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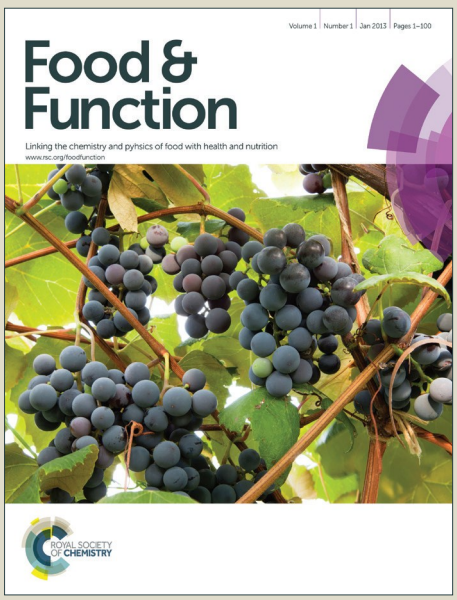

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ARTICLE

Strategies for the release of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides in an enzymatic hydrolyzate of α-lactalbumin

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Bovine α-lactalbumin (α-La) contains numerous dipeptidyl peptidase IV (DPP-IV) inhibitory peptide sequences within its primary structure. *In silico* analysis indicated that the targeted hydrolysis of α-La with elastase should release DPP-IV inhibitory peptides. An α-La isolate was hydrolysed with elastase under different conditions using an experimental design approach incorporating 3 factors (temperature, pH and enzyme to substrate ratio (E:S) ratio) at 2 levels. The hydrolyzate generated at pH 8.5, 50°C, E:S 2.0% (w/w) (H9) displayed the highest mean DPP-IV inhibition value at 3.1 mg mL^{-1} of 75.8 ± 3.7% and had a half maximal DPP-IV inhibitory concentration (IC_{50}) value of 1.20 ± 0.12 mg mL^{-1}. Five α-La-derived DPP-IV inhibitory peptides (GY, GL, GI, NY and WL) predicted to be released *in silico* were identified by liquid-chromatography tandem mass spectrometry (LC-MS/MS) within H9 and its simulated gastrointestinal digestion (SGID) sample. This preliminary study demonstrated the benefit of using a targeted approach combined with an experimental design in the generation of dietary protein hydrolyzates with DPP-IV inhibitory properties.

Introduction

Dipeptidyl peptidase IV (DPP-IV) is an ubiquitous enzyme which is responsible for the cleavage and inactivation of the incretin hormones, glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [1]. In the context of type 2 diabetes management, DPP-IV inhibition may be used as a means to improve the regulation of serum glucose in humans. Various DPP-IV inhibitory drugs (gliptins), have been developed to maintain the insulinotropic activity of the incretins in the post-prandial phase [2].

Dietary components, including food protein-derived peptides, have been shown to play a role in the inhibition of DPP-IV *in vitro* and in certain instances *in vivo* for reviews, see: [3,4,5]. *In silico* studies have shown that selected DPP-IV inhibitory peptide sequences may be found within a wide range of dietary proteins [6,7]. To date, milk proteins appear to be the most frequently studied substrate for the generation of DPP-IV inhibitory peptides. Depending on the enzymatic strategy employed, different milk proteins may appear to be more suitable substrates for the production of DPP-IV inhibitory peptides. For instance, it was recently predicted by Tulipano *et al*. [8] using *in silico* digestion with gastrointestinal proteinases that β-lactoglobulin (β-Lg) should yield a higher number of previously identified DPP-IV inhibitory peptides than α-La. This was further confirmed following *in vitro* digestion of β-Lg and α-La with gastrointestinal enzymes, yielding hydrolyzates with IC_{50} values of 0.74 and 1.70 mg mL^{-1}, respectively [8]. Other *in silico* analysis of the major milk proteins indicated that α-lactalbumin (α-La) displayed the highest level of sequence coverage (43.9%) for previously identified DPP-IV inhibitory peptides as well as the highest DPP-IV inhibitory potency index (i.e., 17.9 10^{-6} μM^{-1} g^{-1}) [7]. It has been suggested that discrepancies between studies to determine the most adequate substrate for the generation of DPP-IV inhibitory peptides may arise from the lack of information on a wide set of DPP-IV inhibitory peptide sequences as well as their potency. For this reason, Lan *et al*., [9] developed a peptide library approach incorporating 337 dipeptides which were evaluated for their *in vitro* DPP-IV inhibitory properties.

It was hypothesized within the present study that selected DPP-IV inhibitory peptide sequences within α-La may be released during hydrolysis by careful selection of the enzyme activity. Therefore, the aim of this study was to predict the most appropriate enzyme(s) for the release of selected DPP-IV inhibitory peptides from bovine α-La and to subsequently validate the release of these peptides *in vitro*. This was achieved by (1) *in silico* prediction of the enzyme activity which should yield the highest number of known DPP-IV inhibitory peptides, (2) hydrolysis of α-La with this enzyme activity using an experimental design (3 factors (temperature, pH and E:S

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ratio) studied at 2 levels) approach to optimize release of the predicted peptides and (3) confirmation of the release of the predicted peptides by liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis. In addition, *in vitro* simulated gastrointestinal digestion (SGID) was used to study the stability of the DPP-IV inhibitory activity in selected hydrolyzates.

Materials and methods

Reagents

Trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (TRIS), sodium phosphate monobasic, sodium phosphate dibasic, citric acid, calcium chloride ($CaCl_2$), sodium chloride (NaCl), Gly-Pro p-nitroanilide (pNA), L, diprotin A (IPI) and porcine DPP-IV (≥ 10 units mg^{-1} protein) were obtained from Sigma-Aldrich (Dublin, Ireland). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was from Pierce Biotechnology (Medical Supply, Dublin, Ireland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Whey protein concentrate 80 (WPC80, 81.1% (w/w) protein) was obtained from Carbery Ingredients (Ballineen, Ireland). Porcine elastase (≥ 5 U mg^{-1} protein) was obtained from My Biosource (catalog # MBS173124, San Diego, CA, USA), pepsin (3000 FCC U g^{-1}) from Biocatalysts (Cefn Coed, Wales, UK) and Corolase PP (2500 UHb g^{-1}) from AB Enzymes (Darmstadtd, Germany). All other reagents were of analytical grade and were obtained from Sigma-Aldrich.

In silico analysis

In silico digestion of bovine α-La with pepsin, trypsin, chymotrypsin, elastase and glutamyl endopeptidase was carried out with the peptide cutter program available in Matlab (version R2014b, MathWorks, Inc, Natick, MA, USA), using the same cleavage rules as per ExPASy. Comparison of the peptide sequences predicted to be released to known DPP-IV inhibitory peptides previously identified in the literature [7,9] was then carried out.

Extraction of α-La from WPC80

α-La was extracted from WPC80 following the method described by Fernández, *et al.* [10], with modifications. Briefly, α-La was selectively precipitated by adjusting the pH of WPC80 to 3.5 with a 1 M citric acid solution. The pellet was washed 3 times and then resolubilized using a 0.1 M $CaCl_2$ solution at pH 7.5 in a 1:1 (w:w) ratio. The α-La extract was then dialyzed with distilled water (18 h, 4°C, snake skin dialysis tubing 10 kDa nominal molecular weight cut-off, Fisher Scientific). The samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C until further analysis.

The protein content of the α-La isolate was determined with the bicinchoninic acid (BCA) method using a Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The α-La content of the isolate was determined by reverse-phase high-performance liquid chromatography (RP-HPLC) as described by Nongonierma and FitzGerald [11], using an α-La standard sample (Sigma-Aldrich). The extraction yield and the purity of the α-La obtained were calculated as described elsewhere [12].

Enzymatic hydrolysis of the α-La isolate and *in vitro* simulated gastrointestinal digestion (SGID) of the hydrolyzates

The outcome of the *in silico* analysis indicated that incubation of α-La with elastase should release the greatest number of previously identified DPP-IV inhibitory peptides, including WL, a potent DPP-IV inhibitory peptide (Table 1). Therefore, digestion of α-La with elastase was carried out following an experimental design approach (yielding hydrolyzates H1-H9). The parameters of the experimental design used for α-La hydrolysis with elastase consisted of the pH, temperature and E:S studied at two levels (Supplementary Table S2). These parameters were specifically chosen as they are key determinants of peptide release during enzymatic digestion. The pH (7.0-8.5) and temperature (37-50°C) range were selected based on the optimum values for elastase as indicated by the supplier. The E:S ratio (1-2% (w/w)) was chosen based on levels which have previously been reported for the hydrolysis of whey proteins with pancreatic enzymes [13]. Each hydrolyzate was generated once with the exception of the central point condition (H5, pH 7.8 (0), 44°C (0) and E:S 1.5% (0)) which was generated as two independent duplicates (H5(1) and H5(2)).

The α-La isolate was resuspended (50 g L^{-1} on a protein basis) in distilled water and was incubated for 30 min in a water bath at the desired temperature and pH as per the experimental design conditions. Elastase was then added to the α-La suspension at the desired E:S and hydrolysis was carried out for 4 h. The pH was maintained constant by adding 0.1 M NaOH with a pH stat (Titrando 843, Tiamo 1.4 Metrohm, Dublin, Ireland). The enzyme was then heat inactivated at 90°C for 20 min. A control sample of unhydrolyzed α-La underwent the same conditions as the hydrolyzate with the exception that it did not contain enzyme.

H9 was subsequently subjected to *in vitro* SGID as described by Walsh *et al.* [14]. Briefly, the hydrolyzate was incubated at 37°C with pepsin (E:S 1%, pH 2.0) for 90 min followed by incubation with Corolase PP (E:S 2.5%, pH 7.5) for another 150 min. The pH was maintained constant during the SGID steps using a pH stat (Metrohm).

All hydrolyzates were freeze-dried post heat-inactivation and stored at -20°C prior to further analysis.

DPP-IV inhibition assay

The protein hydrolyzates were dispersed in HPLC grade water at 3.1 mg mL^{-1} (final concentration). The PP-IV inhibition assay was carried out in triplicate as described earlier [15]. Briefly, 25 µL sample were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA (final concentration 0.200 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0 (25 µL) and Gly-Pro-pNA. The reaction was started by the addition of DPP-IV (final concentration 0.0025 U mL^{-1}). The positive control was diprotin A. The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), and the absorbance of the released pNA was monitored at 405 nm.

Table 1 Peptides predicted to be released *in silico* from α-lactalbumin (α-La) by elastase using the peptide cutter facility of Matlab and peptides identified within the hydrolyzate by reverse-phase ultra-performance tandem mass spectrometry (RP-UPLC MS/MS). The half maximal inhibitory concentration (IC_{50}) value of the peptide is provided when available in the literature.

Peptide sequence*	M_w+H (Da)	Detected by LC-MS/MS		DPP-IV IC_{50} (μM)***	Reference
		H9	SGID H9		
EQL	389.20	✓	✓	-	-
TKC(CEKL)EV**	1050.51	nd	nd	-	-
FREL	564.31	nd	nd	-	-
KDL	375.22	✓	✓	-	-
KGY	367.20	✓	✓	-	-
GGV	232.13	nd	nd	-	-
PEWV	530.26	✓	✓	-	-
C(CS)TTFHTS**	984.37	nd	nd	-	-
GY	239.10	✓	✓	na	[9]
DTQA	434.19	✓	✓	-	-
QNNDS	577.22	nd	nd	-	-
TEY	412.17	nd	✓	-	-
GL	189.12	✓	✓	2615.0	[15]
FQI	407.23	✓	✓	-	-
NNKI	488.28	✓	nd	-	-
WC(CDKFL)KDDQNPHSS**	1920.82	nd	nd	-	-
NI	246.15	✓	✓	-	-
C(MCV)NI**	680.27	nd	nd	-	-
DDDL	477.18	nd	nd	-	-
TDDI	463.20	nd	✓	-	-
KKI	388.29	✓	✓	-	-
DKV	361.21	✓	✓	-	-
GI	189.12	✓	✓	na	[9]
NY	296.12	✓	✓	na	[9]
WL	318.18	✓	✓	43.6	[16]
HKA	355.21	✓	✓	-	-
EKL	389.24	✓	✓	-	-
DQWL	561.27	✓	✓	-	-

nd: not detected; na: not available.

*Peptide sequence with the one letter code.

**A disulphide bridge is present between cysteine (C) residues within the peptide.

***The DPP-IV inhibition reported by Lan, *et al*. [9] were < 35% when the peptides were tested at 100 μM.

For the hydrolyzates displaying DPP-IV inhibition > 50 % at 3.1 mg mL^{-1}, the DPP-IV IC_{50} values were determined by plotting the dose-response curve (at concentrations ranging from 3.1 × 10^{-2} to 3.1 mg mL^{-1}). Each analysis was conducted in triplicate (n = 3).

Determination of the degree of hydrolysis (DH)

The DH was determined following the method of Adler-Nissen [17], essentially as described in Nongonierma and FitzGerald [13]. Each experiment was carried out in triplicate (n = 3).

Gel permeation high performance liquid chromatography (GP-HPLC) and RP-UPLC of the hydrolyzates

The α-La hydrolyzates were analyzed by GP-HPLC as described in Spellman, et al. [18]. The peptide profile of the α-La hydrolyzates was determined by RP-UPLC as described in Nongonierma and FitzGerald [13].

LC-MS/MS of the α-La hydrolyzates

Samples were analyzed by LC-MS/MS using an Acquity UPLC (Waters, Milford, MA, USA) coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF, Impact HD™, Bruker Daltonics GmbH, Bremen, Germany). The MS was fitted with an electrospray ionization (ESI) source used in positive ion mode. Hystar software (Bruker Daltonics) was used to control

the instruments and to acquire data. Chromatographic separation was carried out on an Acquity BEH amide column (Waters) as described by Le Maux, et al. [19]. Fifty micrograms of sample (final concentration: 5 g L^{-1}, injection volume: 10 µL) were injected onto the column. MS measurements were performed over a 70-2500 m/z acquisition range. MS data were processed on Compass DataAnalysis 4.0 SP5 (Bruker Daltonics).

Peptide sequences predicted to be present by *in silico* model were searched for within hydrolyzate samples using the strategy described by Le Maux, et al. [19]. A mass tolerance of 0.1 Da and a time difference between the observed and predicted retention times was set at a maximum of 16 min.

Peptide peak intensities in H9 and its SGID sample were visualized on Venn diagrams. MS data was analyzed using ProfileAnalysis (Bruker Daltonics) and Matlab. More than 500 compounds were detected in each sample but only peaks with an intensity > 5×10^5 were analyzed. The peptides observed within each sample were classified as follows: when peptide peaks had similar intensities in both samples, they were reported at the intersection of the Venn diagram and when peptide peaks within a sample had different intensities compared to the other sample (> 3 fold difference), they were reported outside of the intersection of the Venn diagram.

Statistical analysis

A one way ANOVA was used to assess the effect of the parameters (pH, temperature and E:S) of the experimental design on DPP-IV inhibition when evaluated at 3.1 mg mL^{-1} at a significance level $p < 0.05$. The effects of the experimental design parameters on DPP-IV inhibition were tested ($p < 0.05$) with Matlab using a multi-linear regression (MLR) model (eqn 1) as follows:

$$Y = \beta_0 + \beta_1 pH + \beta_2 T + \beta_3 ES + \varepsilon \quad (1)$$

With Y, the DPP-IV inhibition of the α-La hydrolyzates at 3.1 mg mL^{-1}; β_0 to β_3: the coefficients of the model; pH, T (temperature) and ES (E:S): the z-centered parameters of the experimental design and ε: the residual of the model.

The statistical treatment for DH was carried out with a one way ANOVA followed by a Student Newman-Keuls test used for multiple means comparison ($p < 0.05$). Statistical analyses were carried out with SPSS (version 22, SPSS Inc., Chicago, IL, USA).

Results

***In silico* digestion of α-La**

Hydrolysis of α-La by five different enzyme activities (pepsin, trypsin, chymotrypsin, elastase and glutamyl endopeptidase) showed that elastase allowed the release of the highest number of previously identified DPP-IV inhibitory peptides (Supplementary Table S1 and Table 1). Previous studies have suggested that specific amino acids within peptide sequences correlated with DPP-IV inhibition. For instance W at position 1 and P/A at position 2 are frequently found in DPP-IV inhibitory peptides with IC_{50} values < 200 µM [7,8]. In addition, the presence of P at the C-terminus of peptides has also been reported for DPP-IV inhibitory peptides [20]. The *in silico* analysis did not show any peptides with a P at position 2 or at the C-terminus while two peptides with an A at position 2 were predicted to be released by following *in silico* digestion of α-La with chymotrypsin with low (KAL, (f 108-110)) and high (LAHKALCSEKLDQW, (f 105-118)) specificity. In addition, three peptides with a N-terminal W were identified following *in silico* digestion of α-La with glutamyl endopeptidase (WVCTTFHTSGYDTQAIVQNNDSTE, (f 26-49)), pepsin (WCKDDQNPHSSNICNISCDK, (f 60-79)) and elastase (WL, (f 118-119)).

Five previously identified DPP-IV inhibitory dipeptides (GY, GL, GI, NY and WL) were predicted to be released by *in silico* digestion of α-La by elastase (Table 1). Of the peptides predicted to be released by elastase, WL is a relatively potent DPP-IV inhibitory peptide with an IC_{50} of 43.6 µM [16]. In comparison with elastase, the other enzyme activities (pepsin, trypsin, chymotrypsin and glutamyl endopeptidase) were not predicted to release as many known DPP-IV inhibitory peptides or peptides as potent as WL (Supplementary Table S1). While 4 of the peptides (GY, GL, GI and NY) predicted to be released by elastase were not potent DPP-IV inhibitors, the possibility to release WL with elastase was of interest. Therefore, α-La was digested with elastase to verify if these *in silico* predictions translated *in vitro*.

DPP-IV inhibitory activity of the α-La hydrolyzates generated within the experimental design conditions

As already outlined, α-La was isolated from WPC80. The RP-UPLC profile of the starting WPC80 and the α-La isolate are displayed in Supplementary Fig. S1. The isolate enriched in α-La had a purity and yield of 79.3 ± 0.2 and 40.3 ± 1.4% (w/w), respectively.

The generation of the hydrolyzates was reproducible as indicated by the lack of significant differences ($p > 0.05$) between the two independent duplicates H5(1) and H5(2) in terms of their DH, DPP-IV inhibition at 3.1 mg mL^{-1} and their DPP-IV IC_{50} values (Table 2). The hydrolyzates generated at 37°C (H1-H4) yielded DPP-IV inhibition < 50 % at the highest concentration tested (3.1 mg mL^{-1}), resulting in it not being possible to compare all hydrolyzates based on their DPP-IV IC_{50} value. Therefore, hydrolyzates were compared using the % DPP-IV inhibition observed at 3.1 mg mL^{-1}. The DPP-IV inhibition (at 3.1 mg mL^{-1}) of the hydrolyzates varied between 23.0 ± 6.7 and 75.8 ± 3.7% for H3 and H9, respectively (Table 2). H9 had an IC_{50} value of 1.20 ± 0.12 mg mL^{-1}.

Temperature and E:S significantly ($p < 0.05$) effected DPP-IV inhibition (Table 3). Hydrolysis temperature was found to have the greatest impact on DPP-IV inhibition as indicated by the higher coefficient associated with this parameter (Table 3). The highest DPP-IV inhibition at 3.1 mg mL^{-1} was obtained with hydrolysate H9 (pH 8.5, 50°C and E:S of 2% (w/w)).

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Table 2 Degree of hydrolysis (DH), dipeptidyl peptidase IV (DPP-IV) inhibition of the α-lactalbumin (α-La) hydrolyzates evaluated at 3.1 mg mL^{-1} and DPP-IV half maximal inhibitory concentration (IC_{50}) of the hydrolyzates.

Hydrolyzate	Hydrolysis conditions with elastase*			DH (%)**	DPP-IV inhibition at 3.1 mg mL^{-1} (%)**	DPP-IV IC_{50} (mg mL^{-1})**
	pH	Temperature (°C)	E:S (%)			
H1	7.0 (-1)	37 (-1)	1.0 (-1)	$11.9 \pm 2.5^{a,b}$	34.7 ± 5.9^{a}	> 3.13
H2	7.0 (-1)	37 (-1)	2.0 (1)	$15.0 \pm 0.5^{c,d}$	$38.9 \pm 8.2^{a,b}$	> 3.13
H3	8.5 (1)	37 (-1)	1.0 (-1)	10.9 ± 0.2^{a}	23.0 ± 6.7^{a}	> 3.13
H4	8.5 (1)	37 (-1)	2.0 (1)	$13.4 \pm 0.6^{a,b,c,d}$	$38.8 \pm 2.9^{a,b}$	> 3.13
H5 (1)	7.8 (0)	44 (0)	1.5 (0)	$14.0 \pm 0.5^{b,c,d}$	$54.9 \pm 6.6^{b,c}$	2.07 ± 0.30^{b}
H5 (2)	7.8 (0)	44 (0)	1.5 (0)	$14.8 \pm 0.5^{b,c,d}$	$56.2 \pm 10.7^{b,c}$	$1.61 \pm 0.35^{a,b}$
H6	7.0 (-1)	50 (1)	1.0 (-1)	$12.7 \pm 0.5^{a,b,c}$	$52.8 \pm 7.8^{b,c}$	2.13 ± 0.43^{b}
H7	7.0 (-1)	50 (1)	2.0 (1)	16.2 ± 0.9^{d}	62.7 ± 6.9^{c}	$1.55 \pm 0.38^{a,b}$
H8	8.5 (1)	50 (1)	1.0 (-1)	10.5 ± 0.3^{a}	$55.7 \pm 3.2^{b,c}$	2.15 ± 0.24^{b}
H9	8.5 (1)	50 (1)	2.0 (1)	18.9 ± 2.4^{e}	75.8 ± 3.7^{d}	1.20 ± 0.12^{a}

*The z-centered values of the experimental design parameters are given into brackets.

**Mean ± SD (n=3). Within the same column, values with different superscript letters are significantly different ($p < 0.05$).

Table 3 Multilinear regression (MLR) describing the dipeptidyl peptidase IV (DPP-IV) inhibition observed with α-lactalbumin (α-La) hydrolyzates evaluated at 3.1 mg mL^{-1}, as a function of the parameters of the experimental design.

Parameters	Coefficient Estimate	Standard error	t value	*p**
Intercept	48.59	2.2	22.29	3.38E-06
pH	0.54	2.3	0.23	0.826
Temperature	13.93	2.3	6.02	0.002
E:S	6.25	2.3	2.70	0.043

*$p < 0.05$ are significantly different from 0. Root mean square error: 6.54; R^2: 0.897; *p* model: 0.00664

Physicochemical characteristics of the α-La hydrolyzates

The DH of the hydrolyzates (Table 2) varied between 10.5 ± 0.3 (H8) and 18.9 ± 2.4% (H9). Differences in the peptide profile (Supplementary Fig. S3) and molecular mass distribution (Fig. 1) of the hydrolyzates were seen. The molecular mass distribution profile showed significant protein breakdown in all hydrolyzates. Peptides were broken down to a higher extent in hydrolyzates generated at temperatures > 44°C (H5-H9), with > 75% of the hydrolyzates having a molecular mass < 1 kDa. In the hydrolyzates generated at 37°C (H1-H4), < 70% of the components had a molecular mass < 1 kDa (Fig. 1).

DPP-IV inhibitory activity and physicochemical characteristics of the SGID sample of H9

Since H9 displayed the highest *in vitro* DPP-IV inhibition (Table 2), this hydrolyzate was subjected to SGID. The DPP-IV IC_{50} value for the SGID of H9 was 1.53 ± 0.11 mg mL^{-1}. There was no significant difference ($p > 0.05$) between the IC_{50} value of H9 before and after SGID.

Differences were seen in the peptide profile of H9 following SGID, generally resulting in the presence of more intense hydrophilic peptide peaks (Fig. 2A). A higher proportion of peptides < 1 kDa after SGID of H9 was seen (Fig. 2 B).

Peptide detection within H9 and its SGID sample

More than 500 compounds were detected in either H9 or its SGID sample. The peptides identified within H9 and its SGID sample ranged from 2 to 22 amino acids in length. Of the 28 peptides predicted to be released *in silico* following complete digestion of α-La by elastase, 18 were detected by LC-MS/MS within H9 (Table 1). As already indicated, the most abundant peptides, on the basis of their peak intensities, detected within H9 and its SGID sample were compared using a Venn diagram approach (Fig. 2C). Sixty four high intensity peaks were common between both samples, while 82 and 86 unique peptides were detected for H9 and its SGID, respectively. In addition, several of the peptides detected within H9 and its SGID samples, displayed the features of known-DPP-IV inhibitory peptides consisting of W at the N-terminus, P/A at position 2 or P at the C-terminus [7,8,20] (Supplementary Table S3). However, the knowledge of DPP-IV inhibitory activity of these peptides is not available as these have not been experimentally evaluated.

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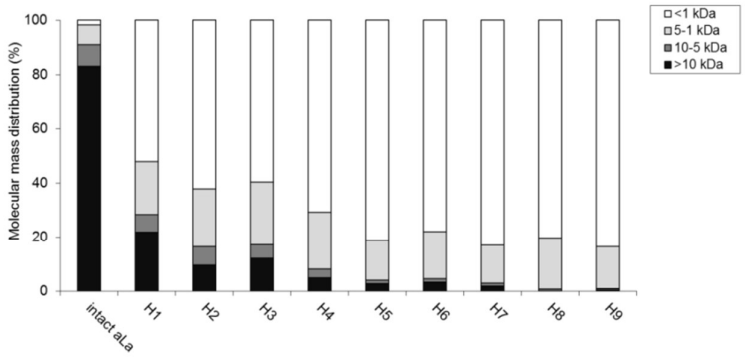

Fig. 1. Molecular mass distribution determined by gel permeation high performance liquid chromatography (GP-HPLC) of the peptides within the α-lactalbumin (α-La) hydrolyzates generated with elastase in the conditions of the experimental design.

Discussion

α-La has been shown to possess several relatively potent DPP-IV inhibitory peptides within its sequence [7]. Therefore, the development of enzymatic strategies for the targeted release of such peptides is of interest. The role of W-containing peptides in *in vitro* DPP-IV inhibition has been demonstrated [21]. Interestingly, α-La is the bovine milk protein with the highest W content. *In silico* digestion of α-La was applied in order to predict the release of peptides which were then compared to known DPP-IV inhibitory peptides. Elastase has been shown to cleave at the C-terminal side of L, A, V and I [22].

It was found in this preliminary study that temperature and E:S had a significant ($p < 0.05$, simplified model) effect on the release of DPP-IV inhibitory peptides, with the temperature having the highest effect (Table 3). This result is similar to an earlier study also showing that hydrolysis temperature played a greater role than the E:S in the release of antioxidant peptides upon hydrolysis of whey proteins with thermolysin [23]. In contrast, Naik, et al. [24] reported that pH and E:S had an effect on the release of antioxidant peptides from whey proteins hydrolysed with trypsin, while temperature did not.

While no significant ($p > 0.05$) effect of hydrolysis pH on DPP-IV inhibition was seen herein, some differences were visible in terms of the physicochemical characteristics of the hydrolyzates generated at different pHs (Fig. 1 and Supplementary Fig. S2). For example, for hydrolyzates generated at 50°C and an E:S of 2%, differences in peptide peak intensities were seen between H7 (pH 7.0) and H9 (pH 8.5), particularly at retention times of 0.6 and 7.7 min.

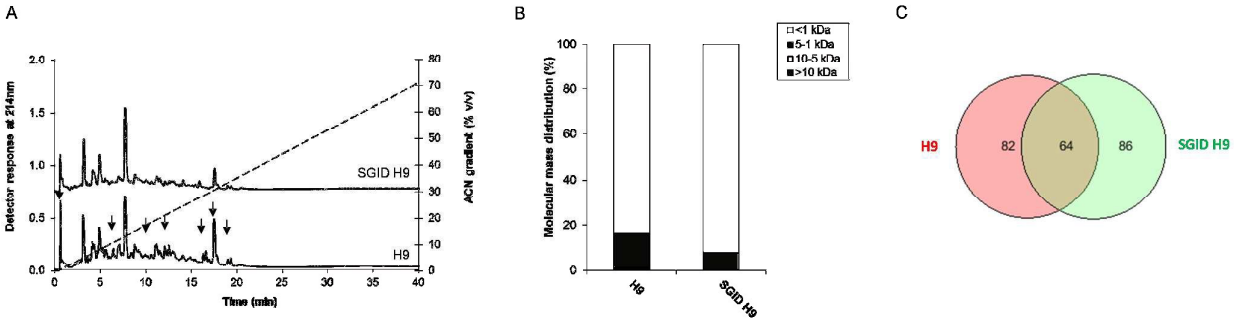

Fig. 2. (A) Reverse phase ultra-performance liquid chromatographic (RP-UPLC) profile, (B) molecular mass distribution by gel permeation high performance liquid chromatography (GP-HPLC) and (C) Venn diagrams of peptide peak intensities within the α-lactalbumin (α-La) hydrolyzate generated at pH 8.5, 50°C, E:S 2% with elastase (H9) and of its simulated gastrointestinal digest (SGID). Peptide peaks highlighted with an arrow on the RP-UPLC profile of H9 differ from the SGID sample.

Food & Function

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Of the 28 predicted sequences, including disulfide linked peptides, only 18 peptides were detected in H9 (Table 1). This may arise from the fact that α-La possesses secondary/tertiary structure, making it challenging to predict peptide release using peptide cutter programs which rely solely on the primary sequence of the protein. Furthermore, *in silico* digestion does not account for partial proteolysis. While combinations of *in silico* and *in vitro* approaches may be beneficial to develop bioactive dietary protein hydrolysates, some limitations exist. Cleavage specificity of enzymes generally only considers major cleavage sites. However, some secondary cleavage sites may exist, which are generally not taken into account in peptide cutter programmes. In addition, side activities may be found within enzyme preparations, which further yield to the release of peptide sequences which were not predicted to be released when using *in silico* digestion methods. Furthermore, protein/peptide digestion is highly dependent on the conditions employed during enzymatic hydrolysis (i.e., temperature, time, pH, E:S ratio, etc.).[25] For all the above reasons, it is quite challenging to directly translate *in silico* predictions to actual *in vitro* peptide release.

Numerous peptides identified within H9 were also detected in its SGID sample (Table 1). Five of these peptides (GY, GL, GI, NY and WL) were previously identified as DPP-IV inhibitors. Similarly, WV (f 24-25), a potent DPP-IV inhibitory peptide (IC_{50} = 65.7 μM [15]) was also identified by LC-MS/MS within the two samples, however, this peptide was only found to be present in low abundance (data not shown). In addition, several peptides within these two samples displayed the features of previously identified DPP-IV inhibitory peptides [7,8,20]. However, these additional peptides may have only been present at low concentrations as most of these appear to originate from other proteins than α-La (Supplementary Table S3). Therefore, it is expected that their contribution to the overall DPP-IV inhibition is minor. Thus, the peptides GY, GL, GI, NY and WL may, in part, be responsible for the DPP-IV inhibitory properties of the hydrolyzates. Quantification of these peptides within the hydrolyzate samples may help to better understand their overall contribution to the DPP-IV inhibition observed and also to determine their extent of release from α-La following enzymatic digestion. However, peptide quantification was not embarked upon during the present study.

To date, two studies have shown that ingestion of DPP-IV inhibitory milk protein hydrolyzates *in vivo* could reduce serum glucose level in small animals [26,27]. A direct link with *in vivo* DPP-IV inhibition had not been established in these studies. While other animal trials carried out with a zein [28], rice [29], porcine [30] and fish skin gelatin hydrolyzates [31,32] showed inhibition of plasma DPP-IV activity. Ultrafiltration fractions of hydrolyzates with DPP-IV IC_{50} values between ~ 1 to 3 mg mL^{-1} have been shown to increase plasma DPP-IV inhibition and reduce glycaemia following an oral glucose tolerance test (OGTT) in small animals [30-32]. H9, had an IC_{50} of 1.20 ± 0.12 mg mL^{-1}. Based on the positive outcomes of previous studies with similar *in vitro* DPP-IV inhibitory potencies, H9 may have the potential to yield positive effects *in vivo*.

No significant modification of the DPP-IV inhibitory properties were seen herein following SGID of H9 (IC_{50} of 1.20 ± 0.12 and 1.53 ± 0.11 mg mL^{-1}, $p > 0.05$, for H9 and its SGID sample, respectively). However, the RP-UPLC and molecular mass distribution profiles of H9 and its SGID sample differed (Fig. 2A and 2B). Furthermore, major differences in terms of peptide peak intensities were observed before and after SGID (Fig. 2C). This indicates that the peptides within H9 were significantly degraded during incubation with the gastrointestinal enzymes.

Conclusion

In silico analyses based on a peptide cutter approach allowed the design of an experimental procedure to specifically release previously identified DPP-IV inhibitory peptides from α-La. Hydrolysis with elastase was predicted to release five previously identified DPP-IV inhibitory peptides (i.e., GY, GL, GI, NY and WL). During *in vitro* enzymatic digestion of α-La with elastase, temperature had the highest effect ($p < 0.05$) on the release of DPP-IV inhibitory peptides followed by the E:S ($p < 0.05$). Several previously identified DPP-IV inhibitory peptides were observed by LC-MS/MS in the DPP-IV inhibitory α-La hydrolyzate, H9. Following SGID, major differences were seen in the peptides within H9. However, potent DPP-IV inhibitory activity was also found within the SGID sample, showing promise in terms of the potential oral application of H9 as an ingredient for serum glucose lowering properties in humans. *In vivo* assessment of the hydrolyzates is required to evaluate their potential role as DPP-IV inhibitors in humans.

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Targeted e*nzymatic digestion to release DPP-IV inhibitory peptides from* α-lactalbumin

α-Lactalbumin

↓

***In silico* prediction**

↓

Digestion with Elastase
Experimental design (pH × E:S × temperature)

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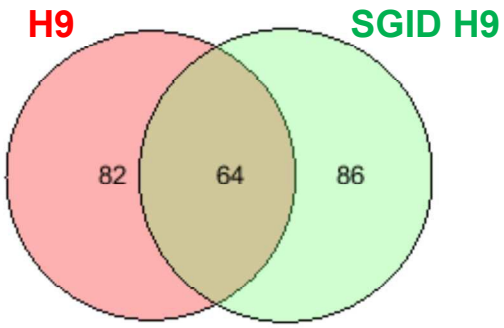
Simulated gastrointestinal digestion (SGID) of H9



Venn diagram on peptides

DPP-IV inhibition maintained after SGID