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# Inhibition of dipeptidyl peptidase IV (DPP-IV) by tryptophan containing dipeptides

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Twenty seven Trp containing dipeptides were evaluated for their ability to inhibit dipeptidyl peptidase IV (DPP-IV), a key enzyme involved in incretin hormone processing. Novel DPP-IV inhibitors were identified comprising of three potent dipeptides (Trp-Arg, Trp-Lys and Trp-Leu) with half maximum inhibitory concentration ($IC_{50}$ values) <45 μM. With the exception of Leu-Trp which was ~20 times less potent than Trp-Leu, their reverse peptide did not inhibit DPP-IV. Trp-Asp was the only peptide studied herein with an N terminal Trp residue which was not a DPP-IV inhibitor. Phosphorylation resulted in an increase in DPP-IV $IC_{50}$, giving values of 482.1 ± 12.9 and >11 000 μM for Trp-Thr and Trp-pThr, respectively. The mode of inhibition of these peptides was studied using Lineweaver and Burk kinetic analysis, which showed both competitive and non-competitive modes of inhibition depending on the peptide sequence. This suggested binding of the peptide inhibitors to different locations on DPP-IV. *In silico* analysis of the milk proteome revealed that some of the DPP-IV inhibitors identified herein may be released from milk proteins following enzymatic digestion. The results are relevant to understanding the mechanism(s) involved in DPP-IV inhibition by short peptides. This in turn may dictate a more targeted approach for the release of potent peptides from milk proteins with the view of developing biofunctional hydrolysates for the management of type 2 diabetes.

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## 1. Introduction

Inhibition of dipeptidyl peptidase IV (DDP-IV) by food derived constituents may represent a dietary related approach for the management/control of type 2 diabetes (T2D). DPP-IV is an enzyme which can be found in different locations of the body including on the surface of various cells and in the circulation.[1] DPP-IV has been identified to play a role in the degradation of incretin hormones such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Those two incretins can enhance insulin secretion from pancreatic beta cells in the presence of nutrients *in vivo*.[2] The degradation of GLP-1 and GIP by DDP-IV results in a loss in their bioactive properties. DPP-IV inhibitory drugs are utilized to prevent incretin degradation *in vivo*, thereby increasing their half-life.[2,3]

Binding of various competitive DPP-IV inhibitors has been described at the active site of DPP-IV, mainly through interactions with an hydrophobic pocket, composed of Tyr-666, Tyr-662, Val-711, Val-656 and Trp-659.[4] However, a secondary binding site for DPP-IV inhibitors has been described for N terminus of the human immunodeficiency virus-1 (HIV) transactivator Tat. This secondary binding site is located close to the active site and involves linear mixed-type or parabolic mixed-type inhibition of DPP-IV.[5] A milk protein-derived dipeptide Trp-Val was recently identified, which behaved as a non-competitive inhibitor of DPP-IV. Using a molecular docking approach, it was shown that Trp-Val was likely to interact with DPP-IV at a secondary binding site located in close proximity to the active site.[6] Another mechanism of DDP-IV inhibition has recently been described for larger peptides (>13 amino acid residues) where peptides may hinder the formation of the active dimer of DPP-IV.[7]

Peptide inhibitors of DPP-IV with various sequences have been reported in the literature.[8] It has been suggested that dipeptides with the sequence Xaa-Pro, Pro-Xaa or Xaa-Ala (with Xaa being an amino acid residue) can act as inhibitors.[9] Different dipeptides with a Pro residue at the C terminus have been identified as DPP-IV inhibitors.[10] However, various peptide sequences without Pro have also been reported as potent inhibitors.[8,11] Recently, Trp and Trp-Val were identified as DPP-IV inhibitors. In contrast, no inhibition could be seen with the reverse peptide Val-Trp and other dipeptides (Arg-Trp, Lys-Trp and Ile-Trp) with Trp at the C terminus.[6,11] Another Trp containing peptide Trp-Pro was reported in a previous study for its DPP-IV inhibitory potential.[12]

Various studies have highlighted the possibility of using food as a natural source of DDP-IV inhibitors. An *in silico* approach was recently used to identify dietary proteins, including milk proteins, which could be potential precursors for peptides with DDP-IV inhibitory properties.[8] A number of

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milk protein hydrolysates have been identified as DPP-IV inhibitors.[11,13–15] Individual milk protein hydrolysates generated with β-lactoglobulin (β-Lg)[14,16–18] α-lactalbumin (α-La),[18] bovine serum albumin (BSA)[18] and lactoferrin (LF)[11,18] have also been identified as potent DPP-IV inhibitors.

It has recently been shown that several Trp-containing dipeptides could inhibit xanthine oxidase (XO), a metabolic enzyme which has been associated with an increase in oxidative stress in humans.[6,19] It has been demonstrated that T2D diabetes can be linked with an elevated oxidative status, which may cause endothelial injuries.[20] There appears to be a limited amount of data available in the literature describing the role of Trp containing peptides as DPP-IV inhibitors and modulators of oxidative stress. In a previous study Trp containing dipeptides (Trp-Val and Val-Trp) have been identified for their radical scavenging activity and Trp-Val was shown to be a DPP-IV inhibitor.[11] A more extensive study of the radical scavenging and DDP-IV inhibitory properties of Trp containing peptides has therefore been undertaken herein. The aim of this study was to evaluate the ability of Trp containing dipeptides to inhibit DPP-IV activity. In addition, the potential antioxidant (radical scavenging activity) role of these peptides was studied.

## 2. Material and methods

### 2.1. Reagents

The synthetic peptides Trp-Asp, Ala-Trp, Pro-Trp, Trp-Trp, Trp-Leu, Leu-Trp, Trp-Tyr, Tyr-Trp, Trp-Asn, Trp-Lys, Trp-Gly, Gly-Trp, Trp-Glu, Glu-Trp and Trp-Ser were obtained from Bachem (Bubendorf, Switzerland) while Trp-Ala, Trp-Arg, Trp-Gln, Trp-Ile, Trp-Phe, Trp-Met, Trp-Cys, Cys-Trp, Phe-Trp, Ser-Trp, Lys-Trp, Asn-Trp and Met-Trp were from Thermo Fisher Scientific (Ulm, Germany) while Trp-Pro, Trp-Thr, Asp-Trp and Trp-pThr (where p represents a phosphorylated residue) were obtained from Genscript (Piscataway, NJ, USA). Tris(hydroxymethyl)aminomethane (TRIS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox™), 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 was from VWR (Dublin, Ireland).

### 2.2. DPP-IV inhibition assay

Peptides were dispersed in HPLC grade water at concentrations ranging from $1.25 \times 10^{-2}$ to 1.25 mg $mL^{-1}$. The DPP-IV inhibition assay was carried out as described by Nongonierma and FitzGerald.[11] Briefly, the test samples (25 μL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA, the reaction substrate (final concentration 0.2 mM). The negative control contained 100 mM Tris–HCl buffer pH 8.0 (25 μL) and the substrate Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 units $mL^{-1}$). All the reagents and samples were diluted in 100 mM Tris–HCl buffer pH 8.0. Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate was incubated at 37 °C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. The DPP-IV half maximum inhibitory concentration ($IC_{50}$) values were determined by plotting the percentage of inhibition as a function of the concentration of test compound.

### 2.3. Determination of the mode of inhibition for DPP-IV inhibitory peptides

Lineweaver and Burk analysis was used as per Nongonierma and FitzGerald[11] to determine the mode of inhibition by DPP-IV inhibitory peptides. The initial rate of pNA release by DPP-IV from Gly-Pro-pNA was measured at different Gly-Pro-pNA concentrations ranging between 0.2 and 0.6 mM in the presence and absence of the DPP-IV peptide inhibitors at their $IC_{50}$ concentration. Km/Kapp and Vmax values were determined from the double reciprocal plots. The mode of inhibition was determined by comparing Km to Kapp values and Vmax obtained in the presence and absence of the DPP-IV inhibitory peptide.

### 2.4. DPPH radical scavenging assay

The DPPH assay was used to determine the proton radical scavenging properties of the peptides which were dispersed in HPLC grade water at concentrations ranging from $1.25 \times 10^{-3}$ to 1.25 mg $mL^{-1}$. The DPPH scavenging assay was carried out essentially according to Nongonierma and FitzGerald.[11] Briefly, the test samples (50 μL) were pipetted onto a 96 well microplate containing a DPPH (final concentration 0.088 mM) solution in 50% (v/v) ethanol. The microplate was incubated at 37 °C for 60 min in a microplate reader, absorbance of the DPPH radical was monitored at 517 nm. Each sample was analysed in triplicate. Trolox was used as a positive control. Scavenging of the DPPH radical was determined with respect to a control containing no scavenger (DPPH solution added with 50 μL water) as described by Liu *et al.*[21] The DPPH scavenging $EC_{50}$ values (concentration of active compound required to observe 50% DPPH scavenging) were determined by plotting the percentage of DPPH scavenging as a function of the concentration of test compound.

### 2.5. Statistical analysis

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

## 3. Results

### 3.1. DPP-IV inhibition of tryptophan containing peptides

Of the 31 Trp containing dipeptides studied, 21 were able to inhibit DPP-IV of which 18 contained a Trp residue at the N terminus (Table 1). The $IC_{50}$ value of the different DPP-IV inhibitory peptides was determined (Table 1). The most potent peptide studied herein was Trp-Arg with an $IC_{50}$ value of 37.8 ± 2.2 μM. Three other peptides with a similar ($P \geq 0.05$) potency to Trp-Arg were determined, these were Trp-Lys, Trp-Leu and Trp-Pro, having $IC_{50}$ values of 40.6 ± 4.0, 43.6 ± 0.9 and 44.5 ± 2.0 μM, respectively. The least potent DPP-IV inhibitor studied was Trp-pThr with an $IC_{50}$ value >11 000 μM (Table 1). With the

 

**Table 1** Inhibitory concentration inducing 50% inhibition ($IC_{50}$) for dipeptidyl peptidase IV (DPP-IV) with Trp containing peptides. $IC_{50}$ values are listed in order of decreasing inhibitory potency for the forward peptides[a]

| Forward peptide | DPP IV $IC_{50}$[b] (μM) | Reverse peptide | DPP IV $IC_{50}$[b] (μM) |
|---|---|---|---|
| Ile-Pro-Ile | 3.5 ± 0.2[a] | na | na |
| Trp-Arg | 37.8 ± 2.2[b] | Arg-Trp | —[c] |
| Trp-Lys | 40.6 ± 4.0[b] | Lys-Trp | —[c] |
| Trp-Leu | 43.6 ± 0.9[b] | Leu-Trp | 993.4 ± 58.6[j] |
| Trp-Pro | 44.5 ± 2.0[b] | Pro-Trp | — |
| Trp-Ala | 92.6 ± 0.8[c] | Ala-Trp | >6000 |
| Trp-Gln | 120.3 ± 1.5[d] | Gln-Trp | nd |
| Trp-Ile | 138.7 ± 8.0[d,e] | Ile-Trp | —[c] |
| Trp-Asn | 148.5 ± 9.3[e] | Asn-Trp | — |
| Trp-Met | 243.1 ± 1.8[f] | Met-Trp | 1691.4 ± 318.7[k] |
| Trp-Tyr | 281.0 ± 1.0[f] | Tyr-Trp | — |
| Trp-Cys | 420.0 ± 21.7[g] | Cys-Trp | — |
| Trp-Thr | 482.1 ± 12.9[g,h] | Thr-Trp | nd |
| Trp-Trp | 554.8 ± 15.1[h,i] | na | na |
| Trp-Ser | 643.5 ± 1.6[i] | Ser-Trp | — |
| Trp-Glu | >2000 | Glu-Trp | — |
| Trp-Phe | >3000 | Phe-Trp | — |
| Trp-Gly | >8000 | Gly-Trp | — |
| Trp-pThr | >11 000 | pThr-Trp | nd |
| Trp-Asp | — | Asp-Trp | — |

[a] nd: not determined; na: not applicable; —: no DDP-IV inhibition. [b] Values represent mean $IC_{50}$ values ± confidence interval ($P = 0.05$) $n = 3$ and triplicate determination. Values with different superscript letters are significantly different ($P < 0.05$). [c] Data taken from Nongonierma *et al.*[6]

exception of Trp-Asp, all peptides with a Trp residue at the N terminus were DPP-IV inhibitors. Three peptides with a Trp residue at the C terminus, Leu-Trp, Met-Trp and Ala-Trp, were also found to be DPP-IV inhibitors.

Most of the DPP-IV peptide inhibitors studied could be found within milk protein sequences with the exception of three peptides (Trp-Pro, Trp-Trp and Met-Trp, Table 2). Lineweaver and Burk kinetic analysis was utilized to determine the type of DPP-IV inhibition with the most potent ($IC_{50}$ values < 1000 μM, Table 1) peptides studied herein. The Lineweaver and Burk double reciprocal plots for Trp-Arg, Trp-Pro, Trp-Lys and Trp-Leu are illustrated in Fig. 1. There was no significant difference in Vmax ($P \geq 0.05$) in the presence and absence of inhibitor for Ile-Pro-Ile, Trp-Leu and Leu-Trp. However, the Km value was significantly different from Kapp values ($P < 0.05$). These peptides therefore behaved as competitive inhibitors of DPP-IV (Fig. 1 and Table 2). In contrast, for the thirteen other peptides (Trp-Arg, Trp-Lys, Trp-Pro, Trp-Ala, Trp-Gln, Trp-Ile, Trp-Asn, Trp-Met, Trp-Lys, Trp-Cys, Trp-Thr, Trp-Trp and Trp-Ser) tested there was no significant difference between Km and Kapp ($P \geq 0.05$). However, Vmax values were significantly different ($P < 0.05$) in the presence and absence of inhibitor. These peptides therefore behaved as non-competitive inhibitors of DPP-IV (Table 2).

### 3.2. Antioxidant activity of the peptides

The antioxidant activity of the peptides was evaluated by determining their ability to scavenge the DPPH radical. All peptides evaluated were able to scavenge DPPH radicals with the exception of Trp-Glu (Table 3). The antioxidant potency of the peptides was evaluated by determining their $EC_{50}$ value. A wide range of $EC_{50}$ values was found. The most potent antioxidant peptide was Cys-Trp with an $EC_{50}$ of 0.23 ± 0.10 mM. The least potent compound was Asp-Trp with an $EC_{50}$ > 20 mM. Of the different antioxidant peptides identified, 21 were also DPP-IV inhibitory peptides (Tables 1 and 3).

**Table 2** Mode of inhibition for dipeptidyl peptidase IV (DPP-IV) and milk derived fragments of Trp containing peptides. Peptides are listed in order of decreasing DPP-IV inhibitory potency for the forward peptides[ac]

| Compound | Type of inhibition[b] | Milk protein fragment |
|---|---|---|
| Ile-Pro-Ile | Competitive | κ-CN (f26–28) |
| Trp-Arg | Non-competitive | LF (f24–25) |
| Trp-Lys | Non-competitive | LF (f268–269) |
| Trp-Leu | Competitive | α-La (f104–105), α-La (f118–119) |
| Trp-Pro | Non-competitive | na |
| Trp-Ala | Non-competitive | LF (f560–561) |
| Trp-Gln | Non-competitive | κ-CN (f76–77), LF (f22–23) |
| Trp-Ile | Non-competitive | $\alpha_{s2}$-CN (f193–194), LF (f125–126) |
| Trp-Asn | Non-competitive | LF (f448–449), LF (f467–468) |
| Trp-Met | Non-competitive | β-CN (f143–144) |
| Trp-Tyr | Non-competitive | β-Lg (f19–20), $\alpha_{s1}$-CN (f164–165) |
| Trp-Cys | Non-competitive | α-La (f60–61), LF (f8–9), LF (f347–348) |
| Trp-Thr | Non-competitive | LF (f138–139) |
| Trp-Trp | Non-competitive | na |
| Trp-Ser | Non-competitive | BSA (f213–214), LF (f361–362) |
| Trp-Glu | nd | β-Lg (f61–62), LF (f549–550) |
| Trp-Phe | nd | LF (f16–17) |
| Trp-Gly | nd | BSA (f134–135) |
| Trp-pThr | nd | LF (f138–139) |
| Trp-Asp | na | $\alpha_{s2}$-CN (f109–110) |
| Arg-Trp | na | LF (f7–8), LF (f21–22) |
| Lys-Trp | na | β-Lg (f60–61) |
| Leu-Trp | Competitive | $\alpha_{s1}$-CN (f198–199) |
| Pro-Trp | na | $\alpha_{s2}$-CN (f108–109), $\alpha_{s2}$-CN (f192–193) |
| Ala-Trp | nd | BSA (f212–213), $\alpha_{s1}$-CN (f163–164) |
| Gln-Trp | na | α-La (f117–118), LF (f23–24), LF (f360–361), κ-CN (f75–76) |
| Ile-Trp | na | α-La (f59–60), LF (f267–268) |
| Asn-Trp | na | na |
| Met-Trp | nd | na |
| Tyr-Trp | na | α-La (f103–104) |
| Cys-Trp | na | na |
| Ser-Trp | na | LF (f137–138), β-CN (f142–143) |
| Glu-Trp | na | α-La (f25–26), LF (f15–16) |
| Phe-Trp | na | BSA (f133–134) |
| Gly-Trp | na | LF (f124–125), LF (f466–467) |
| Asp-Trp | na | LF (f559–560) |

[a] na: not applicable; nd: not determined. [b] Type of inhibition determined using Lineweaver and Burk plots as described in Nongonierma & FitzGerald.[19] [c] CN: casein; β-Lg: beta-lactoglobulin; α-La: alphalactalbumin; LF: lactoferrin; BSA: bovine serum albumin.

## 4. Discussion

It has been suggested that dipeptides with a Pro residue at the C terminus behave as DPP-IV inhibitors.[9] To date, the most potent ($IC_{50}$ < 100 μM) DPP-IV inhibitory peptides identified in the literature appear to contain a Pro or a N-terminal Trp residue.[8]

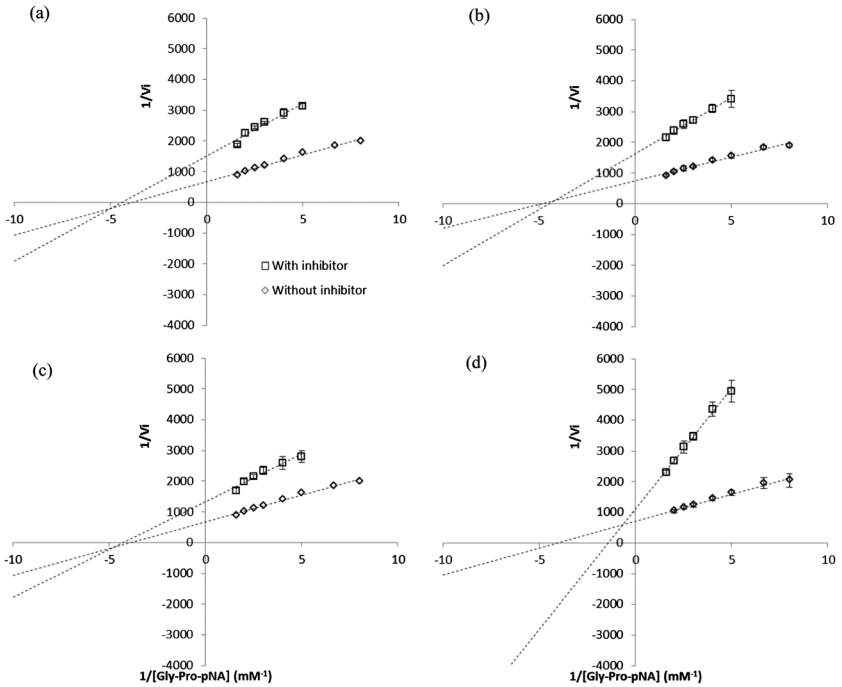


**Fig. 1** Lineweaver and Burk double reciprocal plots for dipeptidyl peptidase IV (DDP-IV) inhibition with Trp containing peptides having an half maximum inhibitory concentration ($IC_{50}$) lower than 45 μM (a) Trp-Arg (b) Trp-Pro, (c) Trp-Lys and (d) Trp-Leu. Values are the mean of three determinations ($n = 3$) ±SD.

Nevertheless, relatively potent DPP-IV inhibitory peptides containing no Pro or Trp residues have also been reported.[11,13,16] Yamada *et al.*[22] studied the structure function relationship of a potent inhibitor isolated from *Aspergillus oryzae* A374 (TMC-2) containing Trp and 2 unusual amino acids ((3*S*)-6,8-dihydroxy-7-methoxy-l,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and (*L*)-dihydroxy-leucine). It was shown that a Trp residue located at the N terminus of the tripeptide resulted in highest DPP-IV inhibition. $IC_{50}$ values of 5.7, 7.0 and 7.9 μM were reported for Trp-Tic, Trp-Tic-Glu and Trp-Tic-Ser, respectively. The Trp-Tic fragment of the tripeptide was shown to be responsible for the DPP-IV inhibitory properties of TMC2. Relatively low DPP-IV inhibition (≤15%) was seen with Trp-Glu, Trp-Phe, Trp-Pro, Trp-Trp, Trp-Tyr and Trp-Val when tested at 10 μM.[22] The most potent peptide studied herein was Trp-Arg ($IC_{50}$ 37.8 ± 2.2 μM, Table 1). This compound has a potency of the same order as previously identified DPP-IV inhibitory peptides. $IC_{50}$ values of 45, 46, 49, 66 and 82 μM for Ile-Pro-Ala-Val-Phe, Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu, Ile-Pro-Ala, Trp-Val and Leu-Pro-Gln, respectively have been reported.[11,13,14,17] However, the presence of Pro and/or Trp residues within a peptide sequence does not guarantee DPP-IV inhibition as demonstrated by the absence of inhibition with Pro-Trp and the other Trp containing non-inhibitory peptides (Table 1).

In a previous study, it was found that Trp-Val was a DPP-IV inhibitor whereas the reverse peptide Val-Trp was inactive. In addition, other dipeptides with a Trp at the C terminus (Arg-Trp, Lys-Trp and Ile-Trp) could not inhibit DPP-IV.[11] All the dipeptides studied herein with a Trp residue at the N terminus (Trp-Arg, Trp-Lys, Trp-Leu, Trp-Pro, Trp-Ala, Trp-Gln, Trp-Ile, Trp-Asn, Trp-Tyr, Trp-Cys, Trp-Thr, Trp-Ser, Trp-Trp Trp-Glu, Trp-Phe, Trp-Gly and Trp-pThr) could inhibit DPP-IV with the exception of Trp-Asp. The reverse peptides were generally not able to inhibit DPP-IV with the exception of Leu-Trp, Met-Trp

**Table 3** Inhibitory concentration inducing 50% scavenging ($EC_{50}$) for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with Trp containing peptides. Peptides are listed in order of decreasing DPPH scavenging activity for the forward peptides[ac]

| Forward peptide | DPPH $EC_{50}$[b] (mM) | Reverse peptide | DPPH $EC_{50}$[b] (mM) |
|---|---|---|---|
| Trp-Cys | 0.26 ± 0.09[b] | Cys-Trp | 0.23 ± 0.10[a] |
| Trp-Tyr | 1.28 ± 0.01[d] | Tyr-Trp | 0.57 ± 0.01[c] |
| Trp-Met | — | Met-Trp | 2.53 ± 0.46[e] |
| Trp-pThr | 2.43 ± 0.11[e] | pThr-Trp | nd |
| Trp-Pro | 2.57 ± 0.03[e,f] | Pro-Trp | >10 |
| Trp-Leu | 2.71 ± 0.03[e] | Leu-Trp | 1.71 ± 0.02[d] |
| Trp-Gln | 3.92 ± 1.29[g,h,i,j] | Gln-Trp | nd |
| Trp-Phe | 4.20 ± 0.27[i,j,k] | Phe-Trp | 3.04 ± 0.86[e,f,g] |
| Trp-Arg | 4.43 ± 1.03[i,j,k] | Arg-Trp | nd |
| Trp-Thr | 4.92 ± 0.03[h,i,j] | Thr-Trp | nd |
| Trp-Asn | 4.92 ± 1.13[h,i,j,k] | Asn-Trp | >5 |
| Trp-Lys | >5 | Lys-Trp | 4.14 ± 0.82[h,i,j] |
| Trp-Gly | >5 | Gly-Trp | 3.68 ± 0.12[f,g,h,i] |
| Trp-Ser | >5 | Ser-Trp | 3.97 ± 0.26[g,h,i,j] |
| Trp-Met | >5 | Met-Trp | nd |
| Trp-Ala | >5 | Ala-Trp | >10 |
| Trp-Ile | >5 | Ile-Trp | nd |
| Trp-Asp | >5 | Asp-Trp | >20 |
| Trp-Trp | >10 | na | na |
| Trp-Glu | — | Glu-Trp | 3.07 ± 0.92[e,f,g] |

[a] na: not applicable.; nd: not determined; —: no inhibition. [b] Values represent mean $IC_{50}$ values ± confidence interval ($P = 0.05$) $n = 3$ and triplicate determination. Values with different superscript letters are significantly different ($P < 0.05$). [c] $EC_{50}$ values for Trolox was of 17.2 ± 5.5 nM.

and Ala-Trp. However, these peptides had a relatively low potency ($IC_{50}$ > 900 μM) which was more than ten times less than that of the corresponding forward peptide (Table 1). These results showed that sequence and not only composition is a major determinant in the DPP-IV inhibitory potential of a peptide.

Larger peptides with a Trp at the N terminus are also able to inhibit DPP-IV in the same fashion as dipeptides. However, a limited number of DPP-IV inhibitory Trp containing peptides larger than 2 amino acids appear to exist in the literature. The Trp containing peptide derived from β-lactoglobulin, Val-Ala-Gly-Thr-Trp-Tyr, with an $IC_{50}$ of 174 μM has been reported.[16]

It was found herein that Trp-pThr could inhibit DPP-IV, however, the potency of this peptide was relatively low. To our knowledge this is the first time that a phosphorylated peptide has been evaluated for its DDP-IV inhibitory activity. The $IC_{50}$ value of Trp-pThr (>11 000 μM) was around 25 times more than that of the non-phosphorylated equivalent Trp-Thr (482.1 ± 12.9 μM), suggesting that phosphorylation was responsible for the decrease in DPP-IV inhibitory activity. This may be explained by the fact that addition of the phospo group to the dipeptide may have interfered with binding to DPP-IV. Binding of peptides to the active site of DPP-IV has been reported to involve both hydrophobic and electrostatic interactions.[23,24] Docking of amino acids and peptides to the active site of DPP-IV also showed interaction of competitive inhibitors with the hydrophobic pocket of DPP-IV.[6] The major reduction in $IC_{50}$ value observed between Trp-pThr and Trp-Thr may arise from differences in the charge of the two peptides. Alterations in peptide charge may modify the electrostatic interaction between the peptide and its binding site on DPP-IV. This may have led to higher electrostatic repulsion between Trp-pThr and DPP-IV, causing a reduction in the DPP-IV inhibitory properties.

Different modes of DPP-IV inhibition were found with the peptides evaluated herein. Most Trp containing peptides were found to be non-competitive inhibitors of DPP-IV (Table 2). The two competitive Trp containing DPP-IV inhibitors, Leu-Trp and Trp-Leu, presumably bind to the active site of DPP-IV. Trp-Pro was described elsewhere as a DPP-IV inhibitor with a linear mixed-type mode of inhibition.[12] This is in agreement with the results reported herein since non-competitive inhibition has been described as a specific case of mixed-type inhibition. Non-competitive inhibition suggests interaction with DPP-IV at a different location from the active site. Similar results have previously been reported with Trp-Val which also behaved as a non-competitive inhibitor of DPP-IV. In addition, utilisation of the Pepsite2 tool showed that Trp-Val was likely to bind near the active site of DPP-IV.[6] Other studies have shown that linear mixed-type or parabolic mixed-type inhibitors, including peptides derived from the N terminus of the HIV transactivator Tat, could bind to a secondary site near the active site of DPP-IV.[5] More knowledge on the secondary binding site of DPP-IV is needed to understand binding of inhibitors at this site. This may allow enhanced prediction and discovery of potent DPP-IV inhibitory peptide sequences.

The peptides evaluated herein had a modest DPPH scavenging activity compared to other peptides mentioned in other studies. $EC_{50}$ values for DPPH scavenging by decapeptides extracted from venison were around 10 μM,[25] 98 μM for the casein derived peptide Tyr-Pro-Tyr-Pro-Glu-Leu and 23 μM for carnosine (Ala-His).[26] $EC_{50}$ values of 0.24 and 0.65 mM have been reported for Trp-Val and Val-Trp, respectively.[11] The most potent DPPH scavengers studied herein were Cys-Trp and Trp-Cys. The presence of Cys may be responsible for the antioxidant activity in these peptides. This is linked to the fact that Cys can reduce the DPPH radical.[27,28] It has been suggested that the antioxidant property of dipeptides depends on peptide sequence rather than its composition. A stronger antioxidant activity has been reported for Trp-Lys compared to Lys-Trp.[29] Similarly, for all peptides, differences were seen between the forward and the reverse peptide for DPPH scavenging (Table 3). In general, higher DPPH $EC_{50}$ values were found with dipeptides having a Trp residue located at the N terminus compared to the reverse peptide. However, for Trp-Asp and Trp-Ala, DPPH $EC_{50}$ was lower than that of their corresponding reverse peptide (Table 3). Interestingly, the phosphorylated peptide Trp-pThr, which had an $EC_{50}$ value of 2.43 ± 0.11 mM, was twice as potent as the non-phosphorylated peptide, Trp-Thr ($EC_{50}$ of 4.92 ± 1.13 mM, Table 3).

Most of the peptides evaluated herein had a dual bioactivity combining antioxidant and DPP-IV inhibitory properties (Tables 1 and 3). In addition, some of the peptides studied herein have already been reported with other bioactive properties. Trp-Trp, Trp-Leu, Ile-Trp, Trp-Val, Ala-Trp,[30] Trp-Pro, Trp-Thr, Trp-pThr,[31] Trp-Tyr[32] and Trp-Gly[33] have been identified as angiotensin converting enzyme (ACE) inhibitors. Combined ACE and DPP-IV inhibitory properties of peptides are highly relevant to the management of T2D as cardiac complications have been associated with many TD2 cases.[9]

With the exception of 3 peptides, all the DDP-IV inhibitory peptides studied herein could be found within the primary sequence of different milk proteins (Table 2). This demonstrates that milk proteins could be a good source for such peptides, especially since milk protein hydrolysates have been identified for their DPP-IV inhibitory properties. The most potent peptides Trp-Arg and Trp-Lys can be found within LF and Trp-Leu within α-La, suggesting that whey proteins are a good source for these DPP-IV inhibitory Trp containing peptides (Tables 1 and 2). Previous studies have identified an LF hydrolysate with potent DPP-IV inhibitory activity having an $IC_{50}$ of 1.088 ± 0.106 mg $mL^{-1}$.[11] *In silico* digestion of LF with the Peptide Cutter programme[34] showed that two peptides with a Trp at the N terminus (Trp-Gln-Trp-Arg and Trp-Cys-Thr-Ile-Ser-Gln-Pro-Glu-Trp-Phe-Lys) could theoretically be released after incubation with trypsin. The LF hydrolysate described earlier was generated with trypsin. It is likely that these 2 peptides with a Trp at their N terminus are DPP-IV inhibitors based on the results presented herein and on previous data from the literature.[12,22] Therefore, it is anticipated that these peptides may contribute to the overall DPP-IV inhibitory activity observed with the LF hydrolysate.

It is generally accepted that short peptides are relatively stable to gastrointestinal digestion. The intestinal stability of Trp containing dipeptides was predicted using the model

developed by Foltz *et al.*[35] It was shown that Trp-Val was moderately stable to intestinal digestion (between 25 and 75% of intact peptide remaining after 60 min simulated intestinal digestion), whereas other Trp containing dipeptides (Arg-Trp, Lys-Trp, Val-Trp and Ile-Trp) were not predicted to be stable.[6] Based on these results, it is anticipated that certain Trp containing dipeptides studied herein may survive gastrointestinal digestion. In addition, some of these peptides may be bioavailable. Permeation of di- and tripeptides through intestinal mucosa models consisting of Caco-2 cells has been demonstrated.[36,37] Recently, it was shown that Trp containing short peptides derived from lactoferricin B (Trp-Gln and Arg-Trp-Gln), could cross Caco-2 cell monolayers.[38] The bioavailability of short peptides has also been reported in humans. It has been shown that short peptides (Leu-Trp, Phe-Tyr, Ile-Trp and Ile-Pro-Pro) could be found intact in the circulation of humans following the consumption of a drink enriched with the lactotripeptide Ile-Pro-Pro.[39] In addition, a relatively long half-time of 2.3 h was reported for Trp-Gln in human plasma.[38] Because DPP-IV is an ubiquitous enzyme and the incretin hormones GLP-1 and GIP have a systemic target (*i.e.*, the pancreas), the bioavailability of DPP-IV inhibitory Trp containing peptides is highly relevant in strategies aiming to increase incretin half-life *in vivo*. Enzymatic strategies to specifically release peptides with a Trp residue at the N terminus may allow development of potent DPP-IV inhibitory hydrolysates. This approach may find application in the development of functional foods with serum glucose lowering activity. There is a need for *in vivo* studies to validate these findings.

## 5. Conclusion

New and potent DPP-IV inhibitory peptides with a Trp at the N terminus have been reported. In general, the reverse peptides were not able to inhibit DPP-IV or had a significantly reduced potency compared to the forward peptide. Interestingly, both competitive and non-competitive modes of inhibition were found depending on peptide sequence, suggesting both direct interaction of the inhibitor with the active site of DPP-IV and binding at a secondary site. More detailed knowledge of DPP-IV binding sites may help to better understand the physicochemical properties of peptides which may positively impact on DPP-IV inhibition. Several of the peptides studied herein were found within the milk proteome, suggesting that these may be enzymatically released from milk proteins. However, other Trp-rich food proteins may also be used as starting substrates for the generation of hydrolysates with potent DPP-IV inhibitory properties. The targeted release of peptides with a Trp residue at the N terminus may be relevant for the generation of food protein hydrolysates with an anti-diabetic biofunctionality.

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