Journal Name

Please cite as: Nongonierma, A. B., Hennemann, M., Paolella, S., & FitzGerald, R. J. (2017). Generation of wheat gluten hydrolysates with dipeptidyl peptidase IV (DPP-IV) inhibitory properties. Food & Function. 8, 2249-2257.

Generation of wheat gluten hydrolysates with dipeptidyl peptidase IV (DPP-IV) inhibitory properties

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/C7FO00165G

www.rsc.org/

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Wheat gluten, a Pro-rich dietary protein, was investigated for its potential to produce dipeptidyl peptidase IV (DPP-IV) inhibitory peptides during enzymatic hydrolysis with Debitrase HYW20. Nine gluten hydrolysates (H1-H9) were generated using a 2 factor × 3 level design of experiments (DOE) including the incubation temperature (40, 50 and 60°C) and the enzyme: substrate ratio (E:S, 0.5, 1.0 and 1.5% (w/w)). Their DPP-IV half maximal inhibitory concentration (IC_{50}) ranged from 0.24 ± 0.02 (H9) to 0.66 ± 0.06 mg mL^{-1} (H2A and H7) and their degree of hydrolysis (DH) from 31.7 ± 0.9 (H7) to 62.2 ± 3.0% (H6). Gluten and H9, the most potent DPP-IV inhibitory hydrolysate, were subjected to simulated gastrointestinal digestion (SGID), yielding Gluten_CorPP and H9_CorPP, respectively. H9_CorPP had a higher DPP-IV inhibitory potency than Gluten_CorPP (i.e., DPP-IV IC_{50} values of 0.33 ± 0.03 vs 1.45 ± 0.26 mg mL^{-1}, respectively). H9 and H9_CorPP both contained relatively potent DPP-IV inhibitory peptides such as Val-Pro-Leu, Trp-Leu and Trp-Pro which were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). In addition, several sequences possessing features of DPP-IV inhibitory peptides, mostly consisting of a penultimate or C-terminal Pro, were identified within H9. The presence of Pro-containing peptides within H9 may contribute to its stability to digestive enzymes. Gluten hydrolysates may have antidiabetic potential for humans.

1 Introduction

Gluten is a group of proteins which is found within several cereals, including wheat, rye and barley. Wheat gluten is composed of gliadins and glutenins, two major storage proteins (prolamins) found in the endosperm, representing up to 85% of the total protein content.[1] Wheat gluten is extensively utilised in the bakery industry as it confers cohesiveness, viscoelastic and gas retention properties to dough.[2] The viscous properties of wheat gluten have been attributed to gliadins while the elastic properties arise from the glutenins.[3] Wheat is a major protein source in the human diet. It is estimated to contribute up to 30% of dietary protein intake.[2] However, people suffering from coeliac disease have been recommended to follow a strict gluten free-diet.[2] Enzymatic hydrolysis of wheat gluten has been proposed to reduce its allergenic potential.[4-6] Interestingly, the process of hydrolysis itself may lead to the release of biologically active peptides.

In silico analysis of wheat gluten proteins has revealed that they contain several known bioactive peptides having potential antioxidant, antihypertensive and antidiabetic properties.[7] The bioactive properties of wheat gluten hydrolysates such as their antioxidant and antihypertensive properties have been demonstrated *in vitro*.[8-10] Numerous dipeptidyl peptidase IV (DPP-IV) inhibitory peptides have been identified within wheat gliadins and glutenins during *in silico* studies.[11,12] DPP-IV is a metabolic enzyme which can bring about the degradation and inactivation of incretins (e.g., glucagon like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP)). Incretins have an insulinotropic action during the post prandial phase, generally resulting in enhanced serum glucose regulation. Inhibition of DPP-IV may lead to the restoration of incretin insulinotropic action particularly in people suffering from type 2 diabetes (T2D).[13] Therefore, DPP-IV inhibition is therapeutically targeted as a means to achieving better serum glucose regulation in T2D sufferers.

Several dietary peptides inhibit DPP-IV *in vitro* and also *in vivo*, in small animals, for reviews see:[14,15]. Velarde-Salcedo et al.[16] showed that an *in vitro* gastrointestinal digest of wheat flour was able to inhibit DPP-IV. To date, limited information is available in the literature in relation to the role of wheat gluten hydrolysates on DPP-IV inhibition. Wheat gluten contains relatively high levels of Pro, Asn, Gln and Arg, but low levels of essential amino acids such as Trp, Lys and Met.[1] Pro residues have frequently been reported in peptides having DPP-IV inhibitory properties. In particular, peptides with a Pro at the penultimate position, which are preferred DPP-IV substrates, display substrate-type inhibition of DPP-IV.[17,18] Furthermore, several peptides having a Pro at the C-terminus

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

have previously been identified as DPP-IV inhibitors.[19,20] Therefore, the utilisation of protein substrates rich in Pro, such as wheat gluten, appears to be of interest for the generation of DPP-IV inhibitory peptides.

The aim of this study was to investigate the generation of DPP-IV inhibitory peptides during enzymatic hydrolysis of wheat gluten. This was achieved by hydrolysing wheat gluten with Debitrase HWY20, a commercial food-grade enzyme preparation. The physicochemical characteristics (degree of hydrolysis (DH), peptide profile and molecular mass distribution) of the gluten hydrolysates were assessed. The stability of the DPP-IV inhibitory properties of the gluten hydrolysates following incubation with digestive enzymes was studied using an *in vitro* simulated gastrointestinal digestion (SGID) protocol. Peptide identification was subsequently carried out by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS) and a bicinchoninic acid (BCA) protein assay kit from Pierce Biotechnology were purchased 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). Tris(hydroxymethyl)aminomethane (TRIS), trifluoroacetic acid (TFA), sodium phosphate monobasic, sodium phosphate dibasic, sodium dodecyl sulphate (SDS), Gly-Pro-pNA, Leu, diprotin A (Ile-Pro-Ile), porcine DPP-IV (≥ 10 units mg^{-1} protein), molecular mass standards (i.e., bovine serum albumin (BSA), β-lactoglobulin (β-Lg), α-lactalbumin (α-La), aprotinin, bacitracin, Leu-Trp-Met-Arg and Asp-Glu), wheat gluten, mass spectrometry (MS) grade water and ACN were purchased from Sigma-Aldrich (Dublin, Ireland). Debitrase HYW20 (11470 U g^{-1}) was provided by Dupont-Danisco (Marlborough, Wiltshire, UK), while pepsin (3000 FCC U g^{-1}) was provided by Biocatalysts (Cefn, Wales, UK) and Corolase PP (2500 UHb g^{-1}), a porcine pancreatic enzyme preparation, was supplied by AB Enzymes (Darmstadt, Germany).

Enzymatic hydrolysis of gluten

The protein content of gluten was determined in triplicate (n=3) using the BCA assay as already outlined earlier.[21]

Gluten was hydrolysed with Debitrase HYW20, using a 2 factor × 3 level design of experiments (DOE). Each factor was centred and reduced (z-centred values). Preliminary experiments showed that hydrolysis times < 6 h were not sufficient to fully hydrolyse gluten with Debitrase HYW20. In addition, pH (6.0-8.0) and substrate concentration (2-10% (w/w)) alterations were studied. Gluten aggregation occurred at pH > 6.0 and concentrations > 2% (w/w). These preliminary experiments showed that it was not practical to adjust the pH of the gluten suspension to the optimal pH for Debitrase HYW20 (pH 7.0) or to study substrate concentrations > 2% (w/w) and hydrolysis times < 6 h. Therefore, two hydrolysis factors, i.e., incubation temperature (T) and enzyme: substrate ratio (E:S), which did not cause gluten to precipitate or induce insufficient gluten hydrolysis, were selected. These factors were studied at three levels (T, 40(-1), 50(0) and 60(+1)°C and E:S, 0.5(-1), 1(0) and 1.5(+1)% (w/w)) (Table 1). These levels were chosen to set the central point conditions of the DOE (i.e., T 50°C and E:S 1%(w/w)) as described previously for the hydrolysis of bovine whey proteins with Debitrase HYW20.[22] Nine different hydrolysates (H1-H9) were generated once with the exception of the hydrolysate at the central point conditions generated in triplicate (H2, H2B, H2C, n=3).

Gluten was suspended (2.0% (w protein equivalent/w)) in distilled water for 60 min in a water bath (Lauda E100, Lauda Brinkmann, Lauda-Königshofen, Germany) set at the hydrolysis temperature as defined by the DOE. The pH of the gluten suspension was pH 5.6. Debitrase HYW20 was subsequently added at the desired E:S ratio as defined in the DOE. Hydrolysis was conducted for 24 h[23] and the enzyme was subsequently heat inactivated in a water bath set at 90°C for 20 min. The negative control corresponded to unhydrolysed gluten which was incubated using the same temperature and time conditions as the hydrolysate but without enzyme addition. Samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C prior to analysis.

In vitro simulated gastrointestinal digestion (SGID) of intact and hydrolysed gluten

Intact gluten and H9 were subjected to SGID *in vitro* as described earlier.[24] 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 (H9_pepsin or Gluten_pepsin) was brought to pH 7.5 using 1 M NaOH and was subsequently hydrolysed with Corolase PP (E:S 1% (w/w)) for 150 min at 37°C, pH 7.5 using a pH stat (Metrohm), yielding H9_CorPP or Gluten_CorPP. Corolase PP contains several intestinal enzyme activities, including trypsin, chymotrypsin, elastase and various exopeptidases.[25] The reaction was terminated by thermal treatment (90°C, 20 min). Samples were freeze-dried and stored at -20°C until utilisation.

Degree of hydrolysis (DH)

The DH of the hydrolysates (H1-H9) was determined in triplicate (n=3) using the TNBS method as already outlined.[26] Absorbance values at 350 nm were measured with a microplate reader (Biotek Synergy HT, Winoosky, VT, USA). The free amino group concentration (AN) was calculated using a Leu standard curve and the absorbance values.[27] The DH was calculated according to Equation 1:

$$\mathrm{DH} = 100 \times \frac{(\mathrm{AN}_2 - \mathrm{AN}_1)}{\mathrm{N}pb} \qquad \text{Equation 1}$$

With AN_1, the free amino group content of the negative control, i.e., unhydrolysed protein (mg g^{-1} protein); AN_2, the

free amino group content of the hydrolysate (mg g^{-1} protein) and Npb, the amino group content of the peptide bonds in the protein substrate (112.1 mg g^{-1} protein for wheat gluten)[28].

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

Gluten hydrolysates were analysed by GP-HPLC as described earlier.[29] A Waters HPLC system (model 600 binary pump, model 2707 autosampler and model 2489 dual λ absorbance detector interfaced with Empower™, Milford, MA, USA) was used for this purpose. Samples were resuspended in the mobile phase (0.1% (v/v) TFA and 30% HPLC grade ACN in HPLC water) at 0.21% (w protein equivalent/v) and filtered through 0.2 µm PTFE syringe filters (VWR). Separation was carried out in isocratic mode at 21°C by injecting 20 µL of sample into 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). The flow rate was set at 0.5 mL min^{-1} for 60 min. The absorbance was monitored at 214 nm. Molecular mass standards (i.e., BSA, β-Lg, α-La, aprotinin, bacitracin, Leu-Trp-Met-Arg and Asp-Glu) were used to calibrate the method (Supplementary Fig. S1). Four molecular mass bands within the resulting chromatogram were defined as > 10, 10-5, 5-1 and < 1 kDa.

Peptide profiling of the hydrolysates using reverse-phase ultra-performance liquid chromatography (RP-UPLC)

The peptide profile of the gluten hydrolysates was determined by RP-UPLC (Acquity, Waters) as described earlier.[30] Solvent A was 0.1% (v/v) TFA in MS grade water and solvent B was 0.1% (v/v) TFA and 80% MS grade ACN in water. The samples were resuspended (0.43% w protein equivalent/v) in solvent A and filtered with 0.2 µm cellulose acetate filters (VWR). Separation was carried out at 30°C at a flow rate of 0.3 mL min^{-1} using 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 (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. Absorbance was monitored at 214 nm.

Dipeptidyl peptidase IV (DPP-IV) inhibition assay

The freeze-dried samples were dispersed in HPLC grade water at concentrations ranging from 1.9×10^{-2} to 1.9 mg mL^{-1} (final concentration expressed in mg protein equivalent mL^{-1}). The DPP-IV inhibition assay was carried out in triplicate as outlined previously.[31] The DPP-IV half maximal inhibitory concentration (IC_{50}) was determined by plotting the percentage inhibition as a function of test compound concentration.

The DPP-IV IC_{50} values as a function of the E:S and temperature were fitted with Matlab (version R2014b, MathWorks, Inc, Natick, MA, USA) using a multilinear regression (MLR) model (Equation 2).[32]

$$Y = \beta_0 + \beta_1 T + \beta_2 ES + \beta_3 T^2 + \beta_4 ES^2 + \beta_5 T \times ES + \varepsilon$$

Equation 2

With Y, the DPP-IV IC_{50} value; β_0 to β_5, the coefficients of the model and ε, the residual of the model.

Table 1. Degree of hydrolysis (DH) and dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration (IC_{50}) of gluten hydrolysates generated within the design of experiments (DOE, H1-H9), H9 digested with pepsin (H9_pepsin), H9_pepsin digested with Corolase PP (H9_CorPP), gluten digested with pepsin (Gluten_pepsin) and Gluten_pepsin digested with Corolase PP (Gluten_CorPP).

Hydrolysate	Variable levels[1]		DH[2,3] (%)	DPP-IV IC_{50}[2,4]
	Temperature (°C)	E:S (%)		(mg mL^{-1})
H1	50 (0)	0.5 (-1)	37.9 ± 1.5	0.53 ± 0.05
H2A	50 (0)	1.0 (0)	59.5 ± 2.5	0.66 ± 0.06
H2B	50 (0)	1.0 (0)	61.6 ± 2.5	0.43 ± 0.04
H2C	50 (0)	1.0 (0)	57.9 ±2.5	0.42 ± 0.04
H3	50 (0)	1.5 (+1)	58.8 ± 0.8	0.37 ± 0.04
H4	40 (-1)	0.5 (-1)	40.0 ± 2.2	0.48 ± 0.02
H5	40 (-1)	1.0 (0)	60.6 ± 1.0	0.34 ± 0.04
H6	40 (-1)	1.5 (+1)	62.2 ± 3.0	0.35 ± 0.03
H7	60 (+1)	0.5 (-1)	31.7 ± 0.9	0.66 ± 0.06
H8	60 (+1)	1.0 (0)	39.1 ± 2.0	0.46 ± 0.06
H9	60 (+1)	1.5 (+1)	55.5 ± 4.6	0.24 ± 0.02
H9_pepsin	-	-	nd	0.40 ± 0.03
H9_CorPP	-	-	nd	0.33 ± 0.03
Gluten_pepsin	-	-	nd	> 1.90
Gluten_CorPP	-	-	nd	1.45 ± 0.26

[1]The z-centred values for each variable of the experimental design are provided in brackets.

[2]Mean ± SD (n=3).

[3]nd: not determined. The SD of the DH for the triplicate determination of H2A, H2B and H2C was 1.9%.

[4]IC_{50}: concentration inducing 50% DPP-IV inhibition, expressed in mg protein equivalent per mL (mg mL^{-1}). The SD of the DPP-IV IC_{50} value for the H2A, H2B and H2C was 0.13 mg mL^{-1}. The IC_{50} value of the positive control Ile-Pro-Ile was 4.02 ± 0.51 µM.

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Liquid chromatography tandem mass spectrometric (LC-MS/MS) analyses of the hydrolysates

Peptide identification in H9 and H9_CorPP was carried out by LC-MS/MS using an Acquity UPLC (Waters) fitted with an Acquity BEH C18, 130 Å column (2.1 mm × 50 mm × 1.7 µm). 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.[33] The MS was fitted with an electrospray ionization (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 mg mL^{-1}. A sample volume of 2 µL was injected in the UPLC. Peptide identification was carried with PEAKS Studio (version 7.5, Bioinformatics Solutions Inc, Waterloo, Canada). The false discovery rate (FDR) and the average local confidence (ALC) were set at 1 and 60%, respectively.

Previously identified DPP-IV inhibitory peptides for which an IC_{50} value was available in the literature as well as peptides displaying features of known DPP-IV inhibitory peptides, i.e., hydrophobic amino acid (Trp (W_1), Leu (L_1), Ile (I_1) or Phe (F_1)) at the N-terminus and/or a Pro (P_2)/Ala (A_2) at position 2 and/or Pro at the C-terminus (P-term) of the peptide,[11,19,34] were searched for within H9 and H9_CorPP.

Statistical analysis

A one-way analysis of variance (ANOVA) at a significance level $p < 0.05$ was used to study the significance of the coefficient of the MLR (Equation 2) with Matlab. The DH and DPP-IV IC_{50} values of the triplicate determination of H2 was studied using an ANOVA which was followed by a Student Newman-Keuls test for multiple means comparison ($p < 0.05$) using SPSS (version 22, SPSS Inc., Chicago, IL, USA).

Results

DPP-IV inhibitory properties of the gluten hydrolysates

Gluten controls were not able to inhibit DPP-IV (IC_{50} > 1.9 mg mL^{-1}). In contrast, all hydrolysates (H1-H9) generated within the DOE displayed DPP-IV inhibitory properties. Their DPP-IV IC_{50} ranged from 0.24 ± 0.02 (H9) to 0.66 ± 0.06 mg mL^{-1} (H2A and H7, Table 1). Hydrolysate generation was reproducible from a DPP-IV inhibition point of view for H2B and H2C ($p > 0.05$, Table 1). Significant differences were seen between H2A and H2B/H2C ($p < 0.05$). However, all these three hydrolysates had DPP-IV IC_{50} values of the same order. These differences may come from the fact that gluten is poorly soluble in water, which may have resulted in some variation in the peptides released during the hydrolysis process.

While statistical treatment indicated that hydrolysis temperature had a significant effect on the DPP-IV IC_{50} value ($p < 0.05$, Table 2), the MLR model was not statistically significant ($p > 0.05$, Table 2).

Physicochemical properties of the gluten hydrolysates

The protein content of the starting gluten preparation was determined to be 61.71 ± 0.66% (w/w). Gluten was hydrolysed to different extents depending on the hydrolysis conditions employed. The DH of the nine hydrolysates generated within the DOE varied between 31.7 ± 0.9 (H7) and 62.2 ± 3.0% (H6, Table 1). The DH of the three hydrolysates (H2A, H2B and H2C) made in the central point conditions were not significantly different ($p > 0.05$, Table 1).

Intact gluten contained mostly proteins having a molecular mass > 10 kDa, which accounted for > 95% of the sample (Fig. 1). Following hydrolysis, proteins were extensively degraded as can be seen in the hydrolysates (H1-H9), which contained < 10% material > 10 kDa (Fig. 1). Enzymatic digestion of gluten resulted in the generation of low molecular mass components, with the material < 1 kDa representing > 50% of the hydrolysates. H2A, H2B and H2C displayed similar molecular mass distribution profiles (Fig. 1).

SGID of gluten and H9

Following digestion with pepsin (Gluten_pepsin), the DPP-IV inhibitory properties of gluten were not modified (DPP-IV IC_{50} > 1.90 mg mL^{-1}, Table 1). However, further digestion with Corolase PP (Gluten_CorPP) induced a decrease in DPP-IV IC_{50} value to 1.45 ± 0.26 mg mL^{-1} (Table 1). The digestion of H9 with pepsin (H9_pepsin) and pepsin and Corolase PP (H9_CorPP) led to an increase in the DPP-IV IC_{50} value (0.40 ± 0.03 and 0.33 ± 0.03 mg mL^{-1}, respectively, Table 1).

The molecular mass distribution profile of the SGID samples is illustrated on Fig. 1. In Gluten_pepsin, proteins (> 10 kDa) were degraded into smaller molecular mass components (< 10 kDa). Proteins were further degraded in Gluten_CorPP. However, both Gluten_pepsin and Gluten_CorPP still contained significant amounts of proteins (> 17% material > 10 kDa). There was a decrease in the proportion of proteins (> 10 kDa) in H9_pepsin. There were no intact proteins remaining in H9_CorPP, which only contained material < 5 kDa (Fig. 1).

Along with differences in molecular mass distribution profiles, H9, H9_pepsin and H9_CorPP displayed different peptide profiles (Supplementary Fig. S2), suggesting that certain sequences within H9 were not stable to digestive enzymes.

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Table 2. Coefficients of the multilinear regression (MLR) model (Equation 2) correlating the dipeptidyl peptidase IV (DPP-IV) half maximal inhibitory concentration (IC_{50}) of the gluten hydrolysates to the parameters of the design of experiments (DOE) and their interactions.

Parameters of the model[1]	Estimate value of the coefficients	Standard Error	t value	p
Intercept	0.48	0.05	9.73	1.95E-04
T	-0.12	0.04	-3.00	0.03
ES	0.03	0.04	0.79	0.46
T^2	-0.01	0.06	-0.12	0.91
ES^2	-0.06	0.06	-0.95	0.38
T×ES	-0.07	0.05	-1.46	0.21
Root mean squared error: 0.097; R^2: 0.719; p-value model = 0.163				

[1]T: temperature and ES: enzyme to substrate ratio.

[2]Coefficients of the parameters having a $p < 0.05$ are significantly different from 0

Peptides identified within H9 and H9_CorPP

Numerous peptide sequences were identified by LC-MS/MS within H9 and H9_CorPP. Those peptides within both samples for which a DPP-IV IC_{50} value had previously been reported in the literature are listed in Table 2. These consisted of di- and tripeptides. Approximately 95% of the DPP-IV inhibitory peptides reported in Table 2 were common between H9 and H9_CorPP. H9 and H9_CorPP contained relatively potent DPP-IV inhibitory peptides such as, Trp-Leu and Trp-Pro. Val-Pro-Leu, a potent DPP-IV inhibitor known as Diprotin B, was also found in H9 and H9_CorPP.

Several peptides identified within H9 had the features of DPP-IV inhibitory peptides. Peptides with DPP-IV inhibitory features, which were identified in α/β-gliadin (accession number P18573), one of the major gluten protein, are listed in Table 3. While several sequences have been identified for their ability to inhibit DPP-IV *in vitro*, their DPP-IV IC_{50} value has not as yet been evaluated. Two main features were seen among these peptides, i.e., having a Pro in the penultimate or C-terminal positions (~ 80% of the peptides, Table 3). Several peptides with an N-terminal Phe or Leu were also present. One peptide with an Ala at the penultimate position was detected while no peptides containing Ile or Trp N-terminal residues were identified.

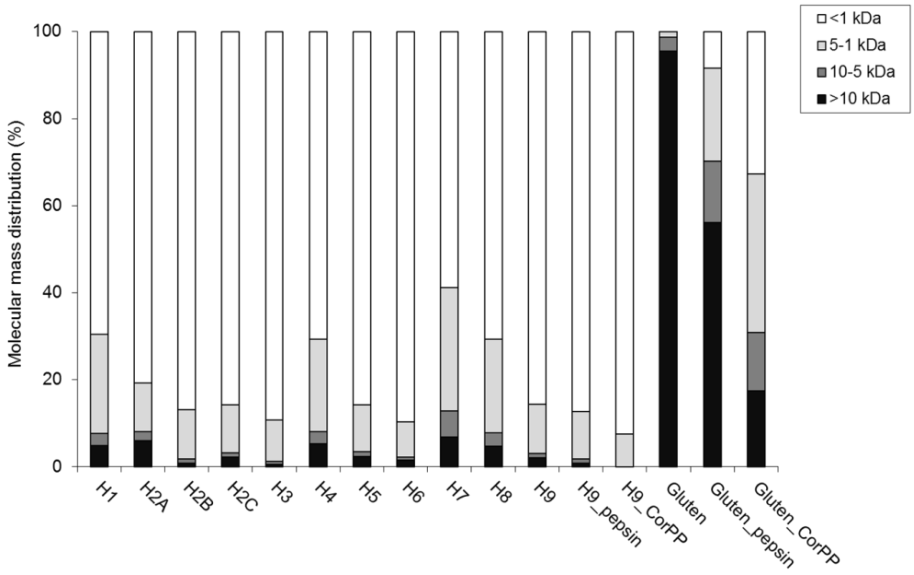

Figure 1. Molecular mass distribution profiles of the gluten hydrolysates generated within the design of experiments (DOE, H1-H9), H9 digested with pepsin (H9_pepsin), H9_pepsin digested with Corolase PP (H9_CorPP), gluten digested with pepsin (Gluten_pepsin) and Gluten_pepsin digested with Corolase PP (Gluten_CorPP).

Table 3. Half maximal inhibitory concentration (IC_{50}) values of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides identified within H9 and H9 digested with pepsin and then Corolase PP (H9_CorPP).

Peptide sequence[1]	Identified in		DPP-IV IC_{50} value (μM)[2]	References
	H9	H9_CorPP		
AL	✓	✓	882	31
AP	✓	✓	7950	20
FL	✓	✓	400	31
FP	✓	✓	3630 ; 683	20,35
HP	✓	✓	2820 ; 903	20,35
L(I)A	✓	✓	91 ; 88 ;300	36,37
LL	✓	✓	192	38
LP	✓	✓	713 ; 2370	17,20
LPL	✓	✓	241	17
LPP	✓	✓	563	35
LQP	✓	✓	1181	19
MP	✓	✓	870	20
PP	✓	✓	5860 ; 4344	20,39
RP	✓	✓	1240 ; 657	20,35
SL	✓	✓	2517	31
SP	✓	✓	5980	20
TP		✓	2370	20
VP	✓	✓	880 ; 381 ;93 ; 785	20,35,36,39
VPL	✓	✓	16	40
VR	✓	✓	826	31
WL	✓	✓	44	41
WP	✓	✓	4530 ; 45	20,41
YL	✓	✓	940	42
YP	✓	✓	3170 ; 658 ; 7564	17,20,39

[1]Peptide sequences abbreviated with the one letter amino acid code. Each peptide sequence could be found within several individual wheat proteins.

[2]DPP-IV IC_{50} : dipeptidyl peptidase IV half maximal inhibitory concentration.

Discussion

Several studies have shown that cereal proteins constitute good starting substrates for the generation of bioactive peptides.[43,44] Gluten, the major protein group in wheat, is a rich source of Pro. Pro residues appear to play a key role in DPP-IV inhibition when they are located in the penultimate or the C-terminal positions of peptides. In addition, *in silico* studies have indicated that wheat gluten contains numerous DPP-IV inhibitory peptide motifs.[11,12] Therefore, gluten was evaluated for its ability to serve as a substrate for the enzymatic generation of DPP-IV inhibitory peptides. The DPP-IV inhibitory properties of barley, buckwheat and oat glutenin hydrolysates have been studied.[45,46] To our knowledge, there is only one previous publication which reported on the DPP-IV inhibitory properties of wheat flour.[16]

The highest DPP-IV inhibitory potency was observed with H9 (DPP-IV IC_{50} = 0.24 ± 0.02 mg mL^{-1}, Table 1). This value is lower than those generally found with other cereal protein hydrolysates. DPP-IV IC_{50} values ranging from 1.83 to 8.15 mg mL^{-1} for tryptic hydrolysates of barley, buckwheat and oat proteins have been reported.[45] Alcalase, Flavourzyme and Corolase PP barley protein hydrolysates had DPP-IV IC_{50} values between 2.45 and 3.57 mg mL^{-1}.[38] However, an oat glutenin Alcalase hydrolysate with a DPP-IV IC_{50} value (0.13 mg mL^{-1}) of the same order as H9 has been described.[45] The DPP-IV inhibitory potency of H9 was ~ 2.5 times higher than that of relatively potent milk[31] and plant (quinoa,[21] hemp, pea, rice and soy)[47] protein hydrolysates evaluated using the same experimental protocol.

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Table 4. Peptides identified within H9, which originate from wheat α/β-gliadin (accession number P18573) and which possess the features of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides.

Peptide sequence[1]	Fragment number[2]	DPP-IV inhibitory peptide features[3]							*In vitro* DPP-IV inhibition[4]	References[4]
		W_1	I_1	L_1	F_1	P_2	A_2	P-term		
AP	(f279-280)							✓	✓	20,36
FE	(f253-254)				✓				✓	36
FP	diverse				✓			✓	✓	20,35
FPG	(f30-32)				✓	✓				
FPGQQQPFPPQQPYPQPQP	(f30-48)				✓	✓		✓		
FPGQQQPFPPQQPYPQPQPFP	(f30-50)				✓	✓		✓		
FPGQQQPFPPQQPYPQPQPFPSQQPY	(f30-55)				✓	✓				
FPPQQPYP	(f37-44)				✓	✓		✓		
FPPQQPYPQPQPFP	(f37-50)				✓	✓		✓		
FPPQQPYPQPQPFPSQQPY	(f37-55)				✓	✓				
FPQ	(f61-63)				✓	✓				
FQ	(f218-219), (f232-233)				✓				✓	36
FQP	(f232-234)				✓			✓		
L(I)A	(f259-260)		✓	✓					✓	36,37
LAL	(f259-261)			✓			✓			
LE	(f261-262)			✓					✓	36
LH	(f198-199)			✓					✓	36
L(I)P	diverse		✓	✓				✓	✓	17,20,36
LPYP	diverse			✓		✓		✓		
LPYPQPQLPYPQP	(f66-78), (f73-85)			✓		✓		✓		
LPYPQPQLPYPQPQLPYPQPQP	(f66-87)			✓		✓		✓		
LQ	diverse			✓						
LQP	(f8-10), (f58-60)			✓				✓	✓	19
L(I)S	(f213-214)		✓	✓					✓	36
L(I)SQ	(f213-215)		✓	✓						
NP	(f12-13), (f238-239)							✓	✓	36
NPQ	(f238-240)					✓				
NPS	(f12-14)					✓				
NPSQQQPQEQVP	(f12-23)					✓				
PP	(f38-39), (f273-274)							✓	✓	20,36,39
PQP	diverse							✓		
QFP	(f29-31)							✓		
QLP	diverse							✓		
QLPYPQPQLPYPQPQP	(f72-87)							✓		
QLPYPQPQP	(f79-87)							✓		
QP	diverse							✓	✓	36
QPFP	diverse					✓		✓		
QPFPPQQPYPQPQPFP	(f35-50)					✓		✓		
QPFPPQQPYPQPQPFPSQQP	(f35-54)					✓		✓		
QPFPPQQPYPQPQPFPSQQPY	(f35-55)					✓				
QPL(I)	(f211-213)		✓			✓				
QPQ	diverse					✓				
QPQP	diverse					✓		✓		

QPY	diverse	✓			
QQFP	(f28-31)		✓		
QQP	diverse		✓		
QQQPFPPQQPYPQPQP	(f33-48)		✓		
QQQPFPPQQPYPQPQPFP	(f33-50)		✓		
QQQPFPPQQPYPQPQPFPSQQP	(f33-54)		✓		
QYP	(f224-226)		✓		
RP	(f89-90)		✓	✓	20,35,36
TLP	(f263-265)		✓		
VP	diverse		✓	✓	20,35,36,39
VPL	(f22-24)	✓		✓	40
VPQ	(f5-7)	✓			
VPV	(f3-5)	✓			
VPVP	(f3-6)	✓	✓		
VPVPQLQPQNPS	(f3-14)	✓			
VPVPQLQPQNPSQQQPQ	(f3-19)	✓			
VPVPQLQPQNPSQQQPQEQVP	(f3-23)	✓			
VPVPQLQPQNPSQQQPQEQVPL	(f3-24)	✓			
VPVPQLQPQNPSQQQPQEQVPLVQ	(f3-26)	✓			
YP	diverse		✓	✓	17,20,36,39
YPQ	diverse	✓			
YPS	(f225-227)	✓			
YPSG	(f225-228)	✓			

[1]Peptides sequences abbreviated with the one letter amino acid code.

[2]diverse: peptide sequence found at more than two locations within wheat α/β-gliadin. Fragment numbers are provided for the mature protein sequence.

[3] Trp (W_1), Leu (L_1), Ile (I_1) or Phe (F_1) at the N-terminus of the peptide; Pro (P_2) or Ala (A_2) at position 2 of the peptide; Pro at the C-terminus (P-term) of the peptide.

[4]Peptides which have been identified as DPP-IV inhibitors in the literature.

DPP-IV inhibitory peptides are generally relatively short (< 10 amino acids), for review, see:[14]. This is in agreement with the fact that hydrolysates displaying the largest proportion of material < 1 kDa (e.g., H3, H5, H6 and H9) were also generally the most potent DPP-IV inhibitory hydrolysates (Table 1). The DPP-IV inhibitory potency of H9 decreased to a small extent following SGID (0.24 ± 0.02 vs. 0.33 ± 0.03 mg mL^{-1}, for H9 and H9_CorPP, respectively, Table 1). The DPP-IV IC_{50} value of Gluten_CorPP (1.45 ± 0.26 mg mL^{-1}, Table 1) was higher than that of the SGID of wheat flour (0.8 mg mL^{-1})[16] but of the same order as that of the SGID of barley, buckwheat or oat flour (0.99-3.91 mg mL^{-1})[45]. The higher DPP-IV inhibitory potency of H9_CorPP compared to Gluten_CorPP suggests that it may be beneficial to prehydrolyse gluten prior to ingestion.

Several known DPP-IV inhibitory peptides were identified within H9 and H9_CorPP (Table 2 and 3), most of which were short (≤ 3 amino acids). Three relatively potent peptides, Val-Pro-Leu, Trp-Leu and Trp-Pro, having DPP-IV IC_{50} values < 50 μM,[40,41] were found both in H9 and H9_CorPP (Table 2). While these peptides may, in part, be responsible for the DPP-IV inhibitory potency of the hydrolysates, other peptides may also play a role in the overall bioactivity. Peptides with a penultimate Pro have been shown to behave as substrate-type DPP-IV inhibitors.[17,18] In addition, the presence of Pro at the C-terminal position also appears to be a good predictor for DPP-IV inhibition activity of peptides.[19,20] Interestingly, a large proportion (~ 80 %) of the peptides originating from α/β-gliadin, which were identified within H9, contained either a penultimate or C-terminal Pro (Table 3).

Debitrase HYW20, does not appear to have been used for the generation of DPP-IV inhibitory food protein hydrolysates, for review, see: Lacroix and Li-Chan.[14] Debitrase HYW20 is an enzyme preparation derived from *Aspergillus niger*, with an unknown cleavage specificity. It has been reported to contain both endo- and exoprotease activities,[22] which may explain the relatively high DH values observed in the hydrolysates (Table 1). Similar DH values up to 43[48] or 64%[49] have been reported for wheat gluten hydrolysates obtained with Flavourzyme and Alcalase, respectively, which are two enzyme preparations also having broad cleavage specificity.

The presence of these Pro-containing peptides possessing DPP-IV inhibitory features within H9 is partly explained by the particularly high Pro content of gluten. In addition, it is likely that the enzyme activities within Debitrase HYW20 also played a role in DPP-IV inhibitory peptide release. The relatively minor reduction of the DPP-IV IC_{50} value of H9 following SGID (H9_CorPP) may arise from the fact that Pro-containing peptides have been reported to be relatively stable to digestive enzymes.[50]

Future work may include bioassay-driven fractionation of H9, confirmatory studies with synthetic peptides and quantification of DPP-IV inhibitory peptides. The study of their technofunctional and additional other bioactive properties of the wheat gluten hydrolysates is also of interest. Wheat gluten hydrolysates have been shown to possess a range of technofunctionalities.[49,51] Furthermore, in the context of coeliac disease, the degradation of gluten epitopes through

the enzymatic hydrolysis used herein is also worthy of further investigation.[48,52,53]

Conclusions

Gluten can be used as a protein substrate for the enzymatic generation of DPP-IV inhibitory hydrolysates. H9, a relatively potent DPP-IV inhibitory hydrolysate was produced on incubation (60°C for 24 h) of gluten with Debitrase HYW20 at an E:S of 1.5%. This hydrolysate contained short (di- and tri-) peptides previously identified as DPP-IV inhibitors. In addition, several peptides possessing DPP-IV inhibitory features, mostly comprising of Pro residues at the penultimate and C-terminal positions, were identified within H9. These may play a role in the overall DPP-IV inhibitory properties of H9. Interestingly, the DPP-IV inhibition activity of H9 was minimally reduced following SGID. In addition, the higher DPP-IV inhibitory potency of H9_CorPP compared to that of Gluten_CorPP, suggests the possibility of a more efficient release of DPP-IV inhibitory peptides on prehydrolysis of gluten prior to ingestion. *In vivo* evaluation of the gluten hydrolysates is required in order to study their antidiabetic potential.

Acknowledgements

The work described herein was partly supported by Enterprise Ireland under Grant Number TC2013-0001. Manon Hennemann was funded by the ERASMUS Program. This study was partly funded by the Science Foundation Ireland Research Infrastructure Fund.

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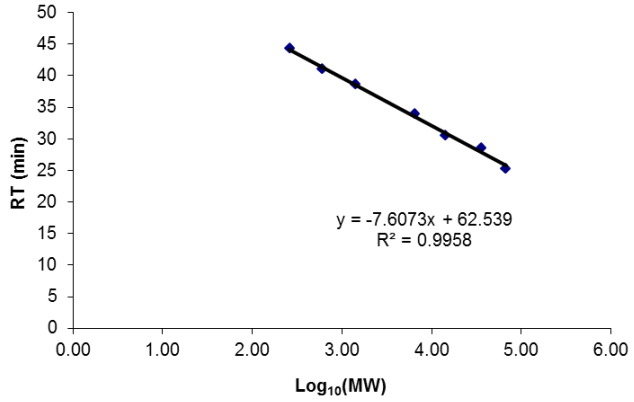
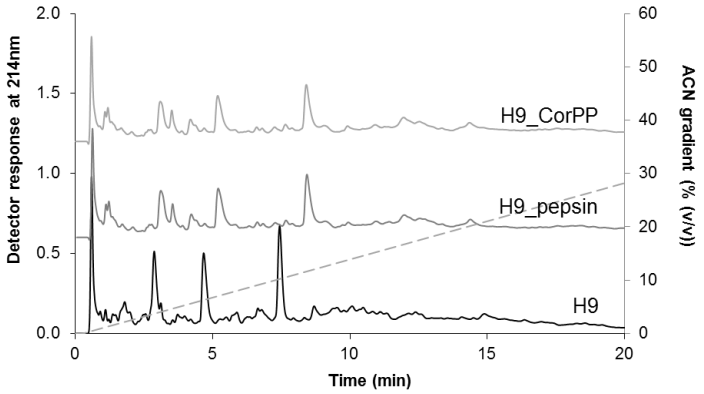
Supplementary data



Supplementary Figure S1. Calibration curve for the gel permeation high performance liquid chromatography (GP-HPLC) method representing the retention time (RT) as a function of the logarithm of the molecular weight (MW) of the standards (i.e., bovine serum albumin, β-Lactoglobulin, α-Lactalbumin, aprotinin, bacitracin, Leu-Trp-Met-Arg and Asp-Glu).



Supplementary Figure 2. Reverse-phase ultra-performance liquid chromatography (RP-UPLC) profile of H9, H9 digested with pepsin (H9_pepsin) and H9_pepsin digested with Corolase PP (H9_CorPP). ACN: acetonitrile.