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Investigation into the potential of commercially available lesser mealworm (*A. diaperinus*) protein to serve as sources of peptides with DPP-IV inhibitory activity

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Summary Food-derived peptides are known to possess inhibitory activity against the dipeptidyl-peptidase IV (DPP-IV) enzyme, a target in the management of type 2 diabetes. While proteins from commonly consumed food commodities have been investigated as precursors of DPP-IV-inhibiting peptides, studies on novel protein sources, such as those from insects, are sparse. This research aimed to determine if DPP-IV inhibitors can be generated upon *in vitro* digestion or enzymatic hydrolysis of lesser mealworm protein isolate and concentrate. Treatment of the proteins with digestive enzymes and proteases generated hydrolysates with varying potency, thermolysin being the most effective at releasing active peptides (IC_{50} = 0.63 and 0.60 mg mL^{-1} for the isolate and concentrate). Ultrafiltration of the thermolysin-treated hydrolysates did not significantly improve the potency. This study shows that DPP-IV inhibitors can be generated from lesser mealworm protein and provides insight on the potential of insects to serve as functional food ingredients.

Keywords Dipeptidyl-peptidase IV inhibitors, hydrolysates, *in vitro* digestion, lesser mealworm (*A. diaperinus*) protein, type 2 diabetes.

Introduction

Insects have generated much interest in recent years as alternative sources of nutrients for both human and animal nutrition (van Huis, 2015). Unlike livestock production, which requires the use of substantial land area and has a considerable negative environmental impact, insect production is more sustainable, requiring less agricultural land, generating less greenhouse gas emissions while presenting a higher feed conversion efficiency (van Huis, 2015). Currently, more than 2000 edible insect species are part of the human diet worldwide (Jongema, 2017), those in the orders Coleoptera, Lepidoptera, Hymenoptera, and Orthoptera being among the most consumed (Feng *et al.*, 2017). From a nutritional point of view, insects are generally considered to contain all major nutrients at levels adequate to meet human requirements (Rumpold & Schlüter, 2013). Presenting a high protein content (15–81%, dry basis) often comparable to that of meat products, and essential amino acid profiles similar to plant proteins (Belluco *et al.*, 2013; Yi *et al.*,2013), common edible insects are often regarded as an interesting alternative to animal proteins. As a result, whole insects, such as yellow mealworm (*T. molitor*) and lesser mealworm (*A. diaperinus*), and protein extracts obtained from them are now being produced commercially.

In addition to being instrumental in a wide range of nutritional and biological processes, dietary proteins can also be precursors of peptides with a variety of biological activities. Over the past few decades, proteins and protein-derived peptides have been widely studied for their potential to help improve human health or complement pharmaceutical drugs in the treatment of chronic diseases (Li-Chan, 2015; Daliri *et al.*, 2017). In recent years, dietary proteins have been found to be precursors of peptides able to inhibit the activity of dipeptidyl-peptidase IV (DPP-IV) *in vitro* and in animal models (Jao *et al.*, 2015; Lacroix & Li-Chan, 2016). This enzyme is involved in the inactivation of the incretins, which are gut-derived hormones recognized to play a pivotal role in glycemic regulation (Filippatos *et al.*, 2014). The inhibition of the DPP-IV enzyme is one of the strategies used in the treatment of type 2 diabetic patients and to date, more than ten DPP-IV inhibitors, often referred to as

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'gliptins', have been approved (Deacon & Lebovitz, 2016). The discovery that natural DPP-IV inhibitors could be produced from dietary proteins has generated great interest in their potential to help improve blood glucose levels. As a result, numerous studies on the production and identification of protein-derived DPP-IV inhibitors have been conducted, resulting in the discovery of more than 150 DPP-IV-inhibiting peptides displaying a wide range of potencies (IC_{50} values ranging from 5 μM to >20 000 μM) (Lacroix & Li-Chan, 2016).

Although the nutritional properties of many common edible insect proteins have been investigated, knowledge on the potential of insect proteins to serve as precursors of peptides with biological activities is limited. To date, only a small number of edible insects have been used to generate bioactive peptides (e.g. Nongonierma & FitzGerald, 2017; Zielińska *et al.*, 2017; Hall *et al.*, 2018; Nongonierma *et al.*, 2018). Therefore, the objective of the present study was to investigate the *in vitro* DPP-IV inhibitory effect of digests and hydrolysates obtained from lesser mealworm (*A. diaperinus*) protein isolate and concentrate, two food-grade ingredients commercially available in The Netherlands.

Materials and methods

Materials

Lesser mealworm protein concentrate (56.5% protein) and isolate (85.9% protein) were purchased from Proti-Farm (Ermelo, The Netherlands). Alcalase® (EC 3.4.21.62, from *Bacillus licheniformis*, ≥2.4 U g^{-1}), Flavourzyme® (from *Aspergillus oryzae*, ≥500 U g^{-1}), pepsin (EC 3.4.23.1, from porcine gastric mucosa, ≥2500 units mg^{-1} protein), pancreatin (from porcine pancreas, 8× USP specifications), papain (EC 3.4.22.2, from papaya latex, ≥10 units mg^{-1} protein), thermolysin (EC 3.4.24.27, from *Geobacillus stearothermophilus*, 30–175 units mg^{-1} protein), recombinant human dipeptidyl-peptidase IV (DPP-IV, EC 3.4.14.5, expressed in S*f*9 cells, ≥4500 units μg^{-1} protein), and diprotin A (Ile-Pro-Ile) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Gly-pro-7-amido-4-methylcoumarin hydrobromide (H-Gly-Pro-AMC ·HBr) was from Bachem (Torrance, CA, USA), while 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from J.T Baker (Thermo Fisher Scientific, Landsmeer, The Netherlands). Novex® 10–20% Tricine Protein Gels and PageBlue™ Protein Staining Solution were obtained from Thermo Fisher Scientific. The Precision Plus Protein™ Dual Xtra Prestained Protein Standards were from Bio-Rad (Veenendaal, The Netherlands). All other chemicals used were analytical grade.

Characterization of the lesser mealworm protein isolate and concentrate by SDS-PAGE

The proteins of the commercial lesser mealworm protein concentrate and isolate were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The un-treated protein concentrate and isolate (50 mg mL^{-1}) were solubilized in 0.01 M Tris-HCl buffer containing EDTA overnight and centrifuged at 10 000 ***g*** for 5 min. The supernatants were combined with 10 μL of tricine SDS sample buffer (2X) and 2 μL of 2-mercaptoethanol to yield final sample concentrations ranging from 1 to 10 mg mL^{-1}. The mixtures were heated at 85 °C for 5 min, then centrifuged at 12 000 ***g*** using an Eppendorf 5414 D centrifuge (VWR, Amsterdam, The Netherlands) for 5 min and 15 μL of the supernatants were loaded in a 10–20% Tricine Protein Gel. Protein bands were revealed using Coomassie blue stain. Molecular weight markers ranging from 5 to 250 kDa were run with the samples.

In vitro digestion

The lesser mealworm concentrate and isolate were submitted to *in vitro* gastrointestinal digestion as described by Lacroix & Li-Chan (2012). In brief, samples were solubilized (30 g L^{-1} in deionized distilled (dd) water) overnight a 4 °C. The protein solutions were then adjusted to pH 2.0 using 6 M HCl and pre-incubated at 37 °C in a water bath shaker (GFL 1086; Burgwedel, Germany). Thereupon, pepsin was added (4% enzyme:substrate (E/S) ratio on a w/w protein basis) and the solutions were incubated for 1 h at 37 °C, after which the pH was increased to 5.3 using 0.9 M $NaHCO_3$. Pancreatin (4% E/S) was added, the pH further adjusted to 7.5 with 1 M NaOH and the solutions were incubated for 2 h at 37 °C. The digested lesser mealworm isolate and concentrate samples were then heated at 80 °C for 15 min to inactivate the pancreatic enzymes, centrifuged using a Avanti J-26 XP centrifuge (Beckman Coulter, Brea, CA, USA) for 10 min at 12 100 ***g*** and the supernatants were collected, filtered (0.45 μm) and freeze-dried. Protein content of the digests was determined by Dumas (FlashEA® 1112 nitrogen and carbon analyser; Interscience, Breda, The Netherlands) using a nitrogen-to-protein conversion factor of 5.60 (Janssen *et al.*, 2017). D-Methionine was used as the standard.

Assessment of different proteases for hydrolysate production

Four commercially available enzyme products with different specificities (Alcalase, Flavourzyme, papain, and thermolysin) were tested for their ability to generate mealworm concentrate and isolate hydrolysates with DPP-IV inhibitory activity. Prior to the screening, the proteolytic activity of each enzyme was measured using a colorimetric assay with the Folin-Ciocalteu reagent as presented in Sigma's Quality Control Test (Procedure for Enzymatic Assay of Protease, Casein as a Substrate. SSCASE1.001). The concentration of each enzyme to be used for the screening was determined based on the amount of enzyme found with Sigma's activity assay to yield 4.1 units of proteolytic activity in 30 mL of a 30 g L^{-1} protein solution. This activity represents an E/S ratio (w/w, protein basis) of 3% for Flavourzyme, the least active of the four proteases. This ratio falls within the range recommended by the manufacturer to obtain effective break down of proteins. For Alcalase, papain and thermolysin, E/S ratios of 0.5%, 2.2% and 0.01%, respectively, were used to obtain the same activity. It should be noted that exopeptidase activity, such as found in the Flavourzyme product, may not be accurately measured by this assay.

Mealworm concentrate and isolate solutions (30 g L^{-1} in dd H_2O, protein basis, un-adjusted pH of 6.7) were pre-incubated in a water bath with a shaker to the desired temperature (55 °C for Alcalase, Flavourzyme and papain, and 70 °C for thermolysin) prior to adding the proteases (4.1 units of proteolytic activity). The mixtures were then incubated for 5 h with constant agitation. The resulting hydrolysates were centrifuged for 10 min at 12 100 ***g*** and the supernatants were collected, filtered (0.45 µm) and freeze-dried, and their protein content determined by the Dumas method (as described in the previous section) prior to being assayed for their effect on DPP-IV activity.

Hydrolysis with thermolysin

Since, among the four enzymes investigated, thermolysin yielded the mealworm protein hydrolsates with the greatest DPP-IV inhibitory activity, this protease was selected for further investigation. Both un-treated mealworm concentrate and isolate were hydrolyzed with the protease, but this time the hydrolysis conditions were adapted, based on the enzyme's manufacturer specification (pH) and the outcome of preliminary trials (E/S ratio and duration of hydrolysis), in order to optimize enzymatic activity and, therefore, the release of active peptides. Mealworm protein solutions (30 g L^{-1} in ddH_2O, protein basis) were adjusted to pH 8.0 using 1 M NaOH and pre-incubated to 70 °C in a water bath with shaker. Thermolysin was then added (1% E/S) and the mixtures were incubated for 1 h. Hydrolysates were then centrifuged, the supernatants were filtered and freeze-dried, and their protein content determined by the Dumas method prior to being assayed for their effect on DPP-IV activity.

Fractionation of thermolysin-treated lesser mealworm concentrate and isolate by ultrafiltration

The mealworm concentrate and isolate hydrolysates obtained by treatment with thermolysin for 1 h were sequentially ultrafiltered using Amicon Ultra-15 Centrifugal Filter Units (Merck, Amsterdam-Zuidoost, The Netherlands) with membranes having molecular weight cutoffs of 10 and 3 kDa. The fractions were freeze-dried prior to being assessed for their effect on DPP-IV activity.

Identification of the peptides present in the thermolysin-treated lesser mealworm concentrate and isolate by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)

Mealworm concentrate and isolate treated with thermolysin for 1 h displaying the highest DPP-IV inhibitory effect were analysed by LC-ESI-MS/MS at the Fred Hutchinson Cancer Research Center (Seattle, WA, USA) to identify their constituent peptides.

The samples (200–500 ng) were diluted in 2% acetonitrile containing 0.1% formic acid and analysed with a Thermo Scientific Easy-nLC II nano HPLC system (Thermo Scientific, Waltham, MA, USA) coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific) mass spectrometer. In-line de-salting was accomplished using a reversed-phase trap column (100 µm × 20 mm) packed with Magic C18AQ (5-µm 200Å resin; Michrom Bioresources, Bruker, Billerica, MA, USA) followed by peptide separations on a reversed-phase column (75 µm × 250 mm) packed with Magic C18AQ (5-µm 100Å resin; Michrom Bioresources) directly mounted on the electrospray ion source. A 60-min gradient from 2% to 40% acetonitrile in 0.1% (v/v) formic acid at a flow rate of 400 nL/min was used for chromatographic separations. The heated capillary temperature was set to 300 °C and a spray voltage of 2750 V was applied to the electrospray tip. The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between MS survey scans in the Orbitrap (automatic gain control (AGC) target value 1 000 000, resolution 240 000, and injection time 250 ms) with MS/MS spectra acquisition in the dual linear ion trap. The 20 most intense ions from the Fourier-transform full scan were selected for fragmentation in the dual linear ion trap by collisional induced dissociation with a normalized collision energy of 35%. Selected ions were dynamically excluded for 15 s with a list size of 500 and exclusion mass by mass width ± 10 ppm.

Data analysis was performed using Proteome Discoverer 2.2 (Thermo Scientific, San Jose, CA, USA). The amino acid sequence of each peptide was identified by comparison with peptide sequences from

A. diaperinus in the Uniprot database. Searches were performed with no enzyme; the precursor ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.6 Da. Variable modifications included oxidation on methionine (+15.995 Da). Data were searched using Sequest HT and all search results were run through Percolator for scoring.

Determination of the extent of hydrolysis

The extent to which the lesser mealworm proteins were broken down during the *in vitro* digestion and hydrolysis with proteases was monitored by determining the content of free amino groups using the TNBS reaction as described by Lacroix & Li-Chan (2012). The amino group content of the hydrolysates and digests was reported in millimoles per gram protein by reference to an L-leucine standard curve.

Determination of DPP-IV inhibitory activity

The effect of the lesser mealworm digests and hydrolysates on DPP-IV activity was tested using the fluorogenic substrate Gly-Pro-AMC according to the method described in the Enzo® DPPIV Drug Discovery Kit Instruction Manual (BML-AK499). Briefly, the samples (10 µL) were pre-incubated for 10 min with 25 µL of assay buffer (0.05 M Tris-HCl, pH 7.5) and 15 µL of DPP-IV (17.3 mU mL^{-1}). The Gly-Pro-AMC substrate (0.01 mM, 50 µL) was then added and the mixture incubated at 37 °C. The fluorescence of the released AMC (excitation: 360 nm; emission: 460 nm) was measured every minute for 20–30 min using a TECAN plate reader (Infinite M200 Pro, Tecan Benelux, Giessen, The Netherlands). Positive and negative controls were also prepared by using the assay buffer in lieu of the sample and in lieu of the sample and enzyme solution, respectively.

The concentrations of samples required to cause 50% inhibition of the enzyme activity (IC_{50}) were determined from the logarithmic regression equations generated by plotting the percent DPP-IV inhibition against the sample concentrations ranging from 0.1 to 1.0 mg mL^{-1} (protein basis, final assay concentration). The peptide diprotin A (Ile-Pro-Ile) was used as reference inhibitor.

Statistical analysis

Significant differences between the samples and/or treatments were established at $P < 0.05$ by conducting one-way analysis of variance using the general linear model and pair-wise comparison with Tukey's method (Minitab Statistical Software Version 18, Minitab Inc., State College, PA, USA). All experiments were conducted at least in triplicate.

Results and discussion

In vitro digestion of lesser mealworm protein concentrate and isolate and effect of the resulting digests on DPP-IV activity

Lesser mealworm protein isolate and concentrate were *in vitro* digested with pepsin and pancreatin. For both, the extent of hydrolysis increased during the peptic phase of the digestion, but more markedly following the addition of pancreatin (Fig. 1a). The breakdown of the proteins appeared to slow down during the last 60 min of pancreatic digestion, as shown by the levelling off of the primary amino groups. The protein isolate and concentrate did not differ significantly in their extent of hydrolysis throughout the course of the digestion. While the digestibility of several insect proteins has been investigated (Ramos-Elorduy *et al.*, 1997), to the authors' knowledge, there is currently no literature in this regard on lesser mealworm protein. Nevertheless, the *in vitro* pepsin-pancreatin digestion of yellow mealworm (*T. molitor*), an insect belonging to the same family as *A. diaperinus* and presenting a similar proximal and amino acid composition (Yi *et al.*, 2013), has recently been investigated in a study by Yi *et al.* (2016). The authors also observed a greater break down of the insect proteins during the *in vitro* duodenal phase of the digestion than during the gastric phase. It can be noted that the un-treated isolate and concentrate also contained some free amino groups (Fig. 1a, 0 min digestion time). Similarly, Yi *et al.* (2016) also observed the presence of free NH_2 groups in undigested *T. molitor* protein fractions and suggested that this might be attributable to the breakdown of the proteins by endogenous enzymes. However, in the presence study, no significant endogenous proteinase activity was measured in the concentrate and isolate.

Unlike what was observed for the extent of hydrolysis, the ability of the samples to inhibit the activity of DPP-IV increased the most rapidly during the first 60 min of digestion with pepsin (Fig. 1b). The inhibitory activity continued to increase slowly during the pancreatic phase of the digestion, but plateaued during the last hour of digestion as shown by the lack of significant differences between the percent DPP-IV inhibition measured at 120 and 180 min, and this for both the mealworm protein isolate and concentrate. The mealworm protein isolate digest obtained after 120 and 180 min of pepsin-pancreatin treatment showed a significantly higher DPP-IV inhibitory activity than the digest obtained from the mealworm protein concentrate. After 180 min of treatment, the isolate and concentrate digested displayed IC_{50} values of 0.53 and 0.71 mg mL^{-1}, respectively (final assay concentration; data not shown). Over the past few years, digests from a number of dietary proteins, including those from

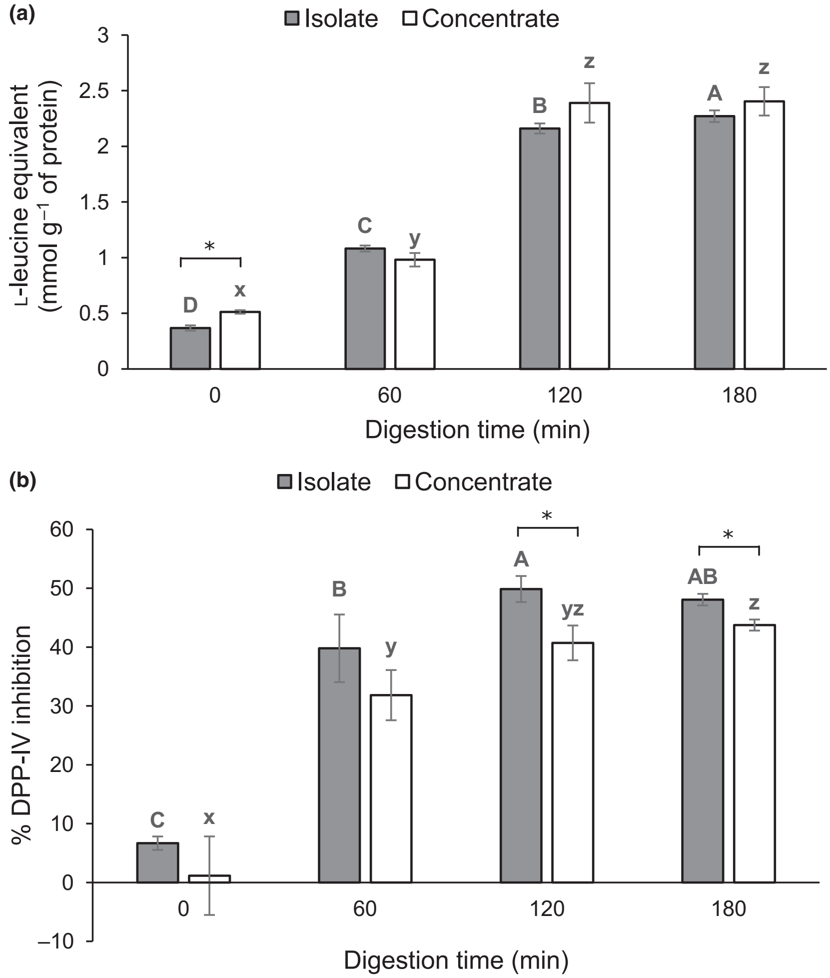

Figure 1 Extent of hydrolysis (mmol L-leucine g^{-1} protein) (a) and DPP-IV inhibitory activity (b) of lesser mealworm isolate and concentrate as a function of time course of *in vitro* digestion. The percent DPP-IV inhibition was determined using 0.5 mg mL^{-1} of sample (protein basis, final assay concentration). Each bar represents the mean and standard deviation of at least three determinations. Bars with different lower or upper case letters or bars connected with an asterisk are significantly different ($P < 0.05$).

milk (Lacroix & Li-Chan, 2012), fish (Guo *et al.*, 2015), hemp (Nongonierma & FitzGerald, 2015), bean (Mojica *et al.*, 2015) and cricket (Hall *et al.*, 2018; Nongonierma *et al.*, 2018), have been shown to present DPP-IV inhibitory activity *in vitro*. This might be partly due to pepsin's known preference for hydrophobic and aromatic residues such as leucine, phenylalanine, tryptophan and tyrosine at the C-terminal of its substrates (Nelson & Cox, 2008), as many potent DPP-IV inhibitory peptides identified to date contain a branched-chain amino acid or an aromatic residue with a polar group in the side-chain (primarily tryptophan) at their N-terminal and/or a proline residue at their P_1 position (Lacroix & Li-Chan, 2016).

Assessment of the potential of various proteases to generate peptides with DPP-IV inhibitory activity from lesser mealworm protein concentrate and isolate

The potential of lesser mealworm protein to serve as precursors of peptides with DPP-IV inhibitory activity was further investigated. The protein isolate and concentrate were hydrolyzed using four commercially available enzyme products with different substrate specificities: Alcalase, Flavourzyme, papain, and thermolysin. In order to compare the efficacy of the enzymes to release DPP-IV-inhibiting peptides, the hydrolysis was performed for 5 h using a concentration of each enzyme yielding the same proteolytic activity (4.1 units of proteolytic activity in 30 mL of a 30 g L^{-1} protein solution).

As shown in Fig. 2b, the enzymatic treatment generated hydrolysates with varying inhibitory activity against the DPP-IV enzyme. The enzyme thermolysin produced the mealworm protein isolate hydrolysate with the highest DPP-IV inhibitory activity (43%) whereas the treatment with Alcalase resulted in the least potent (14%). Conversely, the hydrolysates with the greatest inhibitory activity generated from mealworm protein concentrate were obtained with Alcalase and thermolysin (41% and 46%, respectively). Causing 12% and 8% of DPP-IV inhibition respectively,

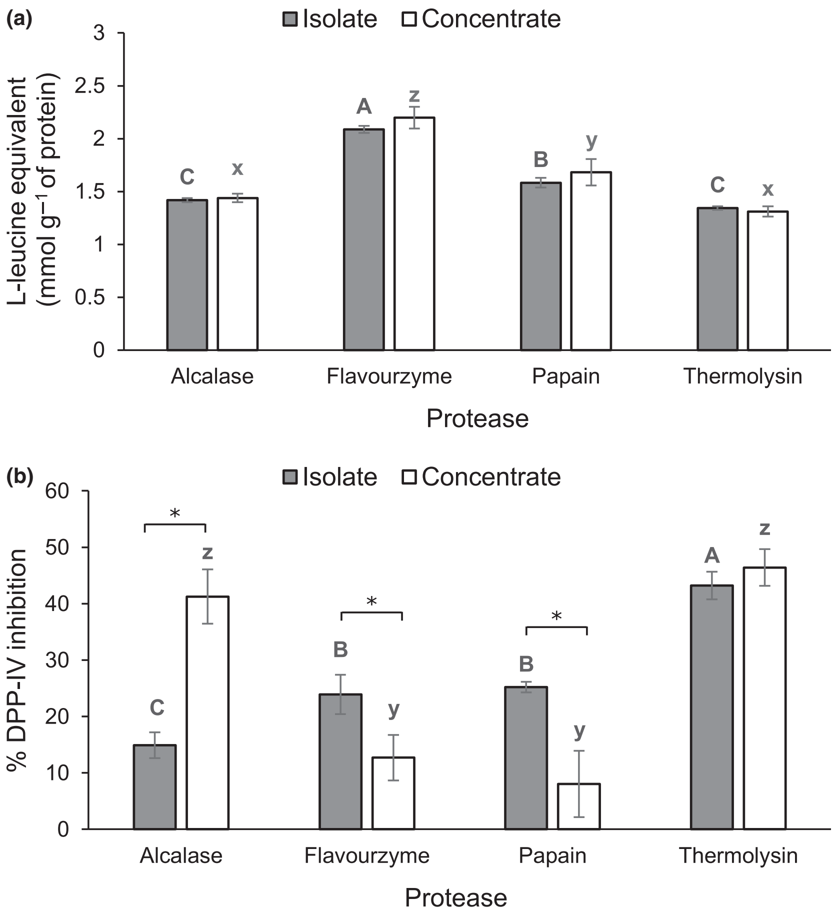

Figure 2 Extent of hydrolysis (mmol L-leucine g^{-1} protein) (a) and DPP-IV inhibitory activity (b) of lesser mealworm concentrate and isolate hydrolysates produced by 5-h treatment with Alcalase, Flavourzyme, papain and thermolysin. The percent DPP-IV inhibition was determined using 0.5 mg mL^{-1} of sample (protein basis, final assay concentration). Each bar represents the mean and standard deviation of at least three determinations. Bars with different lower or upper case letters or bars connected with an asterisk are significantly different ($P < 0.05$).

Flavourzyme and papain were less effective at releasing inhibitory peptides from the protein concentrate. Neither of the two un-treated protein ingredients showed any effect on DPP-IV activity (data not shown). While Alcalase, Flavourzyme and papain have rather broad substrate specificity, the metallo-protease thermolysin is more specific, preferably cleaving substrates with bulky and aromatic residues, such as alanine, leucine, isoleucine, methionine, phenylalanine, and valine, in their P_1' position (Keil, 1992). Thermolysin, like pepsin, has also been previously successfully used to generate peptides with a number of biological activities, including antihypertensive and DPP-IV inhibitory activities (Lacroix & Li-Chan, 2012; Lee & Hur, 2017). Similarly to what was observed during the *in vitro* digestion, the protein isolate and concentrate did not differ significantly in their extent of hydrolysis (Fig. 2a). Hydrolysates generated by Flavourzyme and papain showed the highest extent of hydrolysis while those produced by Alcalase and thermolysin did not differ significantly in their content of free amino groups (Fig 2b).

It is interesting to note that even though the protein concentrate and isolate are both produced from lesser mealworm larvae, and showed similarities in their protein profile (Fig. 3), the hydrolysates obtained from the two substrates by treatment with Alcalase, Flavourzyme, and papain differed in their ability to inhibit the DPP-IV enzyme. This could be due to differences in the peptides released from the protein ingredients during the same enzymatic treatment as shown by the different peptide sequences identified in the thermolysin-treated samples (Table S1). Although the proximate and amino acid compositions of *A. diaperinus* has been reported (Yi *et al.*, 2013), little is known on the insect specific protein composition and few protein sequences are currently available in protein databases. As a result, the ability to identify peptides present in the insect protein hydrolysates by mass spectrometry is limited. Nevertheless, the numerous bands observed between 7 and 75 kDa on the SDS-PAGE electrophoresis profiles of the un-treated protein isolate and concentrate (Fig. 3) illustrate the wide variety of proteins present in the protein ingredients.

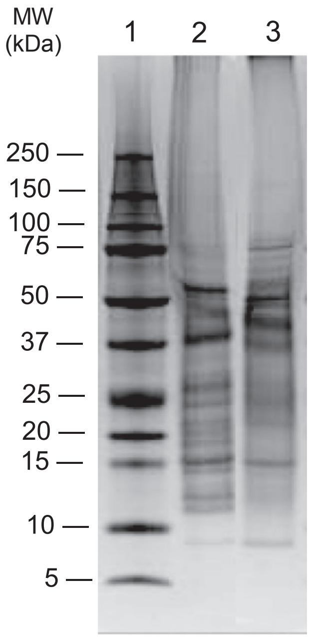

Figure 3 SDS-PAGE electrophoregrams of un-treated lesser mealworm isolate and concentrate. Lanes: 1, molecular weight markers; 2, lesser mealworm protein concentrate; 3 lesser mealworm protein isolate.

Similarly, an array of bands with a wide range of molecular weights (<14–95 kDa) was observed in the supernatant of proteins extracted from *A. diaperinus* in a study by Yi *et al*. (2013). As the exact processes used by the manufacturer to generate the protein concentrate and isolate are unknown, it is difficult to pinpoint the reason(s) behind the variation observed between the inhibitory activity of the protein concentrate and isolate obtained using the same enzymatic treatment. It can, however, be hypothesized that differences in the extraction and purification procedures used to obtain the two ingredients and/or differences in their content of non-protein components, such as fat and chitin, may have affected their hydrolysis. As the un-treated isolate and concentrate displayed no meaningful DPP-IV inhibition, the differences in inhibitory activity is unlikely to be caused by the non-protein constituents themselves.

Ultrafiltration of thermolysin-treated lesser mealworm protein concentrate and isolate and assessment of the resulting fractions on DPP-IV activity

Having generated the hydrolysates with the strongest effect on DPP-IV activity, the thermolysin-treated protein isolate and concentrate were fractionated by ultrafiltration to determine whether a fraction enriched in active peptides could be obtained.

As shown in Table 1, fractionation of the hydrolysates by size did not lead to the generation of fractions with improved DPP-IV inhibitory activity. In fact, none of the fractions were significantly more potent than the whole hydrolysate from which they were obtained from. Only the IC_{50} values of the >10 kDa and 3–10 kDa fractions differed significantly between the two mealworm hydrolysates, those obtained from the protein concentrate hydrolysate being more potent than those from the protein isolate hydrolysate. Although significant, these differences, however, were very small. Similar findings were also reported for hydrolysates generated from other proteins including those from milk (Lacroix & Li-Chan, 2012), and more recently from fish (Zhang *et al*., 2016). Zhang *et al*. (2016) ultrafiltrated a silver carp hydrolysate with DPP-IV-inhibiting properties and found that while fractions smaller than 5 kDa showed slightly improved inhibitory activity, those of higher molecular weight also displayed notable inhibitory effect. None of the fractions collected were as potent as the whole hydrolysate they were generated from. In recent years, a number of DPP-IV inhibitory peptides found to act as competitive inhibitors have been suggested to be in fact substrates for the enzyme, their apparent competitive behaviour being a kinetic artefact resulting from their substrate-like structure. As DPP-IV is known to act *in vivo* on rather large peptides, such as the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), it is possible that peptides present in the higher molecular weight fractions may also act as substrates for the enzyme.

To date, potent peptides of length ranging from 2 to 17 amino acids long have been discovered (Lacroix & Li-Chan, 2016) and while their physicochemical characteristics such as length, hydrophobicity, net charge and isoelectric point have been studied, none other than their amino acid composition have been found to be correlated to their DPP-IV-inhibiting properties (Lacroix & Li-Chan, 2014; Nongonierma *et al*., 2014).

Table 1 Potency (IC_{50} values) of mealworm isolate and concentrate treated with thermolysin for 1 h and of the fractions obtained from them

	IC_{50} (mg mL^{-1})†	
Hydrolysate/fraction	**Isolate**	**Concentrate**
Hydrolysate before ultrafiltration	0.63 ± 0.02 a	0.60 ± 0.02 a
>10 kDa	0.66 ± 0.00 a	0.57 ± 0.01 b
3–10 kDa	0.64 ± 0.02 a	0.57 ± 0.00 b
<3 kDa	0.66 ± 0.05 a	0.54 ± 0.05 a

†IC_{50} values are the means of at least three determinations and reported as final assay concentrations. Within the same row, values with a different letters (a, b) are significantly different ($P < 0.05$). No significant differences were observed between the values within the same column ($P < 0.05$).

The fact that no major difference in DPP-IV inhibitory activity was observed between the peptide fractions obtained in the present study is therefore in agreement with this observation.

The potency of crude hydrolysates reported in the literature typically ranges from μg mL^{-1} to mg mL^{-1} (Lacroix & Li-Chan, 2016). With IC_{50} values of 0.63 and 0.60 mg mL^{-1}, respectively, the mealworm protein isolate and concentrate hydrolysates have an inhibitory activity comparable to that of hydrolysates obtained from proteins of a variety of food commodities, including those from cricket (IC_{50} values = 0.40 to 1.01 mg mL^{-1}) (Nongonierma *et al.*, 2018). *In vitro* digestion of the thermolysin-treated protein isolate and concentrate led to a reduction in their DPP-IV inhibitory activity (IC_{50} values of 1.2 and 1.1 mg mL^{-1} respectively; data not shown). A decreased of inhibitory activity following the simulated *in vitro* digestion of a Protamex™-treated cricket protein isolate was also observed by Nongonierma *et al.* (2018). The authors found that after digestion, the hydrolysate showed a significantly reduced content in large molecular weight peptides and, consequently, a higher content in shorter peptides. These findings suggest that perhaps some of the active peptides originally present in the hydrolysates may be broken down by the digestive enzymes. While *in vitro* data do not necessarily translate *in vivo*, these findings suggest that the DPP-IV inhibiting capacity of mealworm peptides may be reduced in humans.

Compared to food protein hydrolysates, the potency of synthetic DPP-IV inhibitors (e.g. gliptins) currently used for the treatment of type 2 diabetes (IC_{50} in the nM range) (Hunziker *et al.*, 2005) or the tripeptide Ile-Pro-Ile which is to date the most potent sequence found to occur in food proteins (IC_{50} value of 1.6 μg mL^{-1}; data not shown) is much greater. This is, however, to be expected as, unlike pure synthetic compounds or pure single peptides, hydrolysates are composed of a complex mixture of peptides many of which may not present any activity.

Conclusion

Results from the present study showed that peptides with *in vitro* DPP-IV inhibitory activity can be generated from two commercially available protein extracts from lesser mealworm. To the authors' knowledge, this is the first time that proteins from *A. diaperinus* are shown to be precursors of bioactive peptides. Products obtained by *in vitro* gastrointestinal digestion and hydrolysis with thermolysin of both protein ingredients were found to be more effective at inhibiting the DPP-IV enzyme than those generated using Alcalase, Flavourzyme and papain. While the potency of the protein isolate and concentrate hydrolysates produced from the same enzymatic treatment generally differed, active peptides could be generated from both ingredients.

These findings suggest the potential of lesser mealworm protein hydrolysates to serve as functional food ingredients to help improve glycemic regulation. Further research is, however, needed to identify the active peptides responsible for the observed inhibitory activity. In order to exert their effect *in vivo*, DPP-IV inhibitors have to be absorbed in the lumen and reach the capillary bed within the intestinal wall where the enzyme can be located near the cells responsible for the secretion of the incretins. Therefore, the bioavailability of lesser mealworm-derived peptides is a factor that also needs to be explored.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Lesser mealworm derived-peptides identified in the mealworm isolate and concentrate treated with thermolysin for 1 h.