 100 mg sitagliptin with a high fat breakfast did not modify its pharmacokinetics suggesting that no specific interaction occurred with food in this case (Bergman et al., 2007).
However, no studies to our knowledge report on potential sitagliptin-peptide interaction.

The aim of this study was to investigate the potential of a whey-derived protein hydrolysate (WPH) to act as a DPP-IV inhibitor. Fractionation was investigated as a means to enrich for the bioactive components within the hydrolysate. Simulated gastrointestinal digestion (SGID) was investigated to determine bioactive peptide stability to gastrointestinal enzymes. Finally, the effect of various combinations of sitagliptin and whey-derived peptides on DPP-IV activity was studied.

2. Materials and methods

2.1. Materials

Trifluoroacetic acid (TFA), porcine DPP-IV (≥ 10 units/mg protein), para-nitroaniline (pNA), Gly-Pro-pNA, Tris(hydroxymethyl)aminomethane (TRIS), diprotin A (Ile-Pro-Ile) and sitagliptin were obtained from Sigma Aldrich (Dublin, Ireland). Trp-Val and Val-Ala were obtained from Bachem (Bubendorf, Switzerland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). The solid-phase extraction (SPE) cartridge Giga Tubes StrataX (33µm, 85Å Polymeric RP 1 g / 12 mL) were obtained from Phenomenex (Cheshire, UK). The whey protein substrate (WP) was a whey protein isolate purchased from a commercial supplier (Carbery, Ballineen, Ireland). BC pepsin and Corolase PP were obtained from Biocatalysts (Wales, UK) and AB enzymes (Darmstadt, Germany), respectively.

2.2. Hydrolysis of the whey protein substrate

Hydrolysis of the whey protein substrate was carried out as described by Nongonierma & FitzGerald (2012). The starting whey protein substrate WP was 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). A control sample without enzyme was removed from the protein
dispersion and maintained at 50°C for the duration of the hydrolysis reaction. WP was hydrolysed
with Corolase PP at an enzyme to substrate ratio (E:S) of 1 % (w/w) at 50°C for 240 min.
Hydrolysis was carried out at a constant pH of 7.0 using a pH Stat (Titando 843, Tiamo 1.4
Metrohm, Dublin, Ireland). 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.

2.3. SGID of WPH

SGID of WHP was carried out according to Walsh et al. (2004). Briefly, the freeze-dried
WPH was resuspended in water with gentle stirring at 2 % (w/v) on a protein basis at 37°C for 30
min. The sample was incubated at 37°C, pH 2.0 with pepsin at an E:S of 1% (w/w) for 90 min
then at pH 7.5 with Corolase PP at an E:S of 2.5 % (w/w) for another 150 min. The enzyme was
inactivated by heating at 90°C for 20 min and the samples were subsequently freeze-dried and
stored at -20°C until utilisation.

2.4. Fractionation of WPH

The WPH hydrolysate was fractionated using a plate and frame UF unit (Sartoflow Alpha
filtration system, Sartorius, Germany). Fractionation was carried out using membranes having 5
and 2 kDa molecular weight cut-off (MWCO) values. UF was carried out sequentially by
processing WPH through the 5 kDa membrane. The 5 kDa permeate was then ultrafiltered using a
2 kDa membrane. The four fractions (permeates and retentates) collected were freeze-dried and
stored at -20°C until utilisation.

WPH was fractionated by SPE as described by Herraiz & Casal (1995) and Nongonierma &
FitzGerald (2012). Briefly, WPH was resuspended in HPLC grade water at a concentration of 1
% (w/v) and 40 mL was applied on the SPE cartridge. The SPE cartridge was connected to a
vacuum (15 mmHg) manifold (Phenomenex, Cheshire, UK). The SPE cartridge was washed with
HPLC grade water (10 mL) to collect the hydrophilic fraction of WPH. This fraction was freeze-dried and stored at -20°C until utilisation.

2.5. Reverse-phase ultra-performance liquid chromatography and molecular mass distribution of peptides and proteins

The starting substrate (WP) and corresponding hydrolysate (WPH) and UF and SPE fractions were analysed by liquid chromatography using an ultra-performance liquid chromatograph (UPLC Acquity - Waters, Dublin, Ireland) as described by Nongonierma & FitzGerald (2012). Each sample was analysed in duplicate and the chromatographic profiles obtained were similar.

The molecular mass profiles of the proteins and peptides were determined by gel permeation chromatography using high performance liquid chromatography (GPC-HPLC) essentially as described by Spellman, O’ Cuinn & FitzGerald (2009). Each sample was analysed in duplicate and the chromatographic profiles obtained were similar.

2.6. DPP-IV inhibition assay

The test samples were dispersed in HPLC grade water at final concentrations ranging from 12.5 × 10^{-3} to 12.5 µg.mL^{-1} for diprotin A and from 0.6 × 10^{-2} to 0.6 mg.mL^{-1} for the WPH, UF and SPE fractions and SGID sample. The DPP-IV inhibition assay was carried out as described by Lacroix & Li-Chan (2012b). Briefly, test samples (25 µL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing the substrate Gly-Pro-pNA, (final concentration 0.2 mM). The negative control contained 25 µL of 100 mM Tris-HCl buffer pH 8.0 and the substrate Gly-Pro-pNA. The reaction was initiated by the addition of 50 µL 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 (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. The IC$_{50}$
values for DPP-IV were determined by plotting the percentage inhibition as a function of the
concentration of test compound. The mode of inhibition of the different samples was investigated
using Lineweaver and Burk analysis. The initial rate of the reaction (pNA released from Gly-Pro-
pNA) was measured at different Gly-Pro-pNA final concentrations ranging between 0.200 and
0.600 mM in the presence and absence of inhibitors (diprotin A, WPH, UF and SPE fractions and
SGID sample) at their IC<sub>50</sub> concentration. Km (determined without inhibitor) or Kapp (in the
presence of inhibitor) and Vmax values were determined from the double reciprocal plots
(Nongonierma & FitzGerald, 2012; Spanou et al., 2012).

Interactions between sitagliptin and diprotin A, WPH or two whey-derived dipeptide inhibitors
(Trp-Val and Val-Ala) of DPP-IV were studied. This was carried out by determining the extent of
DPP-IV inhibition in the presence of 0.006 or 0.031 ng.mL<sup>-1</sup> (final concentration) sitagliptin. The
final concentrations tested ranged from 12.5 × 10<sup>-3</sup> to 12.5 µg.mL<sup>-1</sup> for diprotin A and were from
0.6 × 10<sup>-2</sup> to 0.6 mg.mL<sup>-1</sup> for WPH, Trp-Val and Val-Ala. The dose response curves obtained for
diprotin A, WPH, Trp-Val or Val-Ala with or without added sitagliptin were compared. The
apparent IC<sub>50</sub> in the presence of 0.006 ng.mL<sup>-1</sup> (final concentration) sitagliptin was determined.

2.7. Statistical analysis

Data analysis was carried out with a one way ANOVA for means comparison. A Student
Newman-Keuls test at a significance level P < 0.05 was performed following the ANOVA using
SPSS (version 9, SPSS Inc., Chicago, IL, USA). Experimental data obtained with the Lineweaver
and Burk plot were fitted by linear regression using SPSS.

3. Results

3.1. Physicochemical properties of the WPH

The molecular weight distribution profiles of the WP and the corresponding WPH along with
its 5 and 2 kDa UF retentates and permeates are shown in Fig. 1. The unhydrolysed starting
material, as expected, consisted of relatively large molecular weight proteins, with more than 80% of the sample being greater than 10 kDa. In contrast, the WPH sample contained 25.6% proteinaceous material > 10 kDa. Upon hydrolysis, the proteins were broken down, resulting in a reduction in the molecular weight of the components within the WP substrate. Analysis of the WPH UF treated samples shows that the higher molecular weight components were retained in the UF membranes resulting in an enrichment of lower molecular weight peptides (< 5 and 2 kDa) in the permeates (Fig. 1).

SGID of the WP hydrolysate was carried out to assess the stability of the peptides to gastrointestinal enzymes. Numerous peptide peaks can be seen on the RP profile of WPH (Fig. 2A). Modification of the peptide composition of WPH occurred during SGID as can be seen from the RP profiles (Fig. 2B). SGID of WPH mainly resulted in the disappearance of some of the peptide peaks eluting at higher retention times (> 15 min). In addition some peptide peaks were more abundant in the hydrophilic region of the RP profile for the SGID sample when compared to the corresponding hydrolysate. The RP profile of the hydrophilic fraction of WHP obtained following SPE is illustrated in Fig. 2C. As expected, this fraction contained peptides which eluted at low ACN concentrations. SPE did not allow collection of sufficient amounts of the hydrophobic fraction to allow determination of an IC$_{50}$ value therefore, IC$_{50}$ was only determined for the hydrophilic SPE fraction.

3.2. DPP-IV inhibition with WPH and associated fractions and SGID sample

The DPP-IV IC$_{50}$ values obtained for the different samples are outlined in Table 1. The IC$_{50}$ for diprotin A, a well-known inhibitor of DPP-IV activity, was of the same order as previously reported in other studies (Lacroix & Li-Chan, 2012b; Uenishi et al., 2012). The UF and the hydrophilic fractions of WPH displayed IC$_{50}$ values which were significantly different ($P < 0.05$) from WPH (Table 1). The retentates had significantly higher ($P < 0.05$) IC$_{50}$ values than the associated permeates. The IC$_{50}$ of the 5 kDa permeate (0.95 ± 0.16 mg.mL$^{-1}$) was significantly
higher \((P < 0.05)\) compared to the 2 kDa permeate \((0.48 \pm 0.11 \text{ mg.mL}^{-1})\). The hydrophilic fraction of WPH had a significantly lower \((P < 0.05)\) IC\(_{50}\) value \((1.11 \pm 0.09 \text{ mg.mL}^{-1})\) than WPH and its 5 kDa retentate. Subjection of WPH to SGID resulted in a significant decrease \((P < 0.05)\) in the IC\(_{50}\) \((1.33 \pm 0.17\) and \(1.02 \pm 0.05 \text{ mg.mL}^{-1}\) for WPH and its SGID sample, respectively). The most potent hydrolysate sample/fraction studied was the WPH 2 kDa permeate. The WPH 2 kDa permeate was approximately 500 times less potent than diprotin A (Table 1).

Lineweaver and Burk analysis was used to study the mode of DPP-IV inhibition by the different inhibitors (diprotin A, WPH and its associated UF and hydrophilic fractions and SGID sample). The double reciprocal plots for WPH, its 5 kDa UF permeate, the hydrophilic fraction from SPE along with the SGID sample are illustrated in Fig. 3. For all samples studied, there was no significant difference in V\(_{\text{max}}\) \((P \geq 0.05)\) without inhibitor or in the presence of inhibitor (diprotin A, WPH, its associated UF and hydrophilic fractions and SGID sample). In contrast, K\(_{\text{m}}\) values (Table 1) were significantly different \((P < 0.05)\) from K\(_{\text{m}}\) values. These results indicate that WPH and its associated UF fractions and SGID sample all behaved as competitive inhibitors of DPP-IV (Table 1).

3.3. DPP-IV inhibition in the presence of sitagliptin

The IC\(_{50}\) value for sitagliptin \((69 \pm 16 \text{ nM} \text{ or } 0.036 \pm 0.08 \text{ ng.mL}^{-1} - \text{Table 1})\) was of the same order as the values previously reported in the literature (Thomas et al., 2008). When sitagliptin was tested on its own at 0.031 ng.mL\(^{-1}\), DPP-IV inhibition was of 59.2 \(\pm 2.7\%\). Sitagliptin and other DPP-IV inhibitors (diprotin A, WPH, Trp-Val and Val-Ala) were combined and presented to DPP-IV. The dose response curves for diprotin A, WPH, Trp-Val and Val-Ala in the presence or absence of 0.031 ng.mL\(^{-1}\) sitagliptin are illustrated on Fig. 4. The DPP-IV inhibition obtained with WPH alone and with WPH in the presence of sitagliptin \((0.031 \text{ ng.mL}^{-1})\) was compared. A higher inhibition \((P < 0.05)\) was observed with the mixture of sitagliptin and WPH at all WPH
concentrations studied in comparison to the inhibition seen with WPH alone (Fig. 4B). Furthermore, the extent of inhibition observed with the mixture of sitagliptin and WPH was significantly higher ($P < 0.05$) than 59.2 ± 2.7 % (the extent of DPP-IV inhibition observed with 0.031 ng.mL\(^{-1}\) sitagliptin) at all WPH concentrations tested. In the presence of diprotin A, Trp-Val and Val-Ala, the DPP-IV inhibition with 0.031 ng.mL\(^{-1}\) sitagliptin was significantly higher ($P < 0.05$) only at the lower concentrations studied (Fig 4A, C and D, respectively). This was due to the fact that the extent of DPP-IV inhibition observed at higher concentrations of diprotin A, Trp-Val and Val-Ala had reached a maximum. Significantly higher DPP-IV inhibition than that observed with 0.031 ng.mL\(^{-1}\) sitagliptin alone was seen with the addition of diprotin A between 1.25 and 12.50 µg.mL\(^{-1}\), and Trp-Val and Val-Ala between 0.1×10\(^{-1}\) and 0.6 mg.mL\(^{-1}\).

Because the extent of DPP-IV inhibition observed with 0.031 ng.mL\(^{-1}\) sitagliptin was higher than 50 %, it was not possible to determine the effect of sitagliptin at this concentration on the apparent IC\(_{50}\) value of diprotin A, WPH, Trp-Val and Val-Ala. For this reason, the apparent IC\(_{50}\) value of diprotin A, WPH, Trp-Val and Val-Ala was determined in the presence of 0.006 ng.mL\(^{-1}\) sitagliptin (Table 2). The extent of DPP-IV inhibition observed with 0.006 ng.mL\(^{-1}\) sitagliptin alone was 39.7 ± 2.0 % . As expected, there was a significant reduction ($P < 0.05$) in the IC\(_{50}\) value for diprotin A (tested at final concentrations ranging from 12.5 × 10\(^{-3}\) to 12.5 µg.mL\(^{-1}\)) and WPH, Trp-Val and Val-Ala (tested at final concentrations ranging from 0.6 × 10\(^{-2}\) to 0.6 mg.mL\(^{-1}\)), in the presence of 0.006 ng.mL\(^{-1}\) sitagliptin. The comparable decrease in IC\(_{50}\) value was the largest with diprotin A (51.8 %) and the lowest with WPH (13.6 %).

4. Discussion

Ingestion of milk proteins, milk-derived peptides and associated amino acids has been shown to increase insulin secretion in humans (Akhavan, Luhovyy, Brown, Cho, & Anderson, 2010; Frid, Nilsson, Holst, & Björck, 2005; Geerts et al., 2011; Luhovyy, Akhavan, & Anderson, 2007; Manders et al., 2006a; Manders et al., 2006b; Morifuji et al., 2010; Power et al., 2009). Previous
studies have highlighted the role of milk protein hydrolysates and peptides in the inhibition of DPP-IV (Lacroix & Li-Chan, 2012a; Uchida et al., 2011). The IC$_{50}$ values described for the whey protein sample herein are of the same order as values previously reported in the literature. A 1 kDa permeate of a salmon gelatin hydrolysate having an IC$_{50}$ of 1.35 mg.mL$^{-1}$ (Li-Chan, Hunag, Jao, Ho, & Hsu, 2012), rice bran hydrolysates with IC$_{50}$ values of 2.3 ± 0.1 and 26.4 ± 2.3 mg.mL$^{-1}$ (Hatanaka et al., 2012) and amaranth glutelin hydrolysates with IC$_{50}$ ranging from 1.2 to 2.0 mg.mL$^{-1}$ (Velarde-Salcedo et al., 2013) have been reported. The milk-derived peptide sequences responsible for DPP-IV inhibition described in the literature are generally di- and tri-peptides (Lacroix & Li-Chan, 2012b), with a molecular mass < 1 kDa. The gel permeation results herein show that peptides less than 1 kDa represent a relatively high proportion (54.7 %) of WPH.

UF fractionation of milk-derived hydrolysates with DPP-IV inhibitory properties has previously been described in the literature where DPP-IV inhibition was higher with some UF fractions in comparison to the parent hydrolysate (Lacroix & Li-Chan, 2012a). However, the most potent fractions were not in all cases lower than 1 kDa. For a whey protein hydrolysate, the fraction between 1 and 3 kDa showed the highest DPP-IV inhibition (Lacroix & Li-Chan, 2012a). This was explained by the fact that some of the higher molecular weight peptides from whey may have been substrates for DPP-IV, thereby interfering with hydrolysis of the synthetic substrate (Gly-Pro-pNA) resulting in an apparently higher extent of inhibition (Lacroix & Li-Chan, 2012a). It was shown for the UF fractions of a salmon gelatin hydrolysate that a greater DPP-IV inhibition was observed with the 1 kDa permeate compared to the higher molecular mass fractions (Li-Chan et al., 2012). The same trend was found herein with the lowest IC$_{50}$ value being observed for the WPH 2 kDa permeate (Table 1), which was significantly enriched in peptides less than 1 kDa (Fig. 1). In agreement with these results, all UF retentates were significantly less potent than the permeates (Table 1). The hydrophilic fraction of WPH also showed a significantly lower IC$_{50}$ value than WPH, suggesting that physicochemical
characteristics other than the molecular mass of the peptides may play a role in DPP-IV inhibition. It has been observed that the DPP-IV inhibitory properties of a peptide are governed by its structure (Hatanaka et al., 2012; Lacroix & Li-Chan, 2012b; Uenishi et al., 2012). Therefore, peptide composition and structure rather than molecular mass appears to govern the overall DPP-IV inhibition observed within a particular sample (Lacroix & Li-Chan, 2012a; Li-Chan et al., 2012).

The mode of action of the different samples was studied. From the Lineweaver and Burk kinetic plots, it was evident that the samples studied behaved as competitive inhibitors of DPP-IV (Fig. 3). This indicates that the hydrolysate and its associated UF and hydrophilic fractions and the SGID sample interacted with the active site of the enzyme. It has been shown that diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) are also substrates for DPP-IV which may explain the apparent competitive type of inhibition observed with these compounds (Rahfeld, Schierborn, Hartrodt, Neubert, & Heins, 1991). Lacroix & Li-Chan (2012a) also observed that some milk-derived peptide inhibitors may have structural similarities with DPP-IV substrates and therefore they may behave as substrates for this enzyme.

SGID of WPH was investigated to study the stability of DPP-IV inhibitory peptides to further hydrolysis by digestive enzymes. The RP profiles obtained suggest that certain peptides present in WPH are degraded following SGID (Fig. 2B). The IC₅₀ of the SGID samples was significantly lower (P < 0.05) than that of WPH (Table 1). Similar results were found in another study where DPP-IV inhibition with tuna-derived peptides was unaffected or increased following SGID (Huang, Jao, Ho, & Hsu, 2012). The fact that some low molecular mass peptides may be responsible for the DPP-IV inhibition observed with WPH is further reinforced by the increase in potency of the SGID sample. As expected, short peptide sequences can be relatively stable to SGID. Out of 228 dipeptides studied, Foltz, van Bure, Klaffke & Duchateau (2009) reported that half of the dipeptides were highly stable to hydrolysis by pancreatic enzymes. The relatively
small size (< 1 kDa) and the gastrointestinal stability of the bioactive peptides within WPH may allow them to cross the gut barrier (Foltz et al., 2007; Gardner, 1983; Morifuji et al., 2010). However, intestinal permeation may not be a limiting factor for the DPP-IV inhibitory activity to be seen as degradation of incretins such as GIP and GLP-1 occurs directly in the gastrointestinal tract (Tulipano et al., 2011). The SGID results herein indicate that the DPP-IV inhibitory properties of WPH may pertain in vivo.

Despite significant differences seen in the IC$_{50}$ values of the WPH and its associated SGID sample and fractions, it is likely that in a physiological situation, these differences will not pertain for certain samples. The IC$_{50}$ values for WPH, the SGID sample and the fractions of WPH are relatively high. The IC$_{50}$ values for WPH is more than 40 times higher than that of Trp-Val and Val-Ala and more than 900 times higher to that of diprotin A and sitagliptin. Overall, the potency of WPH and its associated SGID sample and fractions is modest. Therefore it is anticipated that in vivo, WPH, its associated SGID sample and fractions may display very similar effects in terms of DPP-IV inhibition.

To our knowledge, no data is currently available in the literature describing the combined effects of bioactive peptides and drugs on DPP-IV inhibitory properties. DPP-IV inhibition with different mixtures of whey-derived peptides and sitagliptin was therefore investigated. Trp-Val and Val-Ala were previously identified as whey-derived DPP-IV inhibitors with IC$_{50}$ values of 65.69 ± 2.95 and 168.24 ± 7.96 µM, respectively. In addition, Trp-Val was also shown to have both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide (SO) scavenging properties and Val-Ala had SO scavenging properties (Nongonierma & FitzGerald, 2013). At all concentrations of inhibitors (diprotin A, Trp-Val, Val-Ala or WPH) tested in combination with sitagliptin, the DPP-IV inhibition was higher or equal to that of sitagliptin alone. DPP-IV inhibition was significantly higher ($P < 0.05$) than that of sitagliptin alone only at the highest concentrations of diprotin A, Trp-Val and Val-Ala tested. These results suggested that a certain concentration of these peptides
was required to observe an increased DPP-IV inhibition over that of sitagliptin alone.

Furthermore, it was shown that in the presence of 0.006 ng.mL⁻¹ sitagliptin (39.7 ± 2.0 % DPP-IV inhibition), the apparent IC₅₀ of whey-derived inhibitors was significantly decreased (P < 0.05). This result suggested that there was a combined additive effect of sitagliptin and whey-derived peptides on DPP-IV inhibition. The significance of these results needs to be assessed in humans to determine the implications of these findings for the optimum dosage of sitagliptin for T2D subjects.

Conclusion

A whey-derived hydrolysate with DPP-IV inhibitory properties has been generated. The competitive inhibition of DPP-IV observed herein suggests direct interaction by the hydrolysate and its associated fractions and SGID sample with the active site of DPP-IV. WPH may have potential as a dietary ingredient with applications in enhancement of the half-life of incretins. Determination of the bioactive peptide sequences within WPH would allow investigation of the mode of action of these peptides for their DPP-IV inhibitory properties. Finally, combinations of sitagliptin with different whey-derived peptides showed an additive DPP-IV inhibition. This may have implications for an holistic strategy combining a clinical approach, dietary changes and physical activity in the management of T2D.
Acknowledgements

The work described herein was supported by Enterprise Ireland under Grant Number CC20080001. The authors would like to thank Dr. C. Gaudel and Prof. P. Newsholme for reviewing this manuscript.
References


Table 1 Inhibitory concentration inducing 50% inhibition (IC$_{50}$) for dipeptidyl peptidase IV (DPP-IV) and type of inhibition determined by Lineweaver and Burk analysis in the presence of a whey protein hydrolysate (WPH), its associated ultrafiltration and hydrophilic fractions and simulated gastrointestinal digest (SGID). Values are mean ± confidence interval of triplicate determinations (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPP-IV IC$_{50}$ (mg.mL$^{-1}$)</th>
<th>Kapp or Km (mM Gly-Pro-pNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPH</td>
<td>1.33 ± 0.17$^g$</td>
<td>1.31*</td>
</tr>
<tr>
<td>WPH- 5 kDa Retentate</td>
<td>1.98 ± 0.35$^b$</td>
<td>0.66*</td>
</tr>
<tr>
<td>WPH- 5 kDa Permeate</td>
<td>0.95 ± 0.16$^c$</td>
<td>1.36*</td>
</tr>
<tr>
<td>WPH- 2 kDa Retentate</td>
<td>0.72 ± 0.01$^d$</td>
<td>0.80*</td>
</tr>
<tr>
<td>WPH- 2 kDa Permeate</td>
<td>0.48 ± 0.01$^c$</td>
<td>1.44*</td>
</tr>
<tr>
<td>WPH- hydrophilic</td>
<td>1.11 ± 0.09$^f$</td>
<td>1.74*</td>
</tr>
<tr>
<td>WPH- SGID</td>
<td>1.02 ± 0.05$^e$</td>
<td>1.44*</td>
</tr>
<tr>
<td>diprotin A</td>
<td>0.001454±0.000218$^b$</td>
<td>1.79*</td>
</tr>
<tr>
<td>sitagliptin</td>
<td>0.000036 ± 0.000008$^a$</td>
<td>nd</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>na</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Values represent mean IC$_{50}$ values ± confidence interval (P = 0.05) for triplicate determination (n=3). Values with different superscript letters are significantly different ($P < 0.05$).

Kapp value determined using Lineweaver and Burk plots as described in Nongonierma and FitzGerald (2012). *$P < 0.05$ Kapp (determined in the presence of inhibitor) vs Km (determined without inhibitor).

na: not applicable
nd: not determined
Table 2 Apparent inhibitory concentration inducing 50 % inhibition for dipeptidyl peptidase IV (DPP-IV) in the presence of 0.006 mg.mL\(^{-1}\) sitagliptin (39.7 ± 2.0 % DPP-IV inhibition) and different whey-derived DPP-IV inhibitors and diprotin A. Values are mean ± SD of triplicate determinations (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPP-IV IC(_{50}) (mg.mL(^{-1}))</th>
<th>Apparent DPP-IV IC(_{50}) in the presence of 0.006 ng.mL(^{-1}) sitagliptin (µM)</th>
<th>Reduction in apparent IC(_{50}) compared to inhibitor alone (%)(^{2,3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>diprotin A</td>
<td>0.001454 ± 0.000218(^{a})</td>
<td>4.3 ± 0.6(^{a})</td>
<td>2.1 ± 0.1(^{a})</td>
</tr>
<tr>
<td>Trp-Val</td>
<td>0.020 ± 0.001(^{b})</td>
<td>67.5 ± 2.4(^{b})</td>
<td>40.9 ± 1.4(^{b})</td>
</tr>
<tr>
<td>Val-Ala</td>
<td>0.032 ± 0.001(^{b})</td>
<td>171.3 ± 6.8(^{c})</td>
<td>135.9 ± 1.1(^{c})</td>
</tr>
<tr>
<td>WPH</td>
<td>1.333 ± 0.168(^{c})</td>
<td>na</td>
<td>1.149 ± 0.051(^{d})</td>
</tr>
</tbody>
</table>

\(^{1}\)Values represent mean IC\(_{50}\) values ± confidence interval (P = 0.05) for triplicate determination (n=3). Within the same column, values with different superscript letters are significantly different (P < 0.05).

\(^{2}\) Reduction in apparent IC\(_{50}\) compared to inhibitor alone = \(1 - \frac{\text{Apparent IC}_{50} \text{ with sitagliptin}}{\text{IC}_{50}}\) × 100

\(^{3}\) *P < 0.05 vs DPP-IV inhibition determined with diprotin A, Trp-Val, Val-Ala or WPH alone.

na: not applicable
Figure captions

Fig. 1. Gel permeation profiles of the unhydrolysed whey proteins (WP), the whey protein hydrolysate (WPH) and its ultrafiltered 5 and 2 kDa permeates (Perm) and retentates (Ret).

Molecular mass distribution: □: <1 kDa, ■: 1-10 kDa and ▼: >10 kDa.

Fig. 2. Reverse-phase ultra-performance liquid chromatography (RP-UPLC) of (A) the whey protein hydrolysate (WPH), (B) its associated simulated gastrointestinal digestion (SGID) sample and (C) its hydrophilic fraction obtained following solid-phase extraction.

Fig. 3. Lineweaver and Burk plots of dipeptidyl peptidase IV (DPP-IV) in the presence (◇) and absence (□) of whey protein hydrolysate (WPH) derived inhibitors (A) WPH, (B) WPH 5 kDa permeate, (C) hydrophilic fraction of WPH and (D) simulated gastrointestinal digest (SGID) of WPH. Each point represents the mean of 3 values (n=3).

Fig. 4. Inhibition of dipeptidyl peptidase IV (DPP-IV) by food-derived peptides in the presence (●) and absence (▲) of 0.031 ng.mL⁻¹ sitagliptin (59.2 ± 2.7 % DPP-IV inhibition) combined with (A) diprotin A, (B) whey protein hydrolysate (WPH), (C) Trp-Val and (D) Val-Ala. Each point represents the mean of 3 values (n=3). *: DPP-IV inhibition values determined in the presence and absence of 0.031 ng.mL⁻¹ sitagliptin are significantly different at P < 0.05.
Fig. 1
Fig. 2
Fig. 3
Fig. 4