Release of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from milk protein isolate (MPI) during enzymatic hydrolysis

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Abstract

The release of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from bovine milk protein isolate (MPI) during trypsin hydrolysis was studied using a design of experiments (DOE) approach. A 3 factor×3 level DOE including temperature (40, 50 and 60°C), enzyme to substrate ratio (E:S; 0.50, 1.25 and 2.00% (w/w)) and hydrolysis time (60, 150 and 240 min) was used during the generation of 15 hydrolysates (H1-H15). The degree of hydrolysis (DH) varied between 6.98 ± 0.31 (H8) to 12.75 ± 0.62% (H10). The DPP-IV half maximal inhibitory concentration (IC_{50}) ranged from 0.68 ± 0.06 (H11)/0.68 ± 0.10 (H4) to 1.59 ± 0.11 mg mL^{-1} (H8). Temperature had no effect (p > 0.05) on the DPP-IV IC_{50} value, while an increase in E:S or time significantly decreased DPP-IV IC_{50} value (p < 0.05). The DPP-IV IC_{50} value of 0.69 mg mL^{-1}, predicted by response surface methodology (RSM), to be obtained with an hydrolysate generated at 50.5°C, 2% ES and 231 min (H16) was similar to the experimentally obtained value (DPP-IV IC_{50} = 0.66 ± 0.10 mg mL^{-1}, p > 0.05, n=3). Following simulated gastrointestinal digestion (SGID) of H16 (H16_CorPP), the DPP-IV IC_{50} value increased (p < 0.05) to 0.90 ± 0.07 mg mL^{-1}. There was no significant difference between the DPP-IV IC_{50} value of the SGID of MPI (MPI_CorPP, 0.89 ± 0.11 mg mL^{-1}) and that of H16_CorPP. Potent known DPP-IV inhibitory peptide sequences were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) within H16, some of which were also present within H16_CorPP. MPI hydrolysates may be of interest for serum glucose regulation in humans.

Key words: dipeptidyl peptidase IV inhibition; milk protein isolate; bioactive peptides; trypsin; response surface methodology.
1 Introduction

Intact and hydrolysed milk proteins appear quite promising as antidiabetic agents for humans (for reviews, see: Lacroix & Li-Chan, 2014b; Nongonierma & FitzGerald, 2015b). However, the mechanisms of action explaining their antidiabetic effects are, to date, not fully understood. These may involve insulinotropic effects, inhibition of metabolic enzymes or incretin secretagogue effects (Nongonierma & FitzGerald, 2015a). Dipeptidyl peptidase IV (DPP-IV) is a metabolic enzyme which plays a role in serum glucose regulation in humans. During the post-prandial phase, DPP-IV can cleave incretins including glucagon-like peptide-1 (GLP-1) and glucose inhibitory polypeptide (GIP). Incretin degradation causes a loss in their ability to enhance insulin secretion (insulinotropic action), resulting in a dysregulation in serum glucose level (Drucker, 2006; Juillerat-Jeanneret, 2014). DPP-IV inhibition is used as a target for antidiabetic drugs (i.e., gliptins). Interestingly, there is growing evidence showing that certain food protein-derived peptides also have the ability to inhibit DPP-IV (for reviews, see: Jao et al., 2015; Lacroix & Li-Chan, 2016; Nongonierma & FitzGerald, 2016b). To date, most studies describing the enzymatic generation of DPP-IV inhibitory hydrolysates appear to have been carried out with either casein (CN) or whey protein substrates (Lacroix & Li-Chan, 2016). Previous studies have suggested that peptide mixtures may have additive or antagonistic effects on DPP-IV inhibition (Nongonierma & FitzGerald, 2015c). Utilisation of substrates containing both CNs and whey proteins may therefore allow a better understanding of the overall DPP-IV inhibitory effects of bovine milk protein hydrolysates. In this context, milk protein isolate (MPI), a commercially available protein enriched substrate from bovine milk, was studied herein. Bovine MPI contains ~90% (w/w) protein with the same CN and whey protein ratio as in bovine milk (i.e., 80 and 20% (w/w), respectively). In addition, MPI contains low levels of fat and lactose, which make it very attractive for a wide range of nutritional applications (for review, see: Agarwal, Beausire, Patel, & Patel, 2015).
The bioactive potency of food protein hydrolysates, in the context of enzyme inhibition, is often estimated by their half maximal inhibitory concentration (IC$_{50}$). Most milk protein hydrolysates reported in the literature, to date, appear to display IC$_{50}$ values $\leq 1.5$ mg mL$^{-1}$ (Lacroix & Li-Chan, 2016). An increased need has been recently placed on the development of milk protein hydrolysates with enhanced bioactive potency in order to comply with the requirement for ingestion of physiologically relevant amounts as recommended by regulatory agencies such as the European Food Safety Authority (EFSA). Higher bioactive potency may be achieved by increasing the release of potent bioactive peptides during enzymatic hydrolysis. Several factors, which may be classified as intrinsic (i.e., protein type and concentration, enzyme preparation and concentration, solvent, pH, ionic strength, etc.) or extrinsic (i.e., temperature, pressure, etc.), are known to modify the release of bioactive peptides during the enzymatic hydrolysis of food proteins. This may, in certain instances, be linked with modification of enzyme selectivity for peptide bond cleavage, e.g., as a function of pH or substrate concentration (Butré, Sforza, Gruppen, & Wierenga, 2014; Butré, Sforza, Wierenga, & Gruppen, 2015). An efficient manner to optimise the generation of potent bioactive hydrolysates may be through the utilisation of multifactorial design of experiments (DOE) followed by prediction of optimum hydrolysis parameters with response surface methodology (RSM) (Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Nongonierma, Le Maux, Esteveny, & FitzGerald, 2017; van der Ven, Gruppen, de Bont, & Voragen, 2002).

The aim of this study was to evaluate the role of different parameters on the DPP-IV inhibitory properties of MPI hydrolysed with trypsin. A three factor (temperature, time and enzyme to substrate (E:S) ratio) $\times$ three level DOE was used herein. Physicochemical characteristics (degree of hydrolysis (DH), peptide profile and molecular mass distribution) of the hydrolysates were determined. The model used to develop the RSM was verified by selecting hydrolytic parameters (i.e., temperature, time and E:S) yielding low DPP-IV half maximal inhibitory concentration (IC$_{50}$) within the boundaries of the DOE. The most promising hydrolysate was subjected to an in
vitro simulated gastrointestinal digestion (SGID) protocol to assess the effect of digestive enzymes on DPP-IV inhibitory properties. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify the peptides within selected hydrolysates.

2 Materials and methods

2.1 Reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS) from Pierce Biotechnology was obtained from Medical Supply Company (Dublin, Ireland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (TRIS), sodium phosphate monobasic, sodium phosphate dibasic, sodium dodecyl sulfate (SDS), Gly-Pro-pNA, Leu, diprotin A (Ile-Pro-Ile), porcine DPP-IV (≥ 10 units mg\(^{-1}\) protein), standards for molecular mass distribution (i.e., bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), aprotinin, bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr), mass spectrometry (MS) grade water and ACN were purchased from Sigma Aldrich (Dublin, Ireland). Synthetic peptides (purity ≥ 95%) for MS studies were purchased from Bachem (Bubendorf, Switzerland, Ala-Leu, Trp-Tyr, Trp-Lys, Tyr-Leu, Tyr-Ile and Val-Leu), Thermo Fisher Scientific (Ulm, Germany, Trp-Gln, Leu-Leu, Ile-Leu, Leu-Ile, Ile-Ile and Leu-Pro-Leu) and Sigma-Aldrich (Phe-Leu). MPI (86% (w/w) protein) was obtained from Kerry Ingredients (Listowel, Ireland). Trypsin was provided by Novozymes (PTN 6.0S, Bagsvaerd, Denmark), pepsin by Biocatalysts (Cefn, Wales, UK) and Corolase PP (CorPP) by AB enzymes (Darmstadt, Germany).

2.2 Tryptic hydrolysis of MPI

Three parameters, i.e., temperature, E:S and hydrolysis time, which have been shown to affect
bioactive peptide release during enzymatic hydrolysis of milk proteins (Contreras et al., 2011; van der Ven et al., 2002), were selected. The temperature range studied corresponded to the optimum range for trypsin while hydrolysis time and E:S ratios were selected based on previous studies (Nongonierma & FitzGerald, 2012). MPI was hydrolysed with trypsin using a DOE incorporating the parameters incubation temperature (40, 50 and 60°C), E:S (0.50, 1.25 and 2.00% (w/w)) and hydrolysis time (60, 150 and 240 min) which were evaluated at three (-1, 0 and +1, respectively) different levels (Table 1). Fourteen hydrolysates (H1-H14) were generated once within the DOE. In addition, the central point (H15, 50°C, 1.25% E:S and 150 min) conditions were used to generate three independent MPI hydrolysates (n=3, H15A, H15B and H15C) to verify reproducibility of the hydrolytic reaction.

MPI hydrolysis was conducted as described earlier (Nongonierma et al., 2017). Briefly, MPI (10% (w protein equivalent/w)) was resuspended in distilled water and incubated for 60 min in a water bath (Lauda E100, Lauda Brinkmann, Lauda-Königshofen, Germany) set at 50°C. The pH was then adjusted to 7.0 with 2 M NaOH. Temperature, E:S and hydrolysis time were set as defined by the DOE. At the end of each reaction, the enzyme was heat inactivated (90°C, 20 min) in a water bath. A control sample (unhydrolysed MPI) without enzyme addition was prepared for each hydrolysis temperature and time.

Hydrolysis conditions yielding the generation of the MPI hydrolysate having low DPP-IV IC$_{50}$ value within the boundaries of the DOE were predicted by RSM (section 2.3.). The corresponding MPI hydrolysate was generated in three independent replicates (H16A, H16B and H16C) to externally validate the predictive model and the reproducibility of the hydrolytic reaction process.

All samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C until utilisation.

2.3 RSM for the prediction of DPP-IV IC$_{50}$ as a function of hydrolysis parameters of MPI
The RSM curves were generated within the boundaries of the DOE with a multilinear regression (MLR) model (Equation 1, complete model) developed using Matlab (version R2015b, MathWorks, Inc, Natick, MA, USA) as previously described (Nongonierma et al., 2017).

\[
Y = \beta_0 + \beta_1 T + \beta_2 ES + \beta_3 t + \beta_4 T^2 + \beta_5 ES^2 + \beta_6 t^2 + \beta_7 T \times ES + \beta_8 T \times t + \beta_9 ES \times t + \epsilon
\]  
(Equation 1)

With Y, the DPP-IV IC\textsubscript{50} value; \( \beta_0 \) to \( \beta_9 \): the coefficients of the MLR model; T (temperature), E:S (enzyme to substrate ratio) and t (time): the centred and reduced (z-centred) parameters of the experimental design and \( \epsilon \): the residual of the model.

The complete model (Equation 1) was simplified (simplified model) by taking into account the main parameters (T, ES and t) and their interactions having a \( p \)-value < 0.1 (Contreras et al., 2011). The RSM curves were determined within the boundaries of the DOE. Subsequently, hydrolysis parameters yielding low DPP-IV IC\textsubscript{50} values were determined (Nongonierma & FitzGerald, 2016a; van der Ven et al., 2002).

2.4 In vitro SGID of intact and hydrolysed MPI

In vitro SGID was conducted with unhydrolysed MPI and H16 as outlined in Walsh et al. (2004). Briefly, samples were resuspended in distilled water to 2% (w protein equivalent /w) for 30 min at 37°C and the pH was adjusted to 2.0 using 1 N HCl. Hydrolysis with pepsin (E:S 2.5% (w/w)) was carried out under pH regulation (2.0) with HCl (pH stat Titrando 843, Tiamo 1.4 Metrohm, Dublin, Ireland) for 90 min at 37°C. Pepsin was then heat inactivated (90°C, 20 min). An aliquot of the peptic hydrolysate (MPI_pepsin or H16_pepsin) was brought to pH 7.5 using 1 M NaOH and then hydrolysed with CorPP (E:S 1% (w/w)) for 150 min at 37°C, pH 7.5 using a pH stat (Metrohm), to yield MPI_CorPP or H16_CorPP. The reaction was terminated by thermal treatment (90°C, 20 min). Samples were freeze-dried and stored at -20°C until utilisation.
2.5 DPP-IV inhibition assay

The DPP-IV inhibition assay was carried out in triplicate as outlined by Nongonierma and FitzGerald (2013a). The freeze-dried samples were dispersed in HPLC grade water at concentrations ranging from $1.1 \times 10^{-2}$ to 2.7 mg mL$^{-1}$ (final concentration expressed in mg protein equivalent mL$^{-1}$). Diprotin A, a well-known DPP-IV inhibitor, was used as a positive control at final concentrations ranging from $12.5 \times 10^{-2}$ and 12.5 µg mL$^{-1}$. Essentially, 25 µL sample was pipetted in a 96 well clear microplate (Sarstedt, Dublin, Ireland) containing the reaction substrate, Gly-Pro-pNA (final concentration 0.200 mM). The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 units mL$^{-1}$). All reagents were diluted in 100 mM Tris-HCl buffer pH 8.0. The negative control consisted of 100 mM Tris-HCl buffer pH 8.0 and Gly-Pro-pNA. The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA). Absorbance of the pNA released from the substrate by DPP-IV was monitored at 405 nm. The DPP-IV IC$_{50}$ values were determined by plotting the percentage inhibition as a function of the test compound concentration. Each analysis was conducted in triplicate (n=3).

2.6 DH determination

The DH of the hydrolysates (H1-H16) was determined in triplicate (n=3) using the TNBS method as per Le Maux, Nongonierma, Barre, and FitzGerald (2016). Absorbance values (350 nm) were measured with a microplate reader (Biotek Synergy HT). DH was calculated using Equation 2:

$$DH = 100 \times \frac{(AN_2 - AN_1)}{Npb}$$  \hspace{1cm} (Equation 2)

With AN$_1$, the amino nitrogen content of the unhydrolysed protein (mg g$^{-1}$ protein); AN$_2$, the amino nitrogen content of the hydrolysate (mg g$^{-1}$ protein) and Npb, the nitrogen content of the peptide bonds in the protein substrate (114.34 for MPI).
2.7 Peptide profile of the hydrolysates by reverse-phase ultra-performance liquid chromatography (RP-UPLC)

Peptide profiles were determined by RP-UPLC (UPLC Acquity - Waters, Milford, MA, USA) as described previously (Nongonierma & FitzGerald, 2012). Briefly, samples were resuspended (0.4% w protein equivalent/v) in solvent A (0.1% (v/v) TFA in MS grade water) and filtered with a 0.2 µm cellulose acetate filters (VWR). Peptide separation was carried out at 30°C with a flow rate of 0.3 mL min⁻¹ and an injection volume of 10 µL. An Acquity UPLC BEH C18, 130 Å column (2.1 mm × 50 mm × 1.7 µm) equipped with an Acquity BEH C18 (1.7 µm) vanguard pre-column, both from Waters, were used. Peptides and proteins were eluted using a linear gradient: 0-0.28 min: 100% A; 0.28-60 min: 100-40% A, with solvent B consisting of 0.1 % (v/v) TFA and 80% MS grade ACN in water. Absorbance was monitored at 214 nm.

2.8 Molecular mass distribution of the hydrolysates by gel permeation high performance liquid chromatography (GP-HPLC)

The MPI hydrolysates were analysed by GP-HPLC as described earlier (Spellman, O’Cuinn, & FitzGerald, 2009). An HPLC system (Waters model 600 binary pump, model 2707 autosampler and model 2489 dual λ absorbance detector interfaced with Empower™) was employed to analyse samples. Separation of compounds was conducted in isocratic mode at 21°C using a TSK G2000 SW separating column (600×7.5 mm ID - Tosoh Bioscience, Tokyo, Japan) connected to a TSKGEL SW guard column (75×7.5 mm ID - Tosoh Bioscience) with a mobile phase made of 0.1% (v/v) TFA and 30% HPLC grade ACN in HPLC water. The flow rate was of 0.5 mL min⁻¹ for 60 min. A volume of 20 µL sample (0.22% (w protein equivalent/v) in mobile phase filtered through 0.2 µm PTFE syringe filters (VWR)) was injected. The absorbance was monitored at 214 nm. The molecular mass standards consisted of BSA, β-Lg, α-La, aprotinin, bacitracin, Leu-Trp-Met-Arg, Asp-Glu and Tyr. Molecular mass distribution of compounds > 10, 10-5, 5-1 and < 1
2.9 Peptide identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

Peptide identification in H4, H11, H16 and the SGID of H16 (H16_CorPP) and MPI (MPI_CorPP) was carried out by LC-MS/MS. An Acquity UPLC (Waters) fitted with an Acquity BEH amide 300 C18 column (2.1×50 mm, 1.7 μm, Waters) was used. The UPLC was coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF, Impact HD™, Bruker Daltonics GmbH, Bremen, Germany) using a 50-600 and 50-2500 m/z acquisition range as described earlier (Norris, Poyarkov, O’Keeffe, & FitzGerald, 2014; O’Keeffe & FitzGerald, 2015). The MS was fitted with an electrospray ionisation (ESI) source used in positive ion mode. Data acquisition was performed with Hystar software (Bruker Daltonics). Samples were resuspended in mobile phase A (0.1% formic acid in MS water) to a final concentration of 0.1 g L⁻¹. A sample volume of 2 μL was injected. Peptide identification was carried out with PEAKS Studio (version 7.5, Bioinformatics Solutions Inc, Waterloo, Canada). The false discovery rate (FDR), average local confidence (ALC) and MS/MS tolerance were set at 1, 60% and 0.1 Da respectively. In addition, synthetic peptides (Ala-Leu, Phe-Leu, Leu-Leu/Ile-Leu/Leu-Ile/Ile-Ile, Leu-Pro-Leu, Val-Leu, Trp-Gln, Trp-Lys, Trp-Tyr, Tyr-Leu/Tyr-Ile) were used to spike hydrolysate samples, which were subsequently analysed using the same LC-MS/MS conditions in order to confirm the software identification (data not shown). All other amino acid sequences generated by PEAKS Studio software were manually checked in order to (1) verify that the peptide sequence corresponded with the same amino acid sequence in the main milk proteins and (2) control the peptides fragmentation data as ion series continuity and major peaks identified as b or y ions.

Previously identified DPP-IV inhibitory peptides within the samples were then determined. In addition, features of known DPP-IV inhibitory peptides reported in the literature, which consist in an hydrophobic amino acid (Trp, Leu, Ile or Phe) at the N-terminus and/or a Pro/Ala at position...
2, and/or Pro at the C-terminus (Nongonierma & FitzGerald, 2013b, 2014a; Nongonierma & FitzGerald, 2016a; Tulipano, Faggi, Nardone, Cocchi, & Caroli, 2015) were searched for within the peptides identified.

2.10 Statistical analysis

Differences between mean DH and DPP-IV IC$_{50}$ values were analysed with a one-way analysis of variance (ANOVA) at a significance level $p < 0.05$. Multiple means comparison ($p < 0.05$) was conducted using a post-hoc Student Newman Keuls test with SPSS (version 22, SPSS Inc., Chicago, IL, USA). The lack of significant difference between the experimentally determined and predicted DPP-IV IC$_{50}$ values of H16 was assessed with Matlab using a one-sample Student test (t-test) with a significance of $p < 0.05$.

3 Results and Discussion

3.1 Physicochemical and DPP-IV inhibitory properties of the MPI hydrolysates (H1-H15) generated within the DOE

The molecular mass distribution profiles (Fig. 1) of the hydrolysates H1-H15 showed the presence of components $> 10$ kDa, corresponding to unhydrolysed proteins. However, in all hydrolysates the largest proportion ($70 > %$) of material was $< 5$ kDa, showing that most intact proteins were degraded during the hydrolytic reaction. This was also illustrated by the differences in peptide profiles between the intact and hydrolysed MPI (Fig. 2A). In the hydrolysates (H4 and H11), the peaks corresponding to intact proteins seen in the MPI RP-UPLC profile were only present at low intensity. Hydrolysates contained numerous peptide peaks eluting throughout the chromatogram. When comparing two hydrolysates (H4 and H11) having the same DPP-IV inhibitory properties (Table 1), minor differences in terms of occurrence and intensity of peptide peaks were seen on the RP-profiles (Fig. 2A). The DH of the MPI hydrolysates generated within
the DOE is reported is Table 1. Hydrolysate generation was reproducible from a DH perspective as indicated by the values obtained for the triplicate central point hydrolysates (H15A, H15B and H15C) which were not significantly different ($p > 0.05$). The DH values of the 15 hydrolysates generated within the DOE ranged between 6.98 ± 0.31 (H8) and 12.75 ± 0.62% (H10). These values were of the same order as whey protein hydrolysates generated with PTN6.0S using similar hydrolytic conditions (Pouliot, Wijers, Gauthier, & Nadeau, 1999).

Similarly to the DH, no significant differences ($p > 0.05$, Table 1) were found for the DPP-IV IC$_{50}$ values of H15A, H15B and H15C. Differences were seen in the DPP-IV IC$_{50}$ values of the 15 hydrolysates generated within the DOE (Table 1). The highest DPP-IV IC$_{50}$ value was found with H8 (1.59 ± 0.11 mg mL$^{-1}$) while the lowest was seen with both H4 (0.68 ± 0.10 mg mL$^{-1}$) and H11 (0.68 ± 0.06 mg mL$^{-1}$). The relatively high DPP-IV inhibitory potency of H4 and H11 may be linked with the high E:S (2 %) and the long hydrolysis time ($\geq 150$ min) used during their generation, which resulted in DH values $> 11\%$ (Table 1). Milk proteins are currently the most commonly used substrate for the generation of hydrolysates having DPP-IV inhibitory properties (Nongonierma & FitzGerald, 2016b). *In silico* analyses of individual milk proteins predicted that both CNs and whey proteins contain relatively potent DPP-IV inhibitory peptides (Lacroix & Lee-Chan, 2012; Nongonierma & FitzGerald, 2014a). To date, it appears that hydrolysates generated from substrates containing both CNs and whey proteins have not been extensively investigated for their ability to inhibit DPP-IV. Therefore, bovine MPI, a protein rich-substrate containing both CNs and whey proteins, was hydrolysed with trypsin herein. The range of DPP-IV IC$_{50}$ values (0.62 ± 0.11 to 1.59 ± 0.11 mg mL$^{-1}$, Table 1) obtained with the MPI hydrolysates herein was of the same order as that of tryptic whey (1.00 mg mL$^{-1}$) or CN (1.11 mg mL$^{-1}$)-based hydrolysates (Nongonierma & FitzGerald, 2013a).

The complete and simplified MLR models both gave a good fit of the experimental DPP-IV IC$_{50}$ values as indicated by the non-significant lack of fit ($p > 0.05$, Table 2). Two parameters of the DOE (E:S and t) had an effect on the DPP-IV IC$_{50}$ value ($p < 0.05$, Table 2), while T had no
significant effect ($p > 0.05$). The significance of $t^2$ ($p < 0.05$), was linked with the existence of an optimum time within the DOE boundaries. Both coefficients associated with E:S and $t$ were negative, indicating that an increase in E:S or $t$ would result in a reduction in DPP-IV IC$_{50}$ value. The complete model gave a better correlation between the experimental and predicted DPP-IV IC$_{50}$ values (Table 2 and Supplementary Fig. S1). Therefore, the complete model was employed to build the RSM curves. The RSM curves showed a lower DPP-IV IC$_{50}$ value when either E:S or $t$ were at the +1 (2.00 % E:S and 240 min) vs the -1 (0.50 % and 60 min) level (Fig. 3A, 3B and 3C). In contrast, the temperature value minimally affected the DPP-IV IC$_{50}$ value (Fig. 3A and 3B). Both E:S and time can affect the kinetics of peptide bond cleavage and therefore modify peptide release during enzymatic hydrolysis (Contreras et al., 2011). While the extent of $\beta$-Lg hydrolysis by trypsin has been shown in earlier studies to be temperature dependant (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010; Iametti et al., 2002), no temperature effect could be observed in this study. The lack of a temperature effect may arise from the fact that the temperature range studied was within the optimum range for this trypsin preparation.

3.2 Physicochemical characteristics and DPP-IV inhibitory properties of the MPI hydrolysate (H16) predicted by RSM to yield low DPP-IV IC$_{50}$ values

Hydrolysis conditions predicted to yield DPP-IV IC$_{50}$ values < 0.7 mg mL$^{-1}$ (low DPP-IV IC$_{50}$ value range Supplementary Fig S2) were selected within the boundaries of the DOE as 50.5°C (T = +0.05), 231 min (t = +0.90) and 2% E:S (+1.00). These conditions were used to externally validate the predictive model. The complete and simplified models predicted that this hydrolysate (H16) would yield a DPP-IV IC$_{50}$ value of 0.69 and 0.68 mg mL$^{-1}$, respectively.

The corresponding hydrolysates (H16A, H16B and H16C) had a similar DH (mean value of 12.96 ± 1.51%, $p > 0.05$, Table 1). All three hydrolysates, which contained > 90% material < 5 kDa, displayed similar molecular mass distribution profiles (Fig. 1). The DPP-IV IC$_{50}$ values of H16A, H16B and H16C were not significantly different ($p > 0.05$, Table 1). The experimentally
determined (mean value of 0.66 ± 0.10 mg mL⁻¹) and predicted (0.69/0.68 mg mL⁻¹) DPP-IV IC₅₀ values for H16 were not significantly different (Student test, p > 0.05). These results validated the models developed herein. While the RSM approach has some benefits for predicting optimum hydrolysis parameters during the generation of bioactive peptides (van der Ven et al., 2002), some limitations exists. Notably, the substrate may become limiting during the course of hydrolysis as may be suggested by the low level of variation in the DPP-IV IC₅₀ values (between 0.60 and 0.8 mg mL⁻¹) for a number of hydrolysates generated with different hydrolysis parameters (Table 1 and Supplementary Fig. S2A and S2B). Furthermore, inhibition of trypsin by peptides released during hydrolytic reactions may also occur (Iametti et al., 2002).

3.3 DPP-IV inhibitory properties of H16B and MPI following SGID

Hydrolysate H16B was subjected to the SGID protocol. Hydrolysates H16B before and after pepsin digestion (H16B_pepsin) had similar molecular mass distribution (Fig. 1) as well as peptide profiles (Fig. 2B). The pepsin and CorPP digest (H16B_CorPP) had a different molecular mass distribution (Fig. 1) from H16B, showing > 99% of the material present within H16B_CorPP being < 5 kDa vs. ~ 94% in H16B. Differences were also seen in the RP-UPLC peptide profiles of H16B and H16B_CorPP (Fig. 2B), notably in terms of peptide peaks eluting before 10 and after 15 min. Following SGID of MPI, major differences were seen in the molecular mass distribution, indicating significant protein breakdown in the MPI both after pepsin (MPI_pepsin) and CorPP (MPI_CorPP) digestion (Fig. 1). The MPI proteins were also degraded in MPI_pepsin and MPI_CorPP as indicated by the RP-profiles (Fig. 2C).

A significant increase in the DPP-IV IC₅₀ value of H16B (0.62 ± 0.11 mg mL⁻¹) was seen in H16B_pepsin and H16B_CorPP (0.87 ± 0.05 and 0.90 ± 0.07 mg mL⁻¹, respectively, p < 0.05, Table 1). In contrast, the DPP-IV IC₅₀ value of MPI (> 2.7 mg mL⁻¹) was decreased in MPI_pepsin (2.74 ± 0.53 mg mL⁻¹) and MPI_CorPP (0.89 ± 0.11 mg mL⁻¹, Table 1). No significant differences (p > 0.05, Table 1) were seen between the DPP-IV IC₅₀ values of
MPI_CorPP and H16B_CorPP. Pepsin cleaves proteins and peptides at the C-terminal side of hydrophobic amino acids such as Phe and Leu (Godfrey, 1996). CorPP contains several enzyme activities including trypsin, chymotrypsin, elastase and a range of exopeptidases (Mullally, O'Callaghan, FitzGerald, Donnelly, & Dalton, 1994). Trypsin, chymotrypsin and elastase are mammalian intestinal endoproteinases which preferentially cleave peptide bonds at the C-terminal side of Arg/Lys, bulky hydrophobic amino acids such as Tyr/Trp/Phe/Lys or Leu/Ala/Val/Ile, respectively (Godfrey, 1996). Their cleavage specificity may be affected by hydrolysis temperature. For instance, Cheison et al. (2010) reported modifications of trypsin specificity which resembled that of chymotrypsin when the temperature reached 40-45°C during β-Lg hydrolysis. This was explained by conformational changes in the active site of trypsin, which may have allowed a better accessibility for hydrophobic bulky amino acids. The presence of exopeptidases within CorPP, may have resulted in a further cleavage of peptide bonds within both H16B_CorPP and MPI_CorPP. This hypothesis is consistent with the fact that the RP-UPLC peptide profiles of H16B_CorPP (Fig. 2B) and MPI_CorPP (Fig. 2C) are very similar. In addition, similarities exist between the enzyme activities (i.e., trypsin and chymotrypsin) in PTN 6.0S, the commercial trypsin preparation used during the generation of H16B, and CorPP. In fact certain peptides released by PTN 6.0S were not further degraded following SGID (Table 3).

### 3.4 Peptide identification within MPI hydrolysates

Various MPI hydrolysates (e.g., H4, H11 and H16B) displayed the same DPP-IV IC50 value \( (p > 0.05, \text{Table 1}) \). Despite similarities in their DPP-IV inhibitory potency, some differences in the presence of known DPP-IV inhibitory peptides within these hydrolysates were seen (Table 3). Numerous DPP-IV inhibitory peptides were common between the three samples, some of which were relatively potent, having IC50 values < 100 µM. The most potent peptide identified within these samples was Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr \((\kappa-CN (f51-60))\), which had a reported DPP-IV IC50 value of 40 µM (Zhang, Chen, Ma, & Chen, 2015). All 18 previously
identified DPP-IV inhibitory peptides present within H4 were also observed within H11 and H16B. In addition to these 18 DPP-IV inhibitory peptides identified in H4, H16B also contained a particularly potent DPP-IV inhibitor, Val-Pro-Leu (αs1-CN (f167-169)), known as Diprotin B, having a DPP-IV IC₅₀ value of 15.8 µM (Umezawa et al., 1984), which was not found within H4 or H11. In agreement with trypsin and chymotrypsin specificity, numerous peptides possessing Arg, Lys, Tyr, Trp and Phe residues at their C-terminus were identified within H4, H11 and H16B (Table 3 and 4).

In addition to known DPP-IV inhibitory peptides, the hydrolysates contained peptides possessing the features of DPP-IV inhibitory peptides (i.e., Leu, Ile or Phe at the N-terminus and/or Pro/Ala at position 2, and/or Pro at the C-terminus). The peptides with the above features identified within H16B are summarised in Table 4. H16B did not contain peptides with a Trp at the N-terminus. However, peptides possessing one or several of the other features were identified. While the DPP-IV inhibitory potential of the majority of the peptides reported in Table 4 is unknown, they may play a role in the overall DPP-IV inhibitory activity of H16B. In particular, peptides (possessing a Pro or Ala at the penultimate position) which can act as substrates for DPP-IV are likely to interact with its active site and induce a substrate-type inhibition effect (Nongonierma & FitzGerald, 2014b). Interestingly, a number of substrate-like peptides, mostly arising from CNs, were identified within H16B (Table 4). Leu/Ile residues at the N-terminus of peptides within H16B were also frequently found, arising from either CNs or whey proteins (Table 4). Hydrophobic amino acids such as Leu, Ile and Phe located at the N-terminal position of peptides might interact with the hydrophobic pocket found at the active site of DPP-IV (Engel et al., 2003; Nongonierma, Mooney, Shields, & FitzGerald, 2013), which may explain their DPP-IV inhibitory properties.

All previously identified DPP-IV inhibitory peptides within H16B were also found within H16B_CorPP (Table 3), with the exception of Ile-Pro-Ala-Val-Phe (β-Lg (f78-82)), Ile-Pro-Ala-Val-Phe-Lys (β-Lg (f78-83)) and Val-Ala-Gly-Thr-Trp-Tyr (β-Lg (f15-20)). H16B_CorPP
contained one previously identified DPP-IV inhibitory peptide, Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu (β-Lg (f46-54)), which was not found within H16B. Following SGID of H16B, some of the known DPP-IV inhibitory peptides (i.e., Ile-Pro-Ala-Val-Phe and Ile-Pro-Ala-Val-Phe-Lys) appeared to have been degraded by the hydrolytic activity of enzymes present within pepsin or CorPP preparations. The decrease of DPP-IV inhibitory activity of H16B CorPP compared to H16B (Table 1) may indicate that these peptides play an important role in the overall bioactivity of H16B.

All 17 of the previously identified DPP-IV inhibitory peptides within MPI CorPP were the same as those found within H16B_CorPP (Table 3). This may partly explain why both samples had the same DPP-IV inhibitory potency (Table 1). The benefit of prehydrolysing MPI with PTN 6.0S could not be demonstrated as H16B_CorPP and MPI_CorPP had the same DPP-IV IC₅₀ value (Table 1). In vivo evaluation of MPI and H16 is required to demonstrate the significance of the results reported herein to the potential in vivo DPP-IV inhibitory activity of these hydrolysates. Promising data is available in the literature which demonstrates that milk protein-derived peptides displaying DPP-IV inhibitory properties in vitro have antidiabetic effects in small animals (Hsieh et al., 2016; Uchida, Ohshiba, & Mogami, 2011; Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). In one instance, these antidiabetic effects have directly been linked with a reduction in plasma DPP-IV activity (Hsieh et al., 2016).

4 Conclusions

The DPP-IV inhibitory properties of MPI hydrolysates generated with trypsin were influenced by the E:S and incubation time but not by the temperature. Generation of MPI hydrolysates with relatively low DPP-IV IC₅₀ values was achieved herein. However, the variation in the DPP-IV IC₅₀ value observed following modification of the hydrolytic parameters (temperature, E:S and time) were relatively modest. This may be linked to the fact that DPP-IV inhibitory peptides could not be further released within the hydrolysates, either because of a rate limiting effect of...
the MPI substrate or modification of trypsin activity during the course of hydrolysis. Several
known, potent DPP-IV inhibitory peptides were identified within the MPI hydrolysates, arising
from both CNs and whey proteins. Even though a loss in DPP-IV inhibitory potency was seen in
H16B_CorPP, some of the known DPP-IV inhibitory peptides identified in H16B were stable to
gastrointestinal enzymes. MPI_CorPP had the same DPP-IV inhibitory potency as H16B_CorPP,
which raises the question of the relevance of these findings to the comparative \textit{in vivo} activity of
H16B and MPI.

\textbf{Acknowledgements}

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\textbf{Conflicts of interests}

The authors declare that they have no conflict of interest.
References


Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R.,


Norris, R., Poyarkov, A., O’Keeffe, M. B., & FitzGerald, R. J. (2014). Characterisation of the hydrolytic specificity of *Aspergillus niger* derived prolyl endoproteinase on bovine β-


Table 1. Degree of hydrolysis (DH) and dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration (IC$_{50}$) of the 15 milk protein isolate (MPI) hydrolysates generated within the experimental design (H1-H15), the externally validated samples (H16A, H16B and H16C), H16B digested with pepsin (H16B_pepsin), H16B_pepsin digested with Corolase PP (H16B_CorPP), MPI digested with pepsin (MPI_pepsin) and MPI digested with Corolase PP (MPI_CorPP).

<table>
<thead>
<tr>
<th>Hydrolysate</th>
<th>Variable levels(^1)</th>
<th>DH(^2) (%)</th>
<th>DPP-IV IC$_{50}$(^{2,3}) (mg mL$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>E:S (%)</td>
<td>Time (min)</td>
</tr>
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</tr>
<tr>
<td>H2</td>
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</tr>
<tr>
<td>H3</td>
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</tr>
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<td>240</td>
</tr>
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<td>2.0</td>
<td>240</td>
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<td>240</td>
</tr>
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<td>150</td>
</tr>
<tr>
<td>H15C</td>
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<td>1.25</td>
<td>150</td>
</tr>
<tr>
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<tr>
<td>H16B</td>
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<td>2.0</td>
<td>231</td>
</tr>
<tr>
<td>H16C</td>
<td>50.5</td>
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<td>231</td>
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<td>MPI_pepsin</td>
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</tr>
<tr>
<td>MPI_CorPP</td>
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<td>-</td>
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</tr>
</tbody>
</table>
1Parameters incubation temperature (40, 50 and 60°C), E:S (0.50, 1.25 and 2.00% (w/w)) and hydrolysis time (60, 150 and 240 min) evaluated at three z-centred levels (-1, 0 and +1, respectively).

2Mean ± SD (n=3). Within the same column, values with different superscript letters are significantly different (p < 0.05). nd: not determined.

3IC\textsubscript{50}: concentration inducing 50% DPP-IV inhibition, expressed in mg protein equivalent per mL (mg mL\textsuperscript{-1}). The IC\textsubscript{50} value of the positive control, Ile-Pro-Ile, was 2.63 ± 0.34 µM.
Table 2. Coefficients of the multilinear regression (MLR) model (Equation 2) correlating the dipeptidyl peptidase IV (DPP-IV) inhibition half maximal concentration (IC₅₀) of the milk protein isolate (MPI) hydrolysates to the parameters of the design of experiments (DOE) and their interactions (complete model) and to the main parameters of the DOE and interactions having a p-value < 0.1 (simplified model).

<table>
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<th>Parameters of the model¹</th>
<th>Coefficients</th>
<th>Estimate value</th>
<th>Standard Error</th>
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<th>p</th>
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<td>0.030</td>
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Root mean squared error = 0.0942; R² = 0.935; p-value model = 0.00223; p-value lack of fit = 0.213

Root mean squared error = 0.104; R² = 0.873; p-value model = 0.000128; p-value lack of fit = 0.196

¹T: Temperature; ES: enzyme to substrate ratio and t: time.
²Parameters having a p < 0.05 are significantly different from 0.
Table 3. Peptides identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) within H4, H11, H16 before and after (H16_CorPP) simulated gastrointestinal digestion (SGID) and milk protein isolate after SGID (MPI_CorPP), which have previously been reported in the literature for their dipeptidyl peptidase IV (DPP-IV) inhibitory properties.

<table>
<thead>
<tr>
<th>Peptide sequence¹</th>
<th>Fragment²</th>
<th>Identified in³</th>
<th>DPP-IV IC₅₀ value (µM)⁴</th>
<th>Reference</th>
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<td>AL</td>
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<td>✓</td>
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</tr>
<tr>
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</tr>
<tr>
<td>H16</td>
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<td>✓</td>
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</table>

¹ Peptide sequence
² Fragment
³ Identified in
⁴ DPP-IV IC₅₀ value (µM)
⁵ Reference

(Nongonierma & FitzGerald, 2013a)
(Nevos, Harnedy, O’Keeffe, & FitzGerald, 2017)
(Nongonierma & FitzGerald, 2013a)
(Zhang et al., 2015)
(Silveira, Martinez-Maqueda, Recio, & Hernández-Ledesma, 2013)
(Silveira et al., 2013)
(Lacroix et al., 2014a)
(Connolly, O’Keeffe, Nongonierma, Piggott, & FitzGerald, 2016)
(Nongonierma & FitzGerald, 2014b)
(Silveira et al., 2013)
(Uchida et al., 2011)
(Lan et al., 2015)
(Lacroix, Meng, Cheung, & Li-Chan, 2016)
(Silveira et al., 2013)
(Umezawa et al., 1984)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Identified More Than Twice</th>
<th>Fragment Numbers</th>
<th>Source</th>
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<td>VR</td>
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</tr>
<tr>
<td>WQ</td>
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<td>(Nongonierma &amp; FitzGerald, 2013a)</td>
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<td>(Nongonierma &amp; FitzGerald, 2016c)</td>
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</table>

1Peptide sequences abbreviated with the one letter amino acid code
2CN: casein; LF: lactoferrin; β-Lg: β-lactoglobulin. Diverse: peptide sequences identified more than twice within the major milk proteins. Fragment numbers are provided for the mature protein sequence.
3✓: peptide identified within the hydrolysate; nd: not detected.
4DPP-IV IC<sub>50</sub>: dipeptidyl peptidase IV half maximal inhibitory concentration as reported on the first mention in the literature.
Table 4. Peptide sequences identified within H16, which display features of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides (i.e., Trp (W₁), Ile (I₁), Leu (L₁) or Phe (F₁) at the N-terminus and/or Pro (P₂)/Ala (A₂) at position 2 and/or Pro at the C-terminus (P_C-term)).

<table>
<thead>
<tr>
<th>Peptide sequence¹</th>
<th>Fragment²</th>
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<tr>
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<td></td>
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<td>α₃₁-CN (f133-144)</td>
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<tr>
<td>EPM(+15.99)IGVNEAYF</td>
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<td>α₃₁-CN (f133-145)</td>
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1Peptide sequences abbreviated with the one letter amino acid code; Post translational modification: oxidation (+15.99), phosphorylation (+79.97) and sodium adduct: (+21.98).
BSA: bovine serum albumin; CN: casein; LF: lactoferrin. β-Lg: β-lactoglobulin. Diverse: peptide sequences identified more than twice within the major milk proteins. Fragment numbers are provided for the mature protein sequence.
Figure captions

Figure 1. Molecular mass distribution of (A) the 15 milk protein isolate (MPI) hydrolysates generated within the experimental design (H1-H15), the samples generated using the response surface methodology (RSM) prediction (H16A, H16B and H16C), H16B digested with pepsin (H16B_pepsin), H16B_pepsin digested with Corolase PP (H16B_CorPP), MPI, MPI digested with pepsin (MPI_pepsin) and MPI_pepsin digested with Corolase PP (MPI_CorPP).

Figure 2. Reverse phase ultra-performance liquid chromatography (RP-UPLC) profiles of the milk protein isolate (MPI) hydrolysates (A) H4, H11 and MPI, (B) H16B, H16B digested with pepsin (H16B_pepsin) and H16B_pepsin digested with Corolase PP (H16B_CorPP) and (C) MPI, MPI digested with pepsin (MPI_pepsin) and MPI_pepsin digested with Corolase PP (MPI_CorPP). ACN: acetonitrile.

Figure 3. Response surface methodology (RSM) curves of the dipeptidyl peptidase IV (DPP-IV) half maximal concentration (IC$_{50}$) as a function of (A) temperature (Temp) and enzyme to substrate ratio (ES), (B) time and Temp and (C) ES and time. The RSM was constructed using the complete model and at the central condition (0) of the parameter which is not varied on each figure panel.
Fig. 1
Fig. 2
Fig. 3

(A) DPP-IV IC_{50} (mg mL^{-1})

(B) Time (min)

(C) Time (min)

DPP-IV IC_{50} (mg mL^{-1})
**Supplementary Figure S1.** Predicted dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration (IC$_{50}$) obtained with (A) the complete and (B) the simplified models as a function of the experimentally (observed) determined DPP-IV IC$_{50}$ value of hydrolysates H1-H16. H16 is the external validation sample.
**Supplementary Figure S2.** (A) Proportion of predicted dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration ($IC_{50}$) values within different ranges obtained using the simplified and the complete models. (B) Predicted DPP-IV $IC_{50}$ values used to build the RSM in the simplified and the complete models (predictions are ordered by increasing DPP-IV $IC_{50}$ value).