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In silico approaches to predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV) inhibitors

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Abstract

Molecular docking of a library of all 8000 possible tripeptides to the active site of DPP-IV was used to determine their binding potential. A number of tripeptides were selected for experimental testing, however, there was no direct correlation between the Vina score and their *in vitro* DPP-IV inhibitory properties. While Trp-Trp-Trp, the peptide with the best docking score, acted as a moderate DPP-IV inhibitor (IC_{50} 216 μM), Lineweaver and Burk analysis revealed its action to be non-competitive. This suggested that it may not bind to the active site of DPP-IV as is the assumption in the docking prediction. Furthermore, there was no significant link between DPP-IV inhibition and the physicochemical properties of the peptides (molecular mass, hydrophobicity hydrophobic moment (μH), isoelectric point (pI) and charge). LIGPLOT analysis indicated that competitive inhibitory peptides were predicted to have both hydrophobic and hydrogen bond interactions with the active site of DPP-IV. DPP-IV inhibitory peptides generally had a hydrophobic or aromatic amino acid at the N-terminus, with a marked preference for Trp among the non-competitive inhibitors and a broader range of residues at the N-terminus of the competitive inhibitors (Ile, Leu, Val, Phe, Trp or Tyr). Two of the potent DPP-IV inhibitors, Ile-Pro-Ile and Trp-Pro (IC_{50} values of 3.5 and 44.2 μM, respectively), were predicted to be gastrointestinally/intestinally stable. This work highlights the needs to test the assumptions (i.e. competitive peptide binding) of any integrated strategy of computational and experimental screening, in optimising screening. Future strategies targeting allosteric mechanisms may need to rely more on structure-activity relationship modeling, rather than on docking, in computationally selecting peptides for screening.

Key words: dipeptidyl peptidase IV inhibitors, molecular docking, bioactive peptides, milk, predictive modeling, hydrophobicity

1. Introduction

Different approaches have been suggested for the treatment and management of Type 2 diabetes. Among these, inhibition of dipeptidyl peptidase IV (DPP-IV), an ubiquitous enzyme involved in the cleavage of incretins such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), is being used as a therapeutic strategy in the treatment of Type 2 diabetes . Incretin cleavage by DPP-IV leads to their inactivation, which results in a decrease in insulin secretion from pancreatic beta cells in the presence of nutrients. There is significant evidence in the literature reporting that various dietary protein hydrolysates derived from animal, plant and marine sources can inhibit DPP-IV *in vitro* . In this context, milk protein hydrolysates, have been identified as a source of DPP-IV inhibitory peptides . Specific milk protein-derived peptides and certain free amino acids inhibit DPP-IV to different extents . The DPP-IV inhibitory peptides identified to date within milk proteins differ in their composition, sequence and physicochemical characteristics, including their molecular mass, hydrophobicity, hydrophobic moment (μH), charge and isoelectric point (pI).

A limited number of *in silico* studies have been performed to predict the potential of dietary proteins to act as a source of DPP-IV inhibitory peptides . Recently, the *in silico* digestion of caseins with prolyl oligopeptidase was studied as a means to predict the release of short (≤ 4 amino acid residue) peptides with a Pro at the C terminus. This strategy has allowed the identification of Phe-Leu-Gln-Pro (β-CN (f87–90)), as a potent (IC_{50} 65.3 ± 3.5 μM) DPP-IV inhibitory peptide . *In silico* digestion of caseins with gastrointestinal enzymes has also been used to predict the release of peptides with a Pro at position 2 in order to study the different modes of DPP-IV inhibition by milk protein-derived peptides. These peptides, which were shown to operate differently compared to true DPP-IV inhibitors, behaved as substrate- or prodrug- type inhibitors . Other studies describe the use of molecular docking approaches as a means to better understand the binding of peptides derived from milk and amaranth proteins to DPP-IV. Molecular docking studies have helped in demonstrating that the binding of dietary peptides to alternative locations than the active site involving either a secondary binding site or the dimerization site of DPP-IV can occur.

Despite the increasing number of DPP-IV inhibitory peptide sequences identified to date and advances in *in silico* approaches, the structural features which govern the DPP-IV inhibitory properties of peptides are still poorly understood. It is generally thought that DPP-IV

inhibitors are relatively hydrophobic compounds . However, it was recently shown that the hydrophilic fraction of a whey protein hydrolysate (WPH) could inhibit DPP-IV with a higher potency than WPH *per se* (IC_{50} value of 1.3 ± 0.2 and 1.1 ± 0.4 $mg.mL^{-1}$ for WPH and its hydrophilic fraction, respectively). To our knowledge, only a limited number of *in silico* approaches have been used for prediction of the DPP-IV inhibitory potential of food protein-derived peptides to date. Therefore, the aim of this study was to evaluate the role of molecular docking as a predictive tool for identification of milk protein-derived DPP-IV inhibitory tripeptides. In addition, the relationship between DPP-IV inhibition and key physicochemical characteristics of DPP-IV inhibitory peptides (molecular mass, hydrophobicity, hydrophobic moment, charge and pI) was also evaluated. This allowed evaluation of the potential of *in silico* approaches for DPP-IV inhibitory peptide discovery.

2. Materials and methods

2.1. Reagents

The synthetic peptides Leu-Gln-Pro, Trp-Trp-Trp and Val-Gly-Leu were from Thermo Fisher Scientific (Ulm, Germany). Tyr-Ser, Tyr-Thr and Pro-Tyr were from Genscript (Piscataway, NJ, USA). Tris(hydroxymethyl)aminomethane (TRIS), Gly-Pro-pNA, Diprotin A (Ile-Pro-Ile), ethanol and porcine DPP-IV (≥ 10 $Units.mg^{-1}$ protein) were obtained from Sigma Aldrich (Dublin, Ireland). HPLC grade water and hydrochloric acid were from VWR (Dublin, Ireland).

2.2. DPP-IV inhibition assay

Peptides were dispersed in HPLC grade water at concentrations ranging from 12.5×10^{-3} to 1.25 $mg.mL^{-1}$. The DPP-IV inhibition assay was carried out as described elsewhere . Each sample was analysed in triplicate. The microplate was incubated at 37˚C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. DPP-IV half maximum inhibitory concentration (IC_{50}) values were determined by plotting the percentage inhibition as a function of the concentration of test compound.

Lineweaver and Burk analysis was used to study the mode of inhibition with Leu-Gln-Pro, Trp-Trp-Trp, Leu-Pro-Leu, Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr Leu-Pro-Tyr-Pro-Tyr and Leu-Pro-Leu-Pro-Leu as previously described . The affinity constant (Km, without inhibitor), the

apparent affinity constant (Kapp, with inhibitor) and the maximum rate of the reaction (Vmax) were determined from the double reciprocal plots.

2.3. Computational analysis

Computational analysis was carried out as per . All the possible combinations of the 20 aminoacids (20^3) were used to generate 8000 tripeptides. AutoDock Vina was used to dock 8000 tripeptides and the additional 9 selected tetra to pentapeptides to Protein Data Bank (PDB) 1WCY, the crystal structure of human DPP-IV in complex with Diprotin A . The initial poses of the PDB-formatted structures of peptides were generated using the Open Babel Package, version 2.1.1 . The ligands and the 1WCY receptor was prepared and the 'search space' was determined with AutoDockTools . The Vina score (predicted affinity of the molecule to bind to the PDB structure) was obtained following peptide docking with the PDB structure 1WCY at the active site of DPP-IV. The lower the Vina score, the more favorable the peptide (ligand) interactions with DPP-IV. LIGPLOTs were generated for each peptide with the best Vina docking poses as outlined by Wallace, Laskowski & Thornton . The intestinal stability of the dipeptides was predicted using an amino acid clustering model from . According to this model, dipeptides are rated as 'stable' (> 75 % peptide remaining after 60 min simulated intestinal digestion- SID) 'neutral' (between 25 and 75 % peptide remaining after 60 min SID) or 'unstable' (< 25 % peptide remaining after 60 min SID) with regard to small intestinal stability. Larger peptide sequences (> 2 amino acid) were subjected to simulated gastrointestinal digestion using the peptide cutter program from as described elsewhere . Peptides were considered as stable when they were not predicted to be cleaved by pepsin, trypsin and chymotrypsin and were considered unstable when they were predicted to be cleaved by these enzymes.

2.4. Estimation of the physicochemical characteristics of the DPP-IV inhibitory peptides

The physicochemical characteristics of the 112 different amino acids and peptides evaluated (Table 1 and other milk protein-derived peptides or amino acids previously identified in earlier studies) were estimated using online peptide calculators. The hydrophobicity was estimated by means of the software developed by using the combined consensus scale (CCS) of hydrophobicity. The hydrophobic moment (μH) was calculated with the online software . The peptide charge at pH 8 was estimated with the online calculator, Protcalc . The pI was estimated using the peptide calculator, .

2.5. Statistical analysis

Means comparison was carried out with a one way ANOVA followed by a Student Newman-Keuls test using SPSS (version 20, SPSS Inc., Chicago, IL, USA) at a significance level of $P < 0.05$.

3. Results

3.1. Vina score of the peptides

During a previous study, the docking of the 400 possible dipeptides to the PDB structure 1WCY was reported . As already outlined, the Vina scores for the dipeptides ranged between -9.3 to -4.6 kcal.mol^{-1} for Trp-Trp and Cys-Cys, respectively.

The Vina scores were determined herein for the 8000 possible tripeptides and for 9 selected larger milk-protein derived peptides (4-5 amino acid in length), previously shown to be DPP-IV inhibitors . The frequency of distribution of the Vina scores for the tripeptides is illustrated in Supplementary Fig. S1. Most tripeptides (95.6 %) had a Vina score between -9.0 and -6.0 kcal.mol^{-1}. Only 2.6 % of the tripeptides had a Vina score > -6.0 kcal.mol^{-1} and 1.8 % had a Vina score < -10.0 kcal.mol^{-1}. The tripeptide with the lowest Vina score was Trp-Trp-Trp (-10.8 kcal.mol^{-1}). This low Vina score indicated that this peptide was likely to bind the active site of DPP-IV. Therefore, Trp-Trp-Trp was evaluated for its DPP-IV inhibitory properties *in vitro*. The Vina score of the twelve other tripeptides studied *in vitro* (Table 1) ranged from -.8.6 to -6.3 kcal.mol^{-1}. Within these tripeptides, Ile-Pro-Ile is the positive control for the DPP-IV inhibitory assay and Leu-Pro-Leu was selected on the basis of its structural similarities with Ile-Pro-Ile. Previous studies have already investigated the DPP-IV inhibitory properties of ten of the tripeptides . Val-Gly-Leu (-6.3 kcal.mol^{-1}) and Leu-Gln-Pro (-7.1 kcal.mol^{-1}) were also evaluated for their DPP-IV inhibitory properties *in vitro*.

3.2. DPP-IV inhibition and mode of inhibition by synthetic peptides

The DPP-IV IC_{50} value was determined for Tyr-Ser, Tyr-Thr, Pro-Tyr, Leu-Gln-Pro, Trp-Trp-Trp and Val-Gly-Leu (Table 1). The IC_{50} values of the other peptides assessed herein

were taken from previous studies . The DPP-IV IC_{50} value of the peptides evaluated herein ranged from 216.0 ± 1.2 to > 5,000 μM for Trp-Trp-Trp and Val-Gly-Leu, respectively.
The mode of DPP-IV inhibition was determined by Lineweaver and Burk analysis for Trp-Trp-Trp, Leu-Gln-Pro, Leu-Pro-Leu, Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr Leu-Pro-Tyr-Pro-Tyr and Leu-Pro-Leu-Pro-Leu. There was a significant difference ($P < 0.05$) for Vmax with and without Trp-Trp-Trp and no significant difference ($P \geq 0.05$) between Km and Kapp, showing a non–competitive mode of inhibition for this peptide (Fig. 1a). In contrast, for the other peptides evaluated (Leu-Gln-Pro, Leu-Pro-Leu, Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr Leu-Pro-Tyr-Pro-Tyr and Leu-Pro-Leu-Pro-Leu), there was no significant difference ($P \geq 0.05$) for Vmax with and without inhibitor whereas Km was significantly different ($P < 0.05$) from Kapp (Fig. 1 b, c and d). This indicated that these peptides were competitive inhibitors of DPP-IV. The mode of inhibition for the other peptides listed in Table 1 was obtained from previous studies .

3.3. Prediction of DPP-IV inhibitory potency of the peptides and intestinal stability

The lowest Vina score (-10.8 $kcal.mol^{-1}$) was obtained for Trp-Trp-Trp. The most potent DPP-IV inhibitory peptide, Ile-Pro-Ile (IC_{50} of 3.5 ± 0.2 μM), had a Vina score of -7.4 $kcal.mol^{-1}$. Several peptides had a Vina score lower than that of the positive control Ile-Pro-Ile, some of which were shown to be DPP-IV inhibitors (66 %) while others could not inhibit DPP-IV (34 %).
The LIGPLOTs of the peptides were determined. LIGPLOTs for the competitive DPP-IV inhibitory peptides Ile-Pro-Ile-Gln-Tyr, Phe-Leu-Gln-Pro, Leu-Pro-Tyr-Pro-Tyr and Tyr-Pro-Tyr-Tyr are illustrated in Fig. 2. For all competitive DPP-IV inhibitory peptides studied herein, both hydrogen bonds and hydrophobic interactions were involved in their interaction with the active site of DPP-IV. The interactions with competitive DPP-IV inhibitory peptides appeared to involve common amino acid residues from the active site of DPP-IV. These included hydrophobic interactions with Tyr666, Phe357 and in certain instances Tyr662. Hydrogen bonds between DPP-IV inhibitory peptides and the active site of DPP-IV involved in many instances the amino acid residues Ser209, Arg125 and Tyr5 (Fig. 2). The LIGPLOTS for tripeptides (His-Gln-Pro, Lys-His-Pro, Lys-Tyr-Pro and Ile-Thr-Pro) showing no DPP-IV inhibition is illustrated in Supplementary Fig. S2. As for DPP-IV inhibitory peptides, hydrophobic interactions and hydrogen bonds with residues from the active site of

DPP-IV were seen, indicating that neither the typical mode of binding nor the total binding energy are clear predictors of DPP-IV inhibitory activity.

Fig. 3a and 3b represent the Vina score of the competitive and non-competitive DPP-IV inhibitory peptides, respectively, as a function of their IC_{50} values. The values illustrated in Fig. 3 combine data presented in Table 1. The *in silico* approach described herein consisted of docking the peptides to the active site of DPP-IV. For this reason, only the peptides which were identified as competitive inhibitors (Table 1) were taken into account to predict DPP-IV inhibitory activity using the Vina score. The non-competitive peptides are thought to interact with a secondary binding site of DPP-IV. Therefore, the results obtained following the molecular docking approach described cannot be applied as it assumes docking to the active site of DPP-IV. Competitive DPP-IV inhibitory peptides with a low Vina score generally have a low IC_{50} value (Fig. 3a, Spearman rank correlation coefficient $\rho = 0.49$, $p = 0.02$). However, the correlation between the IC_{50} value and the Vina score was relatively low ($R^2 = 0.41$). As expected, for the non-competitive peptides, there was no relationship between the IC_{50} value and the Vina score (Fig. 3b).

Intestinal and gastrointestinal stability was predicted for the DPP-IV inhibitory dipeptides and peptides > 2 amino acids in length, respectively (Table 1). The predicted intestinal stability varied depending on the sequence of the peptides evaluated. The most potent DPP-IV inhibitory dipeptide predicted to be stable was Trp-Pro with an IC_{50} value of 44.2 ± 2.0 µM. Most peptides > 2 amino acid in length were found to be gastrointestinally unstable, with the exception of Ile-Pro-Ile and Ile-Gln-Pro. The most potent DPP-IV inhibitor which was predicted to be stable was Ile-Pro-Ile with IC_{50} values of 3.5 ± 0.2 µM. The other peptides predicted to be stable had IC_{50} values > 140 µM (Table 1).

3.4. Physicochemical characteristics of the peptides

The physicochemical characteristics (molecular mass, hydrophobicity, µH, pI and charge at pH 8) of the different amino acids and peptides evaluated herein (Table 1) and in previous studies were estimated *in silico*. A plot of the reciprocal of the IC_{50} value as a function of hydrophobicity is represented in Fig 4a and 4b for DPP-IV inhibitory and non-inhibitory peptides, respectively. Most DPP-IV inhibitory peptides (84 %) were hydrophobic (Fig. 4a), i.e., peptides with the lowest DPP-IV IC_{50} (highest $1/IC_{50}$) were generally hydrophobic. However, their DPP-IV potency did not linearly increase with the hydrophobicity. For illustration, Ile-Pro-Ile, the most potent DPP-IV inhibitor studied herein (IC_{50} 3.5 ± 0.2 µM) was not the most hydrophobic compound (hydrophobicity index 5.72). The non-inhibitory

amino acids and peptides presented a wide range of hydrophobicities with 51 % of the compounds being hydrophobic (Fig. 4b). The reciprocal of the IC_{50} value of the amino acids and peptides was also plotted as a function of μH, the molecular mass, pI and charge at pH 8 (Supplementary Fig. S3). There was no obvious relationship between μH, the molecular mass, pI and charge or pH 8 and the DPP-IV inhibitory properties of the amino acids and peptides.

DPP-IV inhibitory peptides generally had a hydrophobic or aromatic amino acid at the N-terminus, with a marked preference for Trp among the non-competitive inhibitors. There was a broader range of residues at the N-terminus of the competitive inhibitors (Ile, Leu, Val, Phe, Trp or Tyr).

4. Discussion

Several peptides have previously been shown to be non-competitive inhibitors of DPP-IV . Using *in silico* approaches, Trp-Val , GIP and GIP-derived peptides and the N-terminus of human immunodeficient virus (HIV-1) Tat(1-9) were predicted to bind to a secondary site located in the neighborhood of the active site of DPP-IV. However, the exact structure of the secondary binding site(s) is still unknown, limiting the utilization of molecular docking for the prediction of non-competitive peptide binding to DPP-IV.

The Vina score was used as a rapid high throughput tool to screen a library of 8000 tripeptides. No relationship was found between the IC_{50} value of non-competitive DPP-IV inhibitors and the Vina score (Fig. 3b). Overall, there was a poor correlation between the Vina score and the DPP-IV inhibitory properties of the tripeptides. Of the 8000 tripeptides, Trp-Trp-Trp had the lowest Vina score. However, because of its experimentally determined non-competitive mode of inhibition, the Vina score could not be linked to its DPP-IV inhibitory properties. When taking into account a larger set of competitive DPP-IV inhibitory peptides, those with a low Vina score generally tended to be potent DPP-IV inhibitors (Fig. 3a). However, the Vina score is not an accurate parameter as confounding factors such as peptide molecular mass may affect docking, with larger peptides generally having lower Vina scores. The predictive aspect of the molecular docking approach described herein is also limited because it can only be applied to competitive inhibitors of DPP-IV. In this context, better knowledge of the secondary binding site(s) structure should help to discriminate non-competitive from competitive inhibitors.

The active site of DPP-IV comprises an hydrophobic S1 pocket and a charged S2 pocket with an overall negative charge playing a role in the recognition of charged N-terminal amino acids . The LIGPLOTs showed several hydrogen bonds and hydrophobic interactions between competitive DPP-IV inhibitory peptides and the active site of DPP-IV. The hydrophobic interactions involved amino acid residues which have previously been reported to contribute to the S1 (Tyr662 and Tyr 666) and S2 (Phe357 and Arg125) sites of DPP-IV . No obvious link between the μH and DPP-IV IC_{50} value could be determined, suggesting that the ability of the peptide to form amphipathic helical structures did not appear to be linked with their DPP-IV inhibitory activity. The pI, charge, μH and molecular mass did not appear to affect peptide binding to DPP-IV. Of all hydrophobic peptides (with an hydrophobicity index > 0) described in Table 1, 77 % were DPP-IV inhibitors. While 53 % of the hydrophilic peptides listed in Table 1 were DPP-IV inhibitors.

The parameters which govern DPP-IV inhibitory properties of peptides are not clearly understood. The stereochemistry appears to play a role in the biological activity of the peptide. Peptides are reported to interact with the active site of DPP-IV from their N-terminal side . The presence of a Trp at the N-terminal side of dipeptides generally yielded more potent DPP-IV inhibitors compared to the associated reverse peptide , but in our analysis these are often non-competitive inhibitors. Ile-Pro was shown to be a DPP-IV inhibitor whereas Pro-Ile was not . Similarly, Pro-Tyr, the reverse peptide of Tyr-Pro (IC_{50} 658.1 μM), was not active (Table 1). Most DPP-IV inhibitors possess an hydrophobic or aromatic amino acid (Ala, Val, Ile, Leu, Met, Phe, Tyr or Trp) at their N-terminal region (Table 1). There were a few exceptions such as Gln-Pro and Asn-Pro, however, these peptides are weak DPP-IV inhibitors (IC_{50} > 4000 μM). It is likely that hydrophobic interactions occur with non-competitive DPP-IV inhibitors, which also generally possess an hydrophobic amino acid (predominantly Trp but also Val) at their N-terminus (Table 1). Several non-inhibitory peptides possessing hydrophobic or aromatic amino acids at their N-terminus were also found (Table 1), indicating that N-terminal hydrophobicity or aromaticity is a desirable characteristic, but not sufficient for inhibition *per se*. Multifactorial approaches combining structural and physicochemical characteristics of the peptides may help to better understand and predict their interaction with DPP-IV. Both chemical (hydrogen bonds and hydrophobic interactions) and structural features have been shown to affect the DPP-IV inhibitory properties of drug inhibitors. The existence of exclusion volumes in the S1 pocket of DPP-IV may restrict the access of bulky amino acids and allow access to smaller amino residues such as Pro, Ala and Gly .

A limited number of peptides (< 4 amino acid residues) were predicted to be intestinally or gastrointestinally stable. The most potent peptide studied herein, Ile-Pro-Ile, was predicted to be intestinally stable. Although the peptides were only evaluated for their stability to gastrointestinal enzymes *in silico*, certain sequences have previously been shown to be intestinally stable *in vitro*. These include Ile-Pro and Trp-Asn which were shown to be intestinally stable (> 75 % peptide remaining after 60 min simulated intestinal digestion) and Trp-Gly and Tyr-Pro which were show to be neutral (between 25 and 75 % peptide remaining after 60 min simulated intestinal digestion) .

Several studies have shown that milk protein-derived hydrolysates can have an insulinogenic action *in vivo* .The mechanisms involved are not yet fully understood. It has been suggested that branched-chain amino acids and bioactive peptides released during food protein digestion or present in food-protein hydrolysates can directly have an insulinotropic activity at pancreatic β-cell level . However, there is growing evidence in the scientific literature suggesting that other mechanisms including DPP-IV and α-glucosidase inhibition may also play a role in the overall serum glucose lowering activity of food protein hydrolysates . To date, there are only a restricted number of food protein-derived peptides which have been suggested to exert DPP-IV inhibitory activity *in vivo*. These include a tryptic β-lactoglobulin (β-Lg) hydrolysate containing the DPP-IV inhibitory peptide Val-Ala-Gly-Thr-Trp-Tyr (β-Lg f15-20)), and a peptide sequence isolated from a water soluble extract of a Gouda type cheese (Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu (β-casein f70-77)) . The *in vitro* IC_{50} value for the DPP-IV inhibitory peptides was 174 and 160 μM for Val-Ala-Gly-Thr-Trp-Tyr and Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu, respectively. No significant differences in terms of plasma insulin were seen between a control and the test group of rats receiving Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu by oral gavage (300 mg kg^{-1} body weight) 30 or 60 min prior to an oral glucose tolerance test (OGTT). However, there was a significant reduction in plasma glucose . Similarly, when mice were fed by oral gavage (300 mg kg^{-1} body weight) with a tryptic β-Lg hydrolysate 30 min before an OGTT, a significant reduction in plasma glucose was observed . Interestingly, the IC_{50} value of Val-Ala-Gly-Thr-Trp-Tyr and Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu is higher than that of the most potent peptides evaluated herein. If the peptides are stable in the gastro-intestinal tract, they may exert their bioactive properties *in vivo* leading to a faster reduction in post-prandial serum glycaemia. These data need to be validated *in vivo* in order to fully understand their impact on the biological activity of DPP-IV in humans.

5. Conclusion

Molecular docking of a tripeptide library to the active site of DPP-IV was investigated in order to predict the DPP-IV inhibitory properties of milk protein-derived peptides. There was no clear relationship between the Vina score and the DPP-IV inhibitory properties of the tripeptides. In addition, it was shown herein that utilization of molecular docking as a predictive tool for the discovery of DPP-IV inhibitory peptides may only be applied to competitive inhibitors. However, it is not easy to predict binding of non-competitive DPP-IV inhibitors computationally, as docking to many possible sites on the protein is likely to yield many false positives as well as possible true signals. Docking, as applied in this study, is a preliminary tool to help focus experimental screening efforts on a smaller number of candidate peptides. Ideally, preliminary treatment of the data needs to be applied in order to distinguish competitive from the non-competitive inhibitory peptides. In our study, this would go against our objectives, since the whole point of docking is to focus experiments (rather than using experiments to validate docking). One potential future solution is to perform an initial screen of a diverse peptide set (most typically using docking to help narrow the search space) and then select a small number of peptides with the strongest competitive inhibition. Molecular evolution of those promising peptides (e.g. tripeptides) by single or double amino acid changes, followed by computational docking and then experimental screening, may lead to a useful diverse library of interesting compounds. However, some caution is needed in relying on docking modeling for DPP-IV, given the relatively low correlations of activity with docking predictions; docking may only provide strong guidance for a subset of enzyme sites.

By studying the physicochemical characteristics of the peptides, it was shown that the presence of an hydrophobic N-terminal amino acid rather than the overall hydrophobicity of the peptide may be linked with its DPP-IV inhibitory properties. However, it was also found that several peptides with an hydrophobic N-terminus appeared to be inactive in terms of DPP-IV inhibition. The structural characteristics of peptides may affect their access to the active site of DPP-IV. In addition, other physicochemical characteristics of the peptides playing a role in DPP-IV inhibitory potential may yet remain to be discovered. An increasing number of potent non-competitive DPP-IV inhibitors has been identified. Therefore, there is a need to develop a better insight on the secondary binding site structure(s) of DPP-IV in order to develop *in silico* strategies which can also take into account this secondary binding site(s) of DPP-IV.

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References

Table captions

Table 1 Half maximum inhibitory concentration (IC_{50}) of dipeptidyl peptidase IV (DPP-IV), Vina scores, predicted gastrointestinal/ intestinal stability, molecular mass, estimated hydrophobic moment (μH), hydrophobicity, isoelectric point (pI), charge at pH 8 and inhibition type for the peptides experimentally determined by Lineweaver and Burk analysis. Tripeptide sequences are written in bold. Peptides and amino acids are listed by increasing IC_{50} value.

Compound	DPP IV IC_{50} (μM)*	Vina Score (1WCY)†	Predicted intestinal stability**	Molecular mass (g mol^{-1})	Hydrophobic moment (μH)§	Hydrophobicity index‡	Isoelectric point (pH units)#	Charge at pH 8##	Mode of inhibition
IPI	3.5	-7.1	stable	341.2	2.88	2.46	5.57	-0.5	competitive
IPIQY	35.2	-8.1	unstable	632.4	3.99	2.74	5.48	-0.5	competitive§§
WR	37.8	-8.2	unstable	360.2	3.34	-1.80	10.73	0.5	non-competitive
WK	40.6	-7.4	neutral	332.2	2.31	-1.60	9.80	0.5	non-competitive
WL	43.6	-8.2	unstable	317.2	1.87	1.45	5.69	-0.5	competitive
WP	44.5	-8.3	stable	301.1	0.93	-1.25	5.75	-0.5	non-competitive
FLQP	65.3	-8.1	unstable	503.3	3.22	0.37	5.50	-0.5	competitive
WA	92.6	-7.7	unstable	275.1	1.43	0.30	5.71	-0.5	non-competitive
LPYPY	108.3	-9.0	unstable	651.3	1.82	-0.40	5.47	-0.5	competitive§§
WQ	120.3	-8.2	neutral	332.1	1.66	-2.20	5.58	-0.5	non-competitive
WI	138.7	-8.1	neutral	317.2	2.19	1.80	5.70	-0.5	non-competitive
IP	149.6	-6.3	stable	228.1	1.5	1.45	5.62	-0.5	competitive
WN	148.5	-8.1	neutral	318.1	1.59	-2.20	5.45	-0.5	non-competitive
YPYY	194.4	-10.2	unstable	604.3	0.9	-1.37	5.36	-0.5	competitive§§
WWW	216.0§	-10.8	unstable	576.2	2.43	-0.90	5.64	-0.5	non-competitive§§
WIQP	237.3	-8.3	unstable	542.3	3.16	-0.37	5.75	-0.5	non-competitive
LPL	241.4	-7.5	unstable	341.2	2.24	2.00	5.58	-0.5	competitive§§
WM	243.1	-7.4	neutral	335.1	1.45	0.49	5.61	-0.5	non-competitive

YPY	243.7	-8.6	unstable	441.2	0.64	-1.40	5.37	-0.5	competitive[§§]
WY	281.0	-8.4	unstable	367.2	1.07	-1.10	5.58	-0.1	non-competitive
LPLPL	325.0	-7.3	unstable	551.4	3.42	1.64	5.58	-0.5	competitive[§§]
WC	420	-7.2	unstable	307.1	1.1	0.80	5.29	-0.7	non-competitive
WT	482.1	-8.1	stable	305.1	0.86	-0.80	5.57	-0.5	non-competitive
WW	554.8	-9.4	unstable	390.2	1.62	-0.90	5.64	-0.5	non-competitive
VLGP	580.4	-7.3	unstable	384.2	2.74	1.50	5.69	-0.5	competitive
WS	643.5	-7.8	stable	291.1	0.99	-0.85	5.60	-0.5	non-competitive
YP	658.1	-8.3	stable	278.1	0.38	-1.45	5.48	-0.5	competitive
LP	712.5	-6.6	neutral	228.1	1.18	1.09	5.63	-0.5	competitive
VR	826.1	-7.4	unstable	273.2	3.61	-0.14	10.73	0.5	non-competitive
LW	993.4	-7.6	unstable	317.2	1.87	0.96	5.53	-0.5	competitive
LQP	1181.1[§]	-7.1	stable	356.2	2.03	1.16	5.63	-0.5	nd
MW	1691.4	-7.2	unstable	335.1	1.45	0.49	5.36	-0.5	nd
WE	> 2,000	-8.4	neutral	333.1	1.55	-2.20	3.27	-1.5	nd
WF	> 3,000	-7.9	unstable	351.2	2	0.95	5.62	-0.5	nd
VRGP	> 3,000	-7.9	unstable	427.3	4.21	-0.57	11.56	0.5	nd
QP	> 4,000	-6.8	stable	243.1	0.97	-2.54	5.47	-0.5	nd
IQP	> 4,000	-6.8	stable	356.2	2.35	-0.20	5.62	-0.5	nd
VGL	> 5,000[§]	-6.3	unstable	287.2	2.62	2.53	5.63	-0.5	nd
AW	> 6,000	-7.6	unstable	275.1	1.43	0.45	5.55	-0.5	nd

YT	> 6,000[§]	-7.9	stable	282.1	0.31	-0.66	5.36	-0.5	nd
WG	>8,000	-7.5	neutral	261.1	1.29	-0.65	5.70	-0.5	nd
NP	> 20,000	-6.3	stable	229.1	0.9	-2.54	5.44	-0.5	nd
DW	-	-7.7	nd	319.1	1.71	-2.20	2.95	-1.5	na
GP	-	-5.5	nd	172.1	0.6	-1.00	5.65	-0.5	na
HQP	-	-7.4	nd	380.2	1.37	-2.76	8.32	-0.5	na
ITP	-	-6.9	nd	329.2	1.55	0.73	5.62	-0.5	na
KHP	-	-6.8	nd	380.2	2.02	-2.90	10.59	0.5	na
KYP	-	-7.6	nd	406.2	1.88	-2.26	10.1	0.5	na
NSLP	-	-7.2	nd	429.2	2.14	-0.52	5.44	-0.5	na
PW	-	-7.4	nd	301.1	0.93	-1.25	5.21	-0.5	na
PY	-[§]	-7.3	nd	278.1	0.38	-1.45	5.17	-0.5	na
VEP	-	-6.5	nd	343.2	1.94	-0.30	3.23	-1.5	na
WD	-	-8.3	nd	319.1	1.71	-2.20	3.18	-1.5	na
YS	-[§]	-7.4	nd	268.1	0.44	-1.05	5.38	-0.5	na
NW	-	-8.0	nd	318.1	1.59	-2.20	5.37	-0.5	na
YW	-	-8.5	nd	367.2	1.07	-1.10	5.41	-0.5	na
CW	-	-7.2	nd	307.1	1.1	0.80	4.94	-0.7	na
SW	-	-7.4	nd	291.1	0.99	-0.85	5.42	-0.5	na
EW	-	-7.6	nd	333.1	1.55	-2.20	3.09	-1.5	na
FW	-	-8.4	nd	351.2	2	0.95	5.42	-0.5	na

GW	-	-7.3	nd	261.1	1.29	-0.65	5.55	-0.5	na
YY	-	-7.7	nd	344.1	0.52	-1.30	5.37	-0.5	na
IQY	-	-7.6	nd	422.2	2.49	-0.10	5.48	-0.5	na

[*]Values represent mean IC_{50} values, n=3 and triplicate determination.
[†]1WCY: crystal structure of human dipeptidyl peptidase IV (DPPIV) complex with Diprotin A.
.[**]The predicted intestinal stability was determined using the model of Foltz *et al*. for the dipeptides and by subjecting the larger peptides (>2 amino acid residues) to the peptide cutter ExPASy as described by Nongonierma and FitzGerald .
[§]The hydrophobic moment was estimated by the hmoment online calculator
[‡]The hydrophobicity was estimated using the combined consensus scale (CCS) of hydrophobicity
[#]The isoelectric point (pI) was estimated by a peptide calculator
[##]the peptide charge was estimated by a peptide calculator
[§§]IC_{50} values and mode of inhibition[§§] experimentally determined within this study by Lineweaver and Burk analysis, other values were taken from previous studies

Figure captions

Figure 1. Lineweaver and Burk representation for dipeptidyl peptidase IV (DPP-IV) inhibitory peptides (a) Trp-Trp-Trp, (b) Leu-Pro-Leu-Pro-Leu, (c) Tyr-Pro-Tyr-Tyr and (d) Leu-Pro-Tyr-Pro-Tyr.

Figure 2. LIGPLOT profiles of the crystal structure of human dipeptidyl peptidase IV (DPP-IV) 1WCY with competitive DPP-IV inhibitory peptides (a) Ile-Pro-Ile-Gln-Tyr, (b) Phe-leu-Gln-Pro (c) Leu-Pro-Tyr-Pro-Tyr and (d) Tyr-Pro-Tyr-Tyr.

Figure 3. Vina score of (a) competitive and (b) non-competitive DPP-IV inhibitors as a function of their half maximum inhibitory concentration (IC_{50}) value

Figure 4. Reciprocal of the dipeptidyl peptidase IV (DPP-IV) half maximum inhibitory concentration (IC_{50}) value of peptides as a function of the hydrophobicity for (a) DPP-IV inhibitory peptides and (b) non-inhibitory peptides. The hydrophobicity was determined using the combined consensus scale (CCS) of hydrophobicity . Values presented on these graphs incorporate the data from this work and values obtained for other milk protein-derived peptides from the literature .

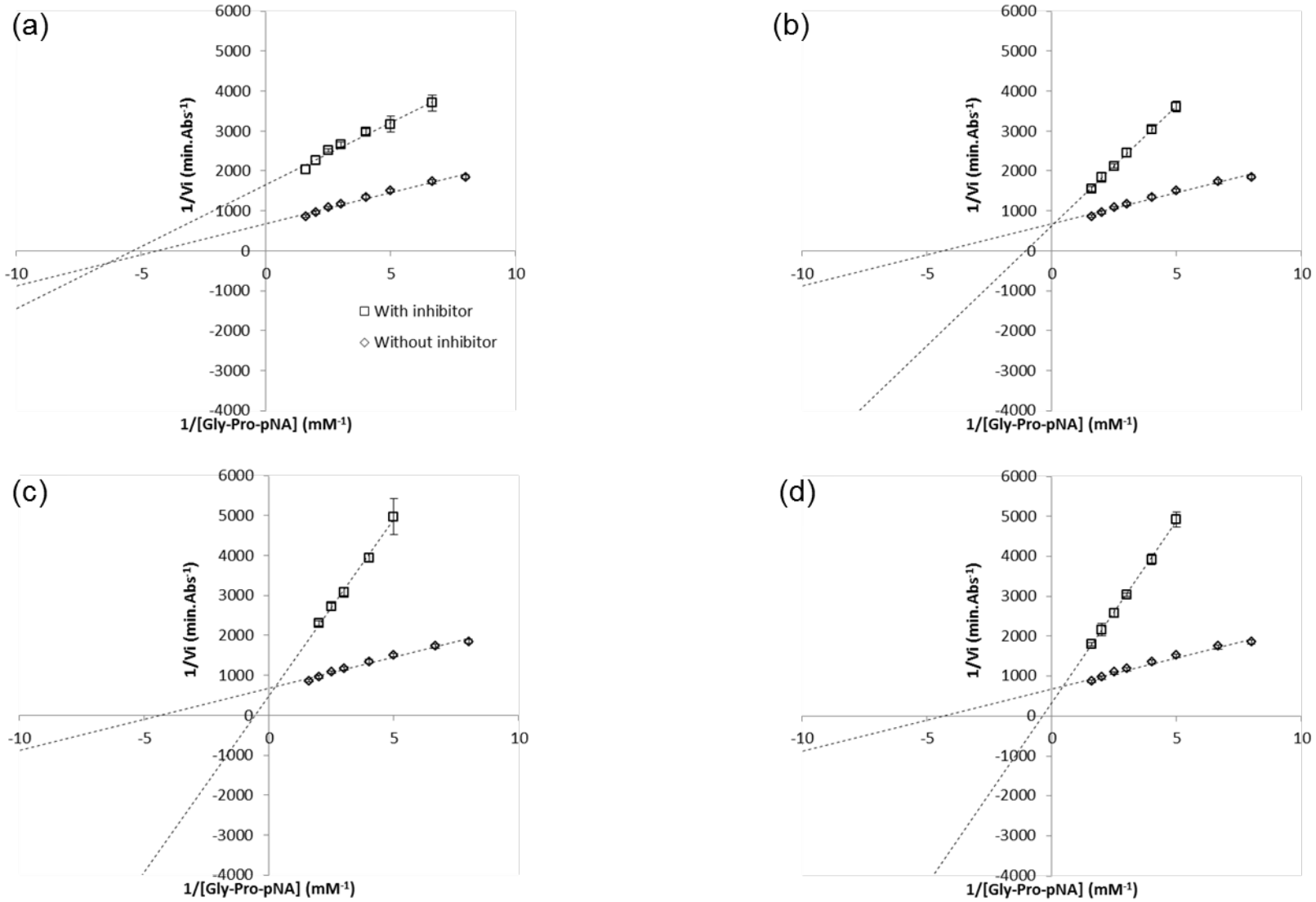

(a)
(b)
(c)
(d)
1/Vi (min.Abs-1)
1/[Gly-Pro-pNA] (mM-1)
With inhibitor
Without inhibitor

Figure 1

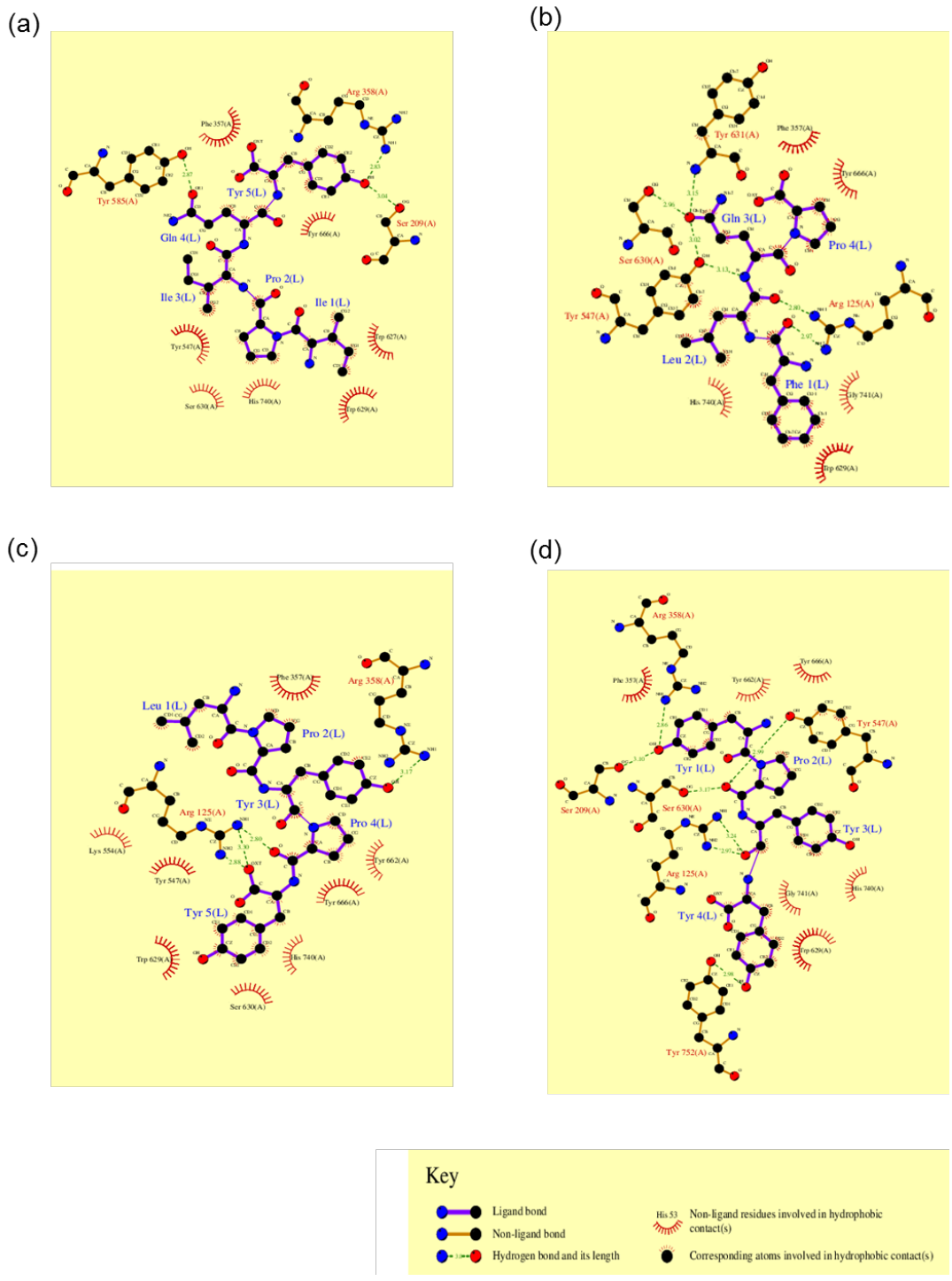

(a)
Arg 358(A)
Phe 357(A)
Tyr 585(A)
Tyr 5(L)
Ser 209(A)
Tyr 666(A)
Gln 4(L)
Pro 2(L)
Ile 3(L)
Ile 1(L)
Trp 627(A)
Tyr 547(A)
Ser 630(A)
His 740(A)
Trp 629(A)
(b)
Tyr 631(A)
Phe 357(A)
Tyr 666(A)
Gln 3(L)
Pro 4(L)
Ser 630(A)
Arg 125(A)
Tyr 547(A)
Leu 2(L)
Phe 1(L)
His 740(A)
Gly 741(A)
Trp 629(A)
(c)
Leu 1(L)
Phe 357(A)
Arg 358(A)
Pro 2(L)
Tyr 3(L)
Arg 125(A)
Pro 4(L)
Lys 554(A)
Tyr 662(A)
Tyr 547(A)
Tyr 666(A)
Tyr 5(L)
Trp 629(A)
His 740(A)
Ser 630(A)
(d)
Arg 358(A)
Phe 353(A)
Tyr 662(A)
Tyr 666(A)
Tyr 547(A)
Pro 2(L)
Tyr 1(L)
Ser 209(A)
Ser 630(A)
Tyr 3(L)
Arg 125(A)
His 740(A)
Gly 741(A)
Tyr 4(L)
Trp 629(A)
Tyr 752(A)
Key
Ligand bond
Non-ligand bond
Hydrogen bond and its length
His 53
Non-ligand residues involved in hydrophobic contact(s)
Corresponding atoms involved in hydrophobic contact(s)

Figure 2

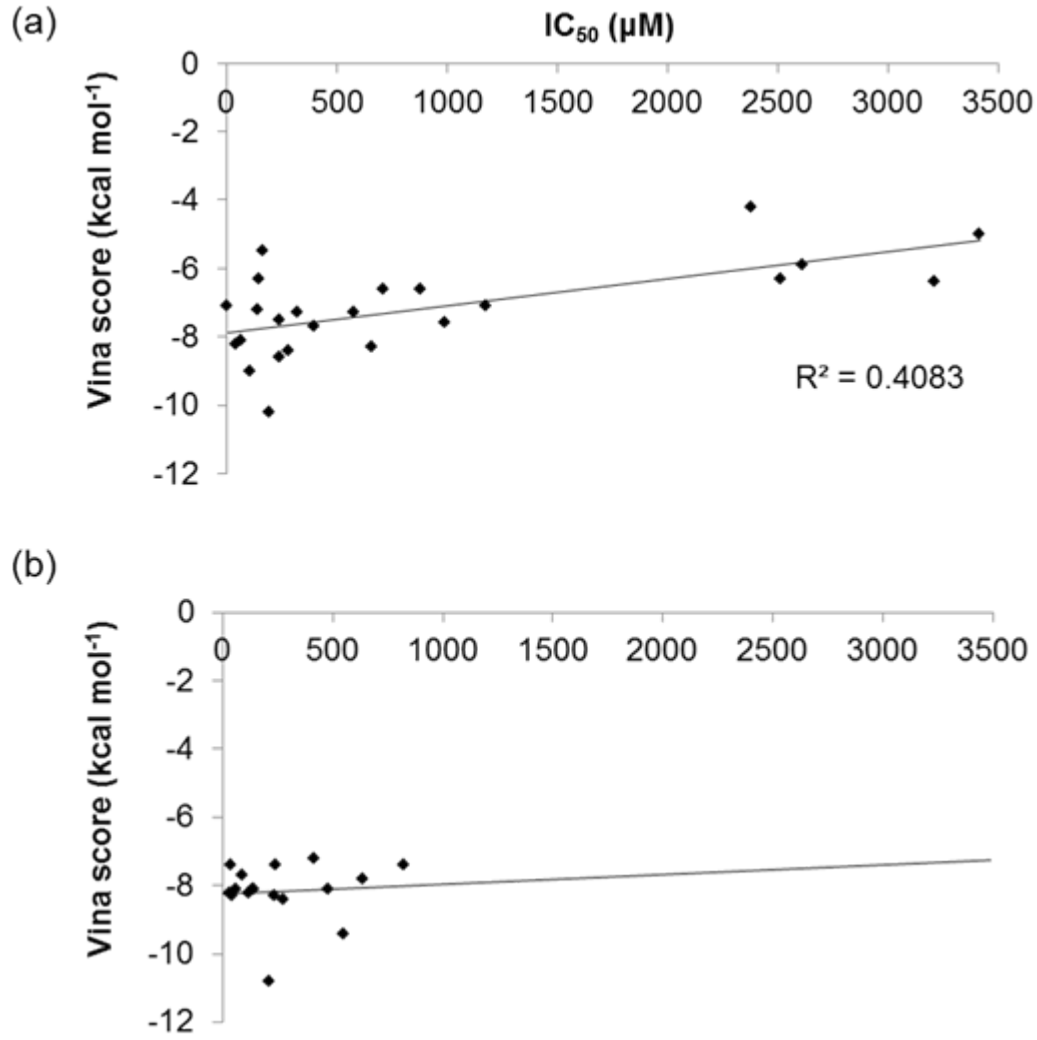

(a)
IC50 (μM)
Vina score (kcal mol-1)
0
-2
-4
-6
-8
-10
-12
0
500
1000
1500
2000
2500
3000
3500
R² = 0.4083
(b)
Vina score (kcal mol-1)
0
-2
-4
-6
-8
-10
-12
0
500
1000
1500
2000
2500
3000
3500

Figure 3

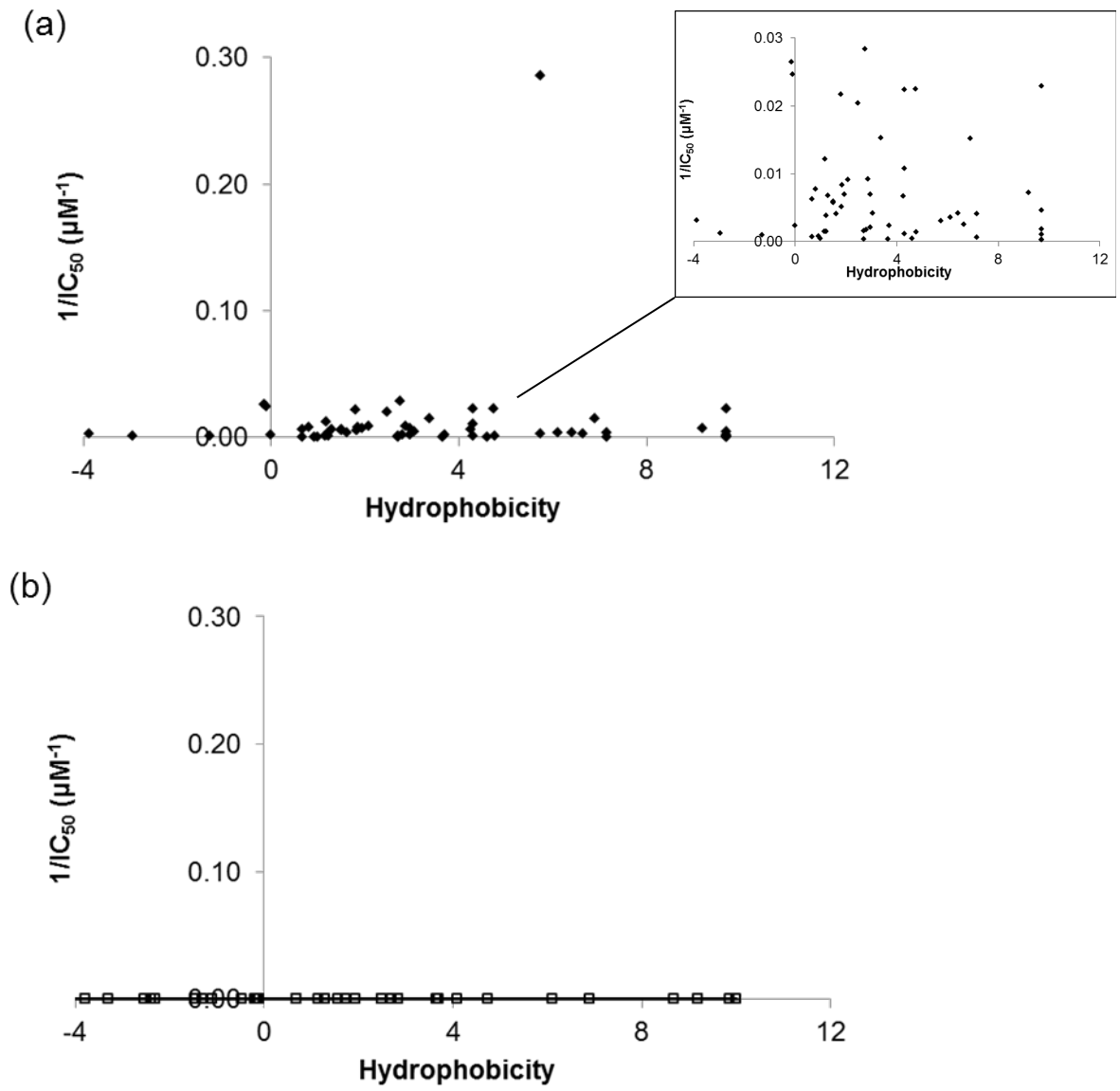
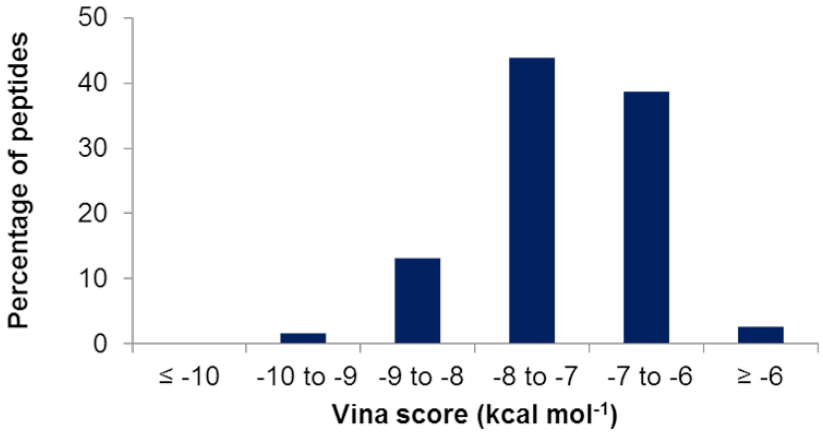

(a)
0.30
0.20
0.10
0.00
1/IC50 (µM-1)
-4
0
4
8
12
Hydrophobicity
0.03
0.02
0.01
0.00
1/IC50 (µM-1)
-4
0
4
8
12
Hydrophobicity
(b)
0.30
0.20
0.10
0.00
1/IC50 (µM-1)
-4
0
4
8
12
Hydrophobicity

Figure 4

Supplementary figures



Supplementary Figure S1. Frequency of distribution of the Vina scores of the 8000 possible tripeptides docked to the protein database bank (PDB) 1WCY, the crystal structure of human dipeptidyl peptidase IV (DPP-IV) in complex with Diprotin A.

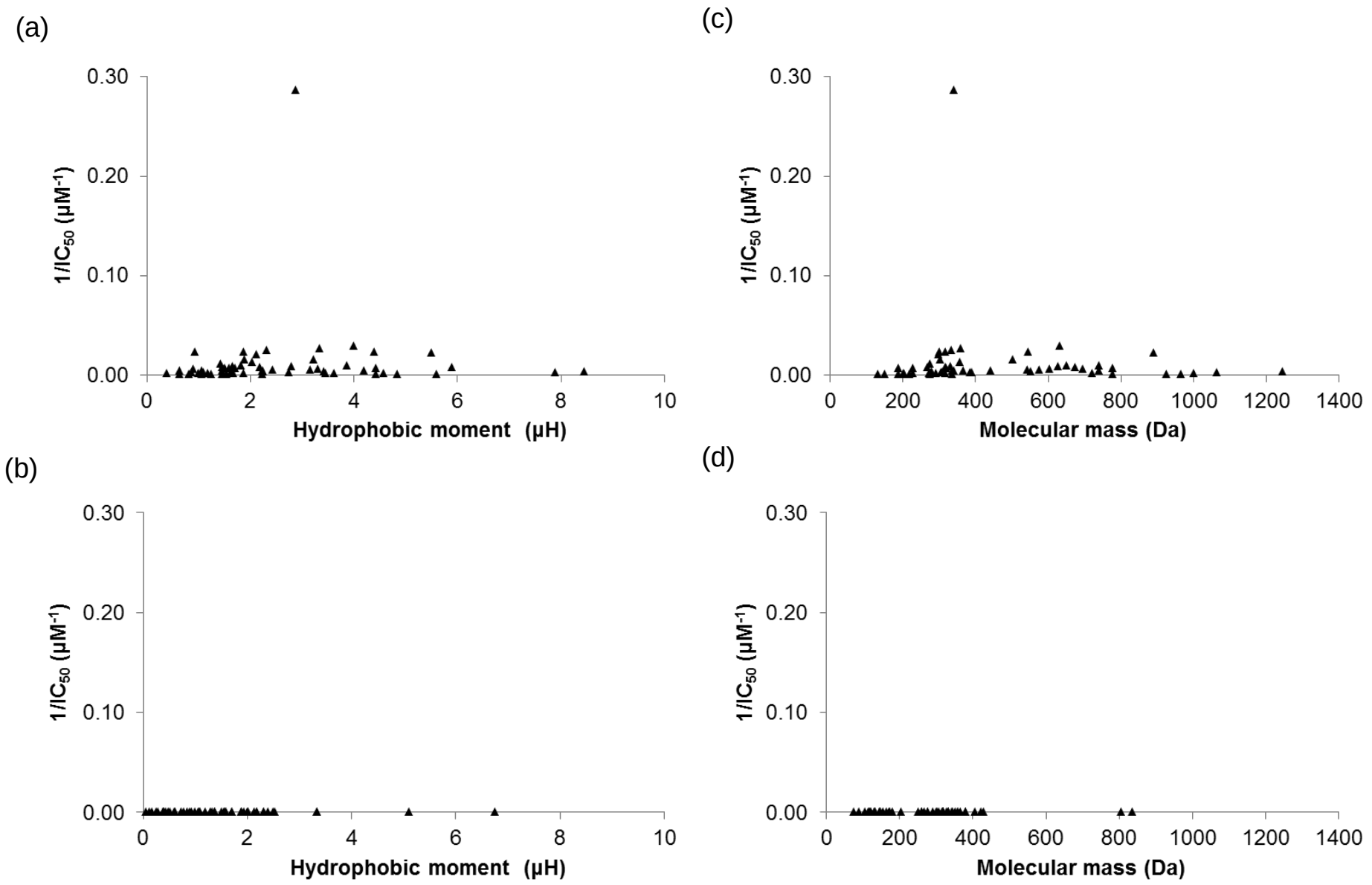
(a)

(b)

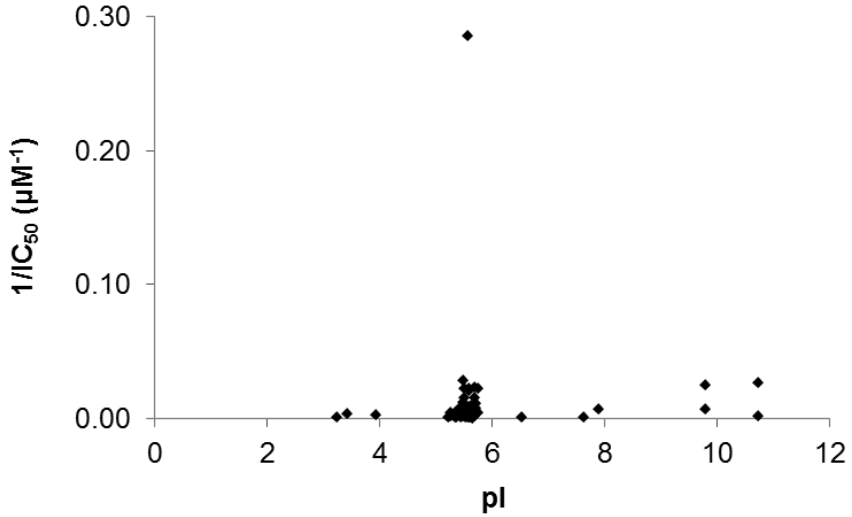
(c)

(d)

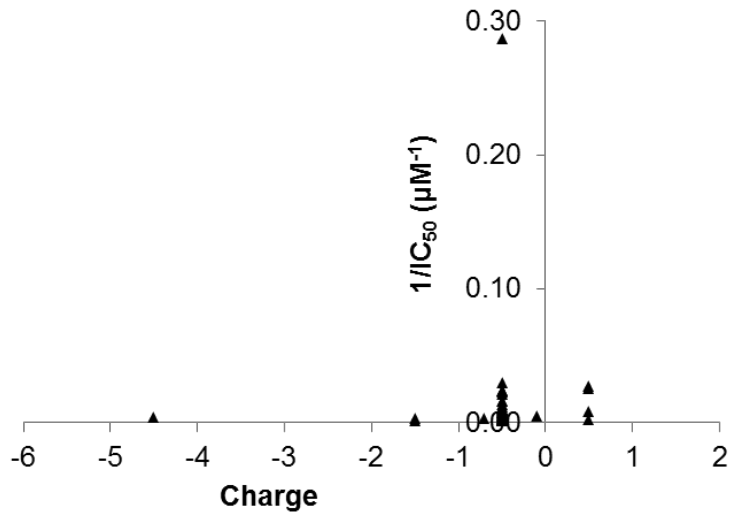
Supplementary Figure S2. LIGPLOT profiles of the crystal structure of human dipeptidyl peptidase IV (DPP-IV) 1WCY with peptides DPP-IV inhibitory peptides (a) His-Gln-Pro, (b) Lys-His-Pro, (c) Lys-Tyr-Pro and (d) Ile-Thr-Pro.


(a)
(c)
(b)
(d)
0.30
0.20
0.10
0.00
1/IC50 (μM-1)
0
2
4
6
8
10
Hydrophobic moment (μH)
200
400
600
800
1000
1200
1400
Molecular mass (Da)

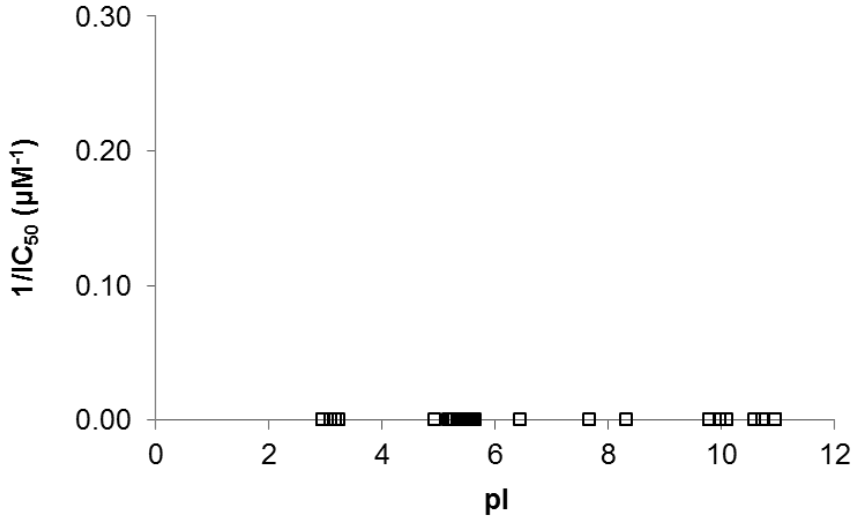
(e)


0.30
0.20
0.10
0.00
1/IC50 (µM-1)
0
2
4
6
8
10
12
pI

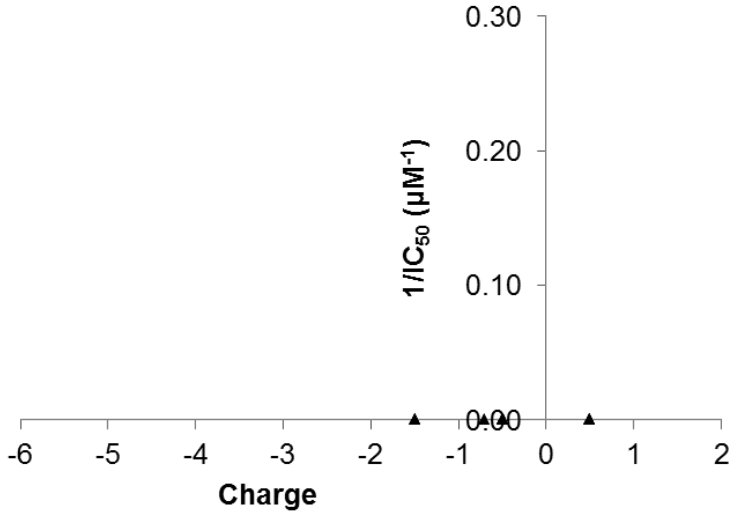
(g)


0.30
0.20
0.10
0.00
1/IC50 (µM-1)
-6
-5
-4
-3
-2
-1
0
1
2
Charge

(f)


0.30
0.20
0.10
0.00
1/IC50 (µM-1)
0
2
4
6
8
10
12
pI

(h)


0.30
0.20
0.10
0.00
1/IC50 (µM-1)
-6
-5
-4
-3
-2
-1
0
1
2
Charge

Supplementary Figure S3. Reciprocal of the dipeptidyl peptidase IV (DPP-IV) half maximum inhibitory concentration (IC_{50}) value of peptides as a function of the hydrophobic moment (μH) for (a) DPP-IV inhibitory peptides and (b) non-inhibitory peptides, the molecular mass for (c) DPP-IV inhibitory and (d) non-inhibitory peptides, the isoelectric point (pI) for (e) DPP-IV inhibitory peptides and (f) non-inhibitory peptides and the charge at pH 8 for (g) DPP-IV inhibitory and (h) non-inhibitory peptidesThe μH, pI and charge at pH8 were estimated with , and Protcalc , repectively. Values presented on these graphs incorporate the data from this work and values obtained for other milk protein-derived peptides in the literature .