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Identification of peptides from edible silkworm pupae *(Bombyx mori)* protein hydrolysates with antioxidant activity

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ABSTRACT

Silkworm (*Bombyx mori*) pupae is a by-product from the silk industry which is rich in protein. Hydrolysates from silkworm pupae generated using Alcalase®, Prolyve®, Flavourzyme® and Brewers Clarex® proteolytic preparations were characterised. The antioxidant activity of the hydrolysates was investigated using *in vitro* antioxidant assays and an *in situ* assay for reactive oxygen species (ROS) reduction using hepatic HepG2. Overall, Alcalase and Prolyve hydrolysates had highest scavenging activities, however, Flavourzyme and Brewers Clarex hydrolysates had enhanced ferric reducing antioxidant power (FRAP) activity compared to the other samples. Furthermore, the Flavourzyme hydrolysate significantly reduced ROS by 40% compared to untreated control HepG2 cells. Peptides identified by LC-MS/MS were synthetised and then tested for their *in vitro* and *in situ* antioxidant activity. Peptides SWFVTPF and NDVLFF showed highest antioxidant activity (ROS reduction, superoxide dismutase (SOD) expression and glutathione (GSH) production activity) in HepG2 cells, and therefore may have potential as natural antioxidants.

1. Introduction

The development of many metabolic disease related conditions has been linked with an increase in inflammation, cellular oxidation, blood glucose and blood pressure level (Hotamisligil & Erbay, 2008). Oxidation plays a beneficial role in cells, where intracellular reactive oxygen species (ROS) can modulate several signalling pathways against inflammation to activate an immune response. However, an imbalance of intracellular ROS can cause cell damage leading to, e.g., cardiovascular disease (CVD) complications in the long term (Cervantes Gracia, Llanas-Cornejo, & Husi, 2017). While synthetic compounds have traditionally been used to treat CVD related conditions, they are generally associated with undesirable side-effects. More recently, bioactive peptides derived from food proteins have been widely studied for their antioxidant properties with the benefit of zero/reduced side-effects. In some instances, these antioxidant peptides can be considered as multifunctional agents as they may additionally contribute to, e.g., reducing inflammation and/or blood pressure (Lammi, Aiello, Boschin, & Arnoldi, 2019).

The European Commision has recently approved the use of specific insect flours as food ingredients, in keeping with the need to identify alternative high quality sources of protein for human nutrition (EFSA, 2021). It is estimated that 100,000 tonnes of silkworm are produced annually in China (Wu, He, Velickovic, & Liu, 2021). Silkworm (*Bombyx mori*) pupa is a by-product of the silk industry and is mainly destined for animal feed (Rahimnejad et al., 2019). However, silkworm pupa is commonly used as a food ingredient in countries such as China, India, Thailand and Korea (Gahukar, 2016). Silkworm pupae have a high nutritional value. They are considered rich in protein (up to 55% dw), lipid, carbohydrate, vitamins and minerals (Tomotake, Katagiri, & Yamato, 2010). Proteins from silkworm have been previously associated with *in vitro* antioxidant properties (Chatsuwan, Puechkamut, & Pinsirodom, 2018) while enzymatic hydrolysis of silkworm proteins has resulted in the generation of hydrolysates with more potent *in vitro* antioxidant properties compared to the intact protein (Liu, Wan, Liu, Zou, & Liao, 2017; Zhou, Ren, Yu, Jia, & Gui, 2017). Furthermore, a range of enzymes may be used during hydrolysis in order to optimise the generation of hydrolysates with different antioxidant potencies (Yang et al., 2013).

The protein hydrolysates generated from silkworm pupae proteins

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may have peptide sequences with potent antioxidant activity. While antioxidant peptide sequences have been identified in other edible insects, such as peptide CTKKHPNC from Asian weaver (Pattarayingsakul et al., 2017), to date, only one study has reported on the *in vitro* antioxidant activity of peptides (FKGPACA and SVLGTGC) from *B. mori* (Zhang et al., 2021). Therefore, the objectives of the present study were (i) to assess the *in vitro* and *in situ* antioxidant capacity of silkworm protein hydrolysates generated using a range of food grade proteolytic enzymes and (ii) to identify, synthetise, and test the most promising peptides derived from the hydrolysates for their *in vitro* and cell-based antioxidant activities.

2. Materials and methods

2.1. Materials

Silkworm flour, obtained from FeedStimulants Ltd. (Zoetermeer, Netherlands), was defatted with n-hexane as per Felix et al. (2020) and stored in sealed polypropylene bags at room temperature until use. The protein content after defatting was 53.00 ± 0.34 (using a nitrogen to protein conversion factor of 4.76 based on Janssen, Vincken, Van Den Broek, Fogliano, and Lakemond (2017). The food-grade enzymes used to generate the hydrolysates were as follows: Brewers Clarex® (37 Anson Units (AU) g^{-1}) was purchased from DSM (Switzerland), Prolyve® 1000 (2.2 AU g^{-1}) was kindly provided by Lyven Enzymes Industrielles (Caen, France) and Alcalase® (2.4 AU g^{-1}) and Flavourzyme® (500 Leucine Amino Peptidase Units (LAPU) g^{-1}) were purchased from Sigma-Merck (Dublin, Ireland). Trinitrobenzensulphonic acid (TNBS) was obtained from Thermo Scientific (Dublin, Ireland). Sulphuric acid (96% w/v) for Nitrogen determination, mass spectrometry (MS) and high-pressure liquid chromatography (HPLC) grade water and acetonitrile (ACN) were from VWR International (Wicklow, Ireland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation kit (Roche, Basel, Switzerland), Eagle's minimum essential medium (MEM), Hanks' balanced salt solution (HBSS), phosphate buffer saline (PBS), fetal bovine serum (FBS), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Radioimmunoprecipitation assay buffer (RIPA) were from Sigma-Merck (Wicklow, Ireland). Individual peptides were synthesised by Genscript (Piscataway, NJ, USA) using a solid phase peptide synthesis methodology. All other chemicals were obtained from Sigma-Merck and were of analytical grade unless otherwise stated.

2.2. Direct enzymatic hydrolysis of silkworm flour

Silkworm flour was suspended in distilled water at 10 % (w/v) and the pH was adjusted to pH 8.0 using 1 M NaOH. The solution was maintained at 50 °C for 30 min after which the pH was readjusted to 8.0, if necessary. Aliquots of the solution were incubated with different enzymes (Alcalase, Prolyve, Flavourzyme and Brewers Clarex) except the control sample which had the same treatment, but without added enzyme. Enzymes were added at 1% (v/w, on a protein basis) and the solutions were maintained at 50 °C for 4 h without pH control (free fall hydrolysis). The solutions were then heated to 80 °C for 20 min to inactivate the enzymes and the hydrolysates were then centrifuged at 2700*g* and 10 °C for 20 min using a Hettich Universal 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatants obtained were freeze-dried and stored at −20 °C prior to further analysis.

2.3. Protein quantification and extent of hydrolysis of control and hydrolysate samples

The protein content of the silkworm derived samples was quantified using the macroKjeldahl method (Connolly, Piggott, & FitzGerald, 2013) using a Nitrogen to protein conversion factor of 4.76 as previously described by Janssen et al. (2017). The protein content of the hydrolysed samples was expressed as protein equivalents. The extent of hydrolysis was determined using the TNBS method, as previously described (Pimentel et al., 2020).

2.4. Reverse phase (RP-)HPLC analysis of the silkworm hydrolysates

RP-HPLC was performed using a Waters HPLC system (Waters, Milford, MA, USA) comprising a 600E binary pump and a 2487 absorbance detector. Mobile phase A was 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water and mobile phase B was 0.1% (v/v) TFA in 80% HPLC-grade ACN. Freeze-dried silkworm hydrolysate supernatants at 1 % (w/v on a protein equivalent basis) were resuspended in mobile phase A, filtered through 0.2 μm cellulose acetate syringe filters and 20 μL was injected onto a Jupiter Proteo column (C18, 250 mm × 4.6 mm ID, 5 mm particle size, 300 Å pore size, Phenomenex, Cheshire, UK). Peptides were eluted at a constant flow of 1 mL min^{-1} for 90 min using the following gradient: 0–4 min: 100% A; 4–59 min:50–50% A-B; 60–70 min: 100% B; 70–75 min 100% A; 75–90 min: 100%. The absorbance was measured at 214 nm.

2.5. In vitro antioxidant analysis

The ferric reducing antioxidant power (FRAP) of the silkworm samples was determined as previously described (Connolly et al., 2013). The $ABTS^{\bullet+}$ radical scavenging assay was carried out as previously described (Kleekayai et al., 2015). The oxygen absorbance capacity (ORAC) assay was carried out as previously described (Harnedy & FitzGerald, 2013).

2.6. Cellular assessment of antioxidant activity

2.6.1. Cell culture

HepG2 cells (European Collection of Authenticated Cell Cultures 85011430) were maintained in MEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) in T175 cell culture flasks at 37 °C under an atmosphere containing 5% CO_2. Cell media was replaced every other day and cells were sub-cultured every 3–4 days before reaching 90% confluence. The HepG2 cells used throughout had <10 passages.

2.6.2. Cell viability

The viability of the HepG2 cells treated with silkworm derived samples was assessed using the MTT I proliferation kit according to the manufacturer's instructions. Briefly, cells were seeded in black, clear bottom 96 well plates (Corning, NY, USA) at a cell density of 1x 10^5 cells per well and left to adhere overnight in the incubator at 37 °C with 5% CO_2. The media was then aspirated, and the plate was washed twice with PBS. The test samples were then added to yield different final concentrations (1.0, 2.5, 5.0, 10.0, 12.5, and 15.0 mg protein mL^{-1}) and the cells were incubated for 24 h, following which the media was aspirated and MTT reagent A (10 μL) was added to the wells. The plate was incubated for 4 h (at 37 °C with 5% CO_2) and then MTT reagent B (100 μL) was added to the wells to dissolve the formazan crystals. The plate was incubated for 16 h and the absorbance was read at 570 nm using a Biotek Synergy plate reader (BioTek Instruments Inc., VT, USA). Sample-treated cells were compared to the control cells i.e., cells with no treatment (100 % viability) and the results were expressed as % cell viability. Silkworm-derived sample concentrations giving a cell viability value below 80% were considered cytotoxic.

2.6.3. Cellular antioxidant assays

The production of ROS by HepG2 cells was quantified as previously described in Kleekayai, Le Gouic, Deracinois, Cudennec, and FitzGerald (2020). Briefly, HepG2 cells were seeded at a density of 6 × 10^4 cells per

well in black, clear bottom 96 well plates which were then incubated at 37 °C in 5% CO_2 for 24 h. The media was then aspirated and the cells were washed with HBSS. The DCFH-DA probe (100 μL), was added to the cells and the plates were placed in the incubator at 37 °C in 5% CO_2 for 30 min. Following this time, 100 μL of the hydrolysates (at different final concentrations 2, 1 and 0.5 mg protein mL^{-1}), or peptides (100 μM) or Trolox (100 μM) were added and the cells were incubated for 1 h. The cells were then washed twice with HBBS, 100 μL of 800 μM AAPH in HBSS was added to the wells and the fluorescence was read using a Biotek Synergy plate reader at excitation and emission wavelengths of 485 and 528 nm, respectively. The results were expressed as % ROS generation (considering that 100 % ROS generation was obtained with cells treated with DCFH-DA and AAPH without test samples).

For superoxide dismutase (SOD) activity analysis, HepG2 cells were incubated in MEM in clear 24 well plates ($1x10^5$ cells $well^{-1}$) for 24 h at 37 °C in 5% CO_2. The media was then aspirated and the cells were incubated with 100 μM (final concentration) of the different synthetic peptides or with Trolox (100 μM, final concentration) for a further 24 h. The media was aspirated and 800 μM AAPH in HBSS was added into each well, and the plates were then incubated for 90 min at 37 °C to induce oxidation. Following that time, the media was aspirated and cells were lysed with 1x RIPA buffer for 15 min and centrifuged at 14,000*g*, 4 °C for 15 min. The supernatant was discarded and the pellet was washed twice with HBSS. SOD activity was measured using the SOD Assay Kit (Sigma-Aldrich) following the manufacturer's instructions.

For GSH determination, HepG2 cells were seeded (0.5×10^5 cells mL^{-1}) in white 96 well plates (Corning). Cells were left to adhere 16 h at 37 °C and 5% CO_2, then MEM media was removed, peptides and Trolox (100 μM) were added, and the plates were incubated for a further 24 h under the same conditions. Supernatants were decanted, and the cells were washed twice with HBSS before adding 800 μM of AAPH for 90 min at 37 °C with 5% CO_2. Cell media was removed and GSH was determined using the GSH-Glo™ Glutathione Assay kit (Promega Gmbh, MA, US), following the manufacturer's instructions.

2.7. Identification of peptides by UPLC–ESI-MS/MS

Peptide identification was performed using an Acquity UPLC system (Waters) coupled to a Bruker micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) using the methodology previously described by O'Keeffe, Conesa, and FitzGerald (2017), with some modifications. Briefly, the hydrolysate samples were diluted to a final concentration of 0.1 mg mL^{-1} in mobile phase A (MS water containing 0.1% (v/v) formic acid (FA)) and were separated on an Acquity BEH 300 C18 column (2.1 × 50 mm, 1.7 μm; Waters, Dublin, Ireland). Separation was performed using a linear gradient from 0 to 80% of mobile phase B (80% (v/v) acetonitrile with 0.1% (v/v) FA) at a flow rate of 0.2 mL min^{-1} during 53 min. The MS and tandem MS full scans were performed in positive mode over an *m/z* range of 100–2500. Electrospray conditions were as follows: capillary voltage was 4500 V; capillary temperature, 220 °C; dry gas (N_2) flow, 8.0 L min^{-1}; nebulizer, 1.8 bar; collision energy was 7 eV; collision cell radio frequency (RF) was 1500 Vpp and transfer time was 100 μs. The system was calibrated with an electro spray ionisation (ESI) low molecular mass tune mix calibrant (Agilent Technologies, CA, US). All MS/MS data was analysed using Peaks Studio 8.5 (Bioinformatics Solutions Inc, Ontario, Canada) against the SwissProt database for the Protostomia taxa and an *in house* database for *B.mori* proteins. Precursor error mass was set at 0.5 Da and the score threshold for peptides (−10lgP) was set at 20. The peptide sequences obtained were then manually verified using Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany). Specific peptide sequences were selected for synthesis and were then maintained at −20 °C until use.

2.8. Statistical analysis

Data were reported as means ± standard deviation (SD) of at least three independent replicates. One-way analysis of variance (ANOVA) with Tukey or Dunnett test was used to analyse the results using GraphPad Prism, version 6.00 for Windows. Significant levels were defined as $p < 0.05$.

3. Results and discussion

3.1. Silkworm protein hydrolysate properties

The conditions of hydrolysis used in this study were incubation at pH 8.0, 50 °C and 4 h with no pH control (free fall pH). An initial pH value of 8.0 was selected based on the solubility of the sample at this pH (approx. 50%; (Felix et al., 2020)). Additionally, the optimal pH and temperature of most of the enzymes used was at alkaline pH, approx. pH 8.0 and at 50 °C (with the exception of Brewers Clarex which had an optimal pH at 6.0). The duration of hydrolysis (4 h) was based on a previous report from Nongonierma, Lamoureux, and FitzGerald (2018) in which the authors optimised the duration of the hydrolysis process (60, 150 and 240 min) using response surface methodology during the generation of insect protein hydrolysates. This incubation period led to a higher extent of hydrolysis and to a greater amount of lower molecular weight peptides in the hydrolysates. Enzymatic hydrolysis of the silkworm flour was performed using four different enzyme preparations, i.e., with Alcalase and Prolyve from *Bacillus licheniformis* and the fungal enzymes Flavourzyme from *Aspergillus oryzae* and Brewers Clarex from *Aspergillus niger*.

Following enzyme inactivation, the samples were centrifuged in order to remove insoluble material and to avoid possible interferences from carbohydrates. The samples hydrolysed with Prolyve and Alcalase had the highest protein content (56.45 ± 1.10 and 57.21 ± 0.38 %, respectively) followed by the Brewers Clarex, the control and the Flavourzyme samples (Table 1). The range of protein contents found in the hydrolysates herein (42.12 ± 0.12–57.21 ± 0.38 %) was lower than that reported previously for silkworm protein hydrolysates i.e., up to 79 % protein (Anootthato, Therdthai, & Ritthiruangdej, 2019). However, this may be due to the use of a different N to protein conversion factors. All the samples were initially adjusted to the same pH (8.0) and following enzyme addition, the pH was not controlled (free fall). A decrease in pH value (during free fall hydrolysis conditions) is usually observed. However, the extent of the pH drop is highly dependent on the type of enzyme, the concentration added and their specificity for cleavage of the protein substrate (Spellman, Kenny, O'Cuinn, & FitzGerald, 2005; Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). Alcalase is a broad specificity serine endopeptidase enzyme preparation and therefore it leads to quite extensive hydrolysis resulting in a significant reduction in pH during non-pH stat controlled hydrolysis reactions. However, the pH reduction in the Brewers Clarex and Flavourzyme hydrolysates was less extensive as these enzymes cleave more selectively. In addition, Flavourzyme contains exopeptidase activities and hydrolysis with exopeptidases is well documented to lead to lower extents of pH reduction.

Table 1
Protein equivalent content of supernatants obtained from silkworm flour following incubation at 50 °C for 4 h with different enzyme preparations.

Enzyme	Protein equivalent (% w/w)*	Initial pH	Final pH
Control#	44.65 ± 0.01^{c}	8.00	7.90
Alcalase	56.45 ± 1.10^{a}	8.00	7.20
Prolyve	57.21 ± 0.38^{a}	8.00	7.33
Flavourzyme	42.12 ± 0.12^{d}	8.00	7.92
Brewers' Clarex	47.49 ± 1.08^{b}	8.00	7.92

* Values expressed as protein equivalent following enzymatic hydrolysis, the Nitrogen to protein factor used was 4.76 based on Janssen et al. (2017).

Control: silkworm flour subjected to similar treatment to the hydrolysates but without added enzyme. Values in protein equivalent column represent mean ± SD (n = 3). Superscript letters denote significant differences ($p < 0.05$).

Furthermore, the overall solubility of the silkworm flour was relatively low approx. 50% (Felix et al., 2020). This was possibly due to a high content in carbohydrate (>30% in the defatted flour) and this may have led to a relatively low accessibility of the enzymes for the proteins in the starting substrate. Incubation with Alcalase and Prolyve led to a more extensive hydrolysis (19.54 ± 2.23 and 15.43 ± 0.94 mg amino N g^{-1}, respectively; Fig. 1). The Alcalase and Prolyve treated samples showed the highest content of released amino groups with 19.54 ± 2.23 and 15.43 ± 0.94 mg amino N g^{-1} protein, respectively. The control sample (without enzyme) had 9.65 ± 0.18 mg amino N g^{-1} protein which indicated that partial autolysis may have occurred in the sample during incubation at pH 8.0 due to the presence of endogenous enzymes in the flour sample or due to low levels of alkaline hydrolysis. This observation was also reported in cricket protein where the control sample (without enzyme addition) was reported to have a degree of hydrolysis value of 4.5 % (Hall, Johnson, & Liceaga, 2018). The total amino N values in the samples incubated with Brewers Clarex and Flavourzyme were not significantly higher than the control suggesting that minimal additional hydrolysis occurred on incubation with these enzyme preparations. For the Flavourzyme hydrolysate this might be related to the properties of the enzyme which mainly contains *exo*-proteinase activities and therefore it has a limited effect on the breakdown of proteins compared to the endoproteinase activities in Alcalase and Prolyve. This was also observed in a previous study by Anootthato et al. (2019), who reported that when silkworm pupae was hydrolysed with Alcalase (containing both endo and *exo*-proteinase activity) it yielded a significantly higher degree of hydrolysis (DH) than when it was hydrolysed with Neutrase (exoproteinase activity), i.e., 9.61 vs 2.91% DH, respectively). In another study, hydrolysis of silkworm chrysalis for 5 h with Alcalase yield to a higher extent of hydrolysis (DH: 30%) compared with incubation with Flavourzyme (DH: <5%) under similar conditions (Yang et al., 2013). Nevertheless and in agreement with the study herein, of the enzymes tested, Alcalase appears to generally yield the highest extent of hydrolysis in silkworm pupae hydrolysates (Yoon, Wong, Chae, & Auh, 2019). This may be related to its broad substrate specificity, i.e., consisting of subtilisin activity along with the presence of a glutamyl endopeptidase side activity (Spellman, O'Cuinn, & FitzGerald, 2009).

Fig. 2 shows the chromatographic profiles of the samples obtained by RP-HPLC. All hydrolysate profiles differed from the control which contained a limited number of low intensity peaks eluting between 3.0 and 6.5 min. Hydrolysates obtained following incubation with Alcalase and Prolyve had similar profiles containing a range of hydrophilic and hydrophobic peptides with a highly intense peak eluting between 5.7 and 6.0 min. The Flavourzyme hydrolysate contained additionally 3 major peaks eluting at 10, 12 and 15 min, which were also eluted in the Alcalase and Prolyve hydrolysates at a much lower intensity, whereas the common peak eluting approx. at 6 min in Alcalase, Prolyve and Brewers Clarex was not as intense in the Flavourzyme hydrolysate. The sample incubated with Brewers Clarex showed a similar peptide pattern to Alcalase and Prolyve up to min 10 of elution, after which not many major peaks could be detected. These differences in the chromatographic profiles were attributed to differences in the enzyme specificities of the hydrolytic preparations employed herein.

3.2. *In vitro* bioactivities of silkworm hydrolysates

3.2.1. *Antioxidant activity*

The *in vitro* antioxidant activity of the hydrolysates was tested using three different assays, i.e., the FRAP, ORAC and ABTS assays. It was assumed given the very low level of enzyme added to generate the hydrolysates, i.e., 1 % (w/v) that the contribution to the antioxidant activity by the enzymes, if any, would be negligible. Hydrolysates obtained with Flavourzyme and Brewers Clarex had the highest FRAP values (1471.19 ± 32.48 and 1397.10 ± 14.02 μmol TE g $protein^{-1}$, respectively) followed by Alcalase, Prolyve and the control (Fig. 3). Water soluble silkworm proteins (unhydrolysed) was previously reported to have a FRAP value of 54.20 ± 0.13 μg TE g^{-1} protein (corresponding to 0.216 μmol TE g^{-1} protein) (Chatsuwan et al., 2018). The results in this study indicate that enzymatic hydrolysis increases the antioxidant FRAP antioxidant activity of silkworm protein. However, it appears that a high extent of hydrolysis may reduce the FRAP activity. For instance, the FRAP activity of a pepsin hydrolysate of the seaweed *Porphyra dioica* was significantly higher than for the same sample further digested with Corolase PP (Pimentel et al., 2020). This may explain why the Flavourzyme and Brewers Clarex samples (which had a lower extent of hydrolysis) showed higher antioxidant activity in the FRAP assay than the Alcalase and Prolyve samples with a higher extent of hydrolysis (Fig. 2). This difference in FRAP activity could also depend on the nature of the sample, e.g., it has been reported that an hydrolysate generated from *Schistocerca gregaria* had the lowest Fe^{2+} chelating activity (IC_{50} 0.101 ± 0.02 mg mL^{-1}) while the same hydrolysis treatment applied to *Gryllodes sigillatus* gave an IC_{50} value 0.456 ± 0.07 mg mL^{-1} (Zielińska, Baraniak, & Karaś, 2017).

The highest ORAC values in this study were obtained for the Alcalase and Prolyve hydrolysates while the Flavourzyme and Brewers Clarex hydrolysates had the lowest ORAC values. When compared to the control sample (no added enzyme) the hydrolysate generated with Prolyve was the only sample with a significantly higher ($p < 0.05$) ORAC value. Several reports have shown that a high extent of hydrolysis is associated with higher amounts of low molecular weight peptides resulting in enhanced ORAC activity. For instance, a <3 kDa fraction of an ultrafiltered silkmoth protein hydrolysate generated with Alcalase was reported to possess significantly higher ORAC and DPPH values (1950 and 275 μmol TE g^{-1}, respectively), than other ultrafiltered fractions with higher molecular weights (Liu et al., 2017).

The antioxidant activity measured by the ABTS assay followed the same trend as the ORAC values. The Alcalase, Prolyve and the control samples had the highest ABTS scavenging activity while the Flavourzyme and Brewers Clarex hydrolysates had significantly lower ABTS activity. No information appears to have been reported to date regarding the ABTS scavenging activity of silkworm protein derived hydrolysates. However, the water-soluble proteins extracted from

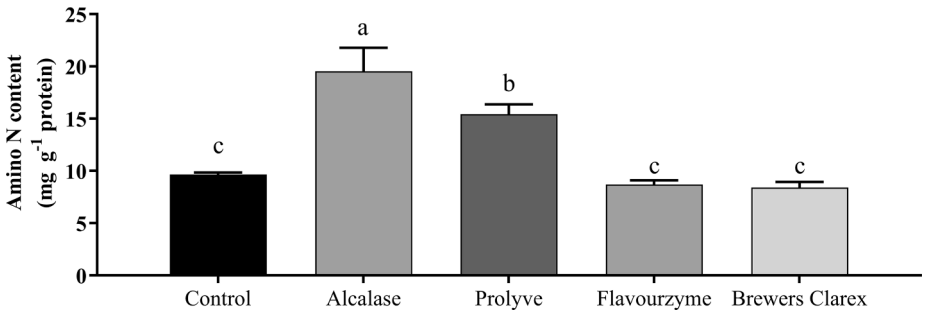

Fig. 1. Amino Nitrogen content of the silkworm flour control sample (with no added enzyme) and its corresponding hydrolysates following 4 h incubation at 50 °C. Different letters denote significant differences between samples. Error bars represent mean ± SD (n = 3).

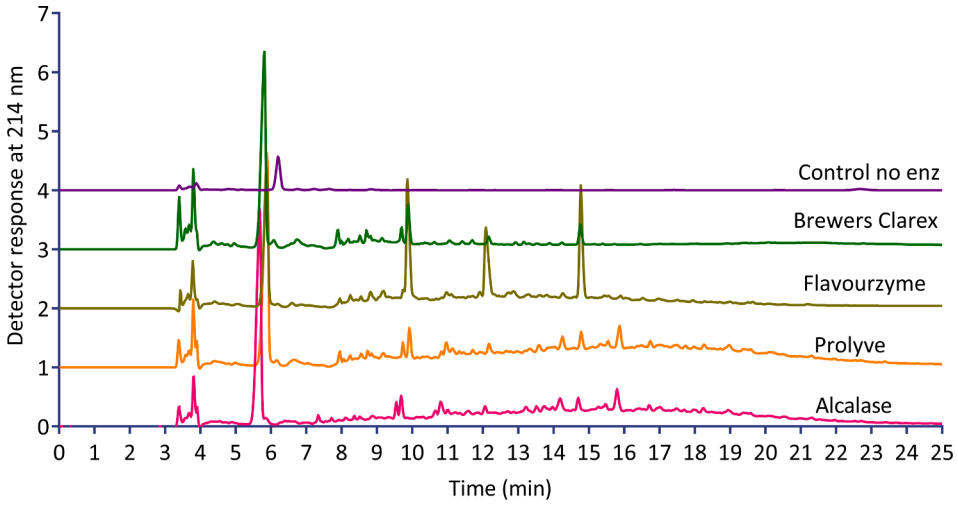

Fig. 2. Reverse phase high pressure liquid chromatography (RP-HPLC) profiles of silkworm protein hydrolysates and control (without enzyme) obtained on incubation for 4 h at 50 °C with different enzyme preparations.

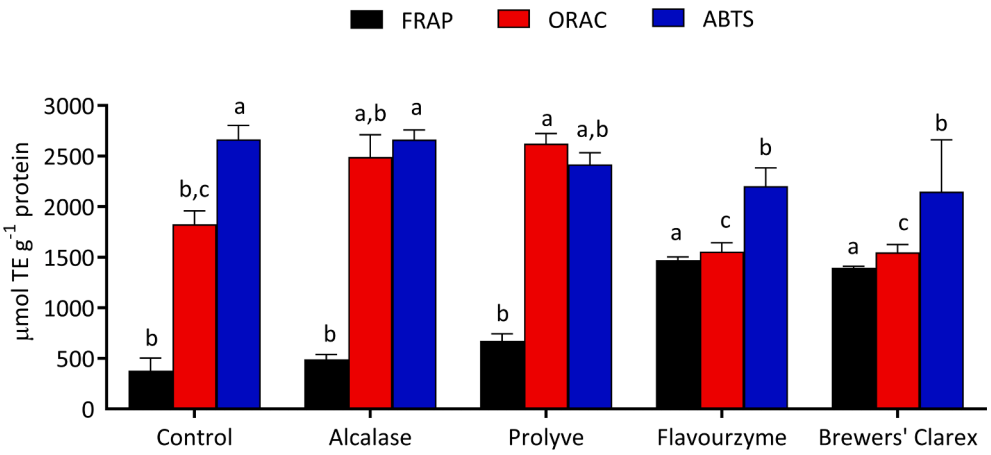

Fig. 3. *In vitro* antioxidant activity of silkworm protein hydrolysates and control (without enzyme) obtained on incubation for 4 h at 50 °C with different enzyme preparations, measured using the ferric reducing antioxidant power (FRAP), the oxygen radical absorbance capacity (ORAC) and the 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. Different letters denote significant ($p < 0.05$) differences between samples within the same assay. Error bars represent mean ± SD (n = 3).

B. mori showed ABTS EC_{50} values of 16.57 µg mL^{-1} (Chatsuwan et al., 2018). Enzymatic treatment of silkworm pupae with an alkaline protease significantly improved the scavenging activity of the sample compared to the untreated sample when measured by the DPPH, Fe^{2+} chelating and total reducing power assays (Zhou et al., 2017). This was not found in the present study since the control sample had similar and even higher values for antioxidant activity. However, it is important to point out that in this study, the control sample was subjected to the same treatment as the hydrolysates except for the addition of enzyme and it was shown from the extent of hydrolysis analysis that autolysis may have occurred. Similar results were reported for a cricket protein control which showed higher ABTS values than the corresponding hydrolysates (Hall et al., 2018).

Overall, the specificity of the enzymes used herein had an impact on the antioxidant activity obtained where a low extent of hydrolysis seems to be more favourable for iron chelation while a high extent of hydrolysis seems to lead to hydrolysates capable of scavenging radicals. This is in agreement with another study that investigated the effect of six different enzyme preparations, i.e., Flavourzyme, Alcalase, Protamex, trypsin, papain and pepsin on the scavenging activity of silkworm chrysalis where the Alcalase hydrolysate yielded the highest DPPH scavenging activity (Yang et al., 2013).

3.2.2. Cellular assessment of antioxidant activity

While *in vitro* antioxidant assays are useful for estimation of antioxidant activity, in some instances *in vitro* results do not translate to *in vivo*. Therefore, an *in situ* antioxidant activity assay was performed using the hepatic cell line HepG2 with the aim of obtaining a more physiologically relevant antioxidant assessment.

Fig. 4A shows the viability of HepG2 cells when treated with the silkworm derived hydrolysates for 24 h. All samples showed a dose-response trend in the viability of the cells with higher cytotoxicity being observe at higher sample concentrations. Concentrations lower than 2.5 mg mL^{-1} of all samples resulted in good viability for the HepG2 cells while cells treated, e.g., with 5 mg mL^{-1} of the Alcalase hydrolysate had <80% viability, therefore, the sample was considered toxic at that concentration. All samples significantly reduced cell viability when tested at 10 mg mL^{-1} with the exception of the silkworm control sample (silkworm flour at pH 8.0 with no added enzyme). It was observed that cells treated with the Prolyve hydrolysed protein showed a lower toxicity (especially at 15 mg mL^{-1}) than the other silkworm derived hydrolysates. Based on Fig. 4A the final concentrations of the silkworm hydrolysates selected for the *in situ* determination of antioxidant activity in HepG2 were 0.5, 1 and 2 mg mL^{-1}.

Fig. 4B shows the % ROS reduction effect of silkworm hydrolysates in HepG2 cells following induction with the oxidant AAPH. The results showed that, contrary to the results observed *in vitro,* the hydrolysates had a higher percentage of protection against ROS than the control at a cellular level. A dose dependent trend was observed for all the samples where concentrations as low as 0.5 mg mL^{-1} of the silkworm-derived

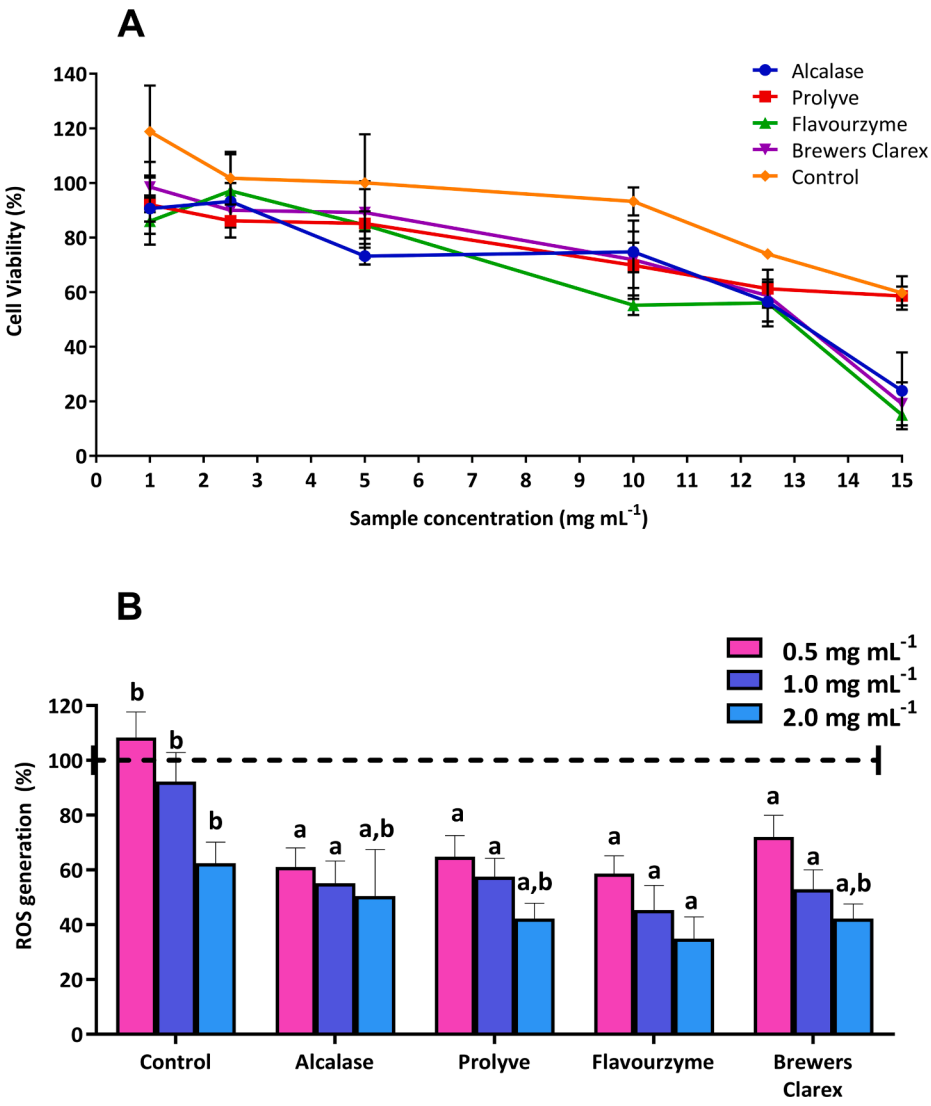

Fig. 4. Cell viability of HepG2 cells treated with different concentrations of silkworm derived samples (A) and intracellular reactive oxygen species (ROS) generation in 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) induced HepG2 cells treated with 0.5, 1 and 2 mg protein mL^{-1} (final concentration) of silkworm control and hydrolysed samples (B). Values reported are mean ± SD (n = 3). Different letters denote significant differences at $p < 0.05$ between samples within each sample concentration. Broken line indicates 100 % ROS generation in untreated cells induced with AAPH.

hydrolysates could reduce the formation of ROS by up to 40 %. The lowest mean ROS generation value was obtained for the cells treated with the Flavourzyme hydrolysate at 2 mg mL^{-1} (34.93 ± 7.9 %), however, no significant differences ($p > 0.05$) were found between the hydrolysate samples at all concentrations tested. To the best of our knowledge, no cellular antioxidant activity values appear to have been reported to date for silkworm protein hydrolysates. Therefore, the peptides within the hydrolysates were identified using LC-MS/MS and the peptides identified were further tested for their cellular antioxidant activity.

4. Identification of peptides by UPLC-MS/MS

The control and the hydrolysates were submitted to UPLC-MS/MS analysis in order to identify their peptide sequences. Both the Alcalase and Prolyve hydrolysate showed the highest percentage of coverage within the proteins from *B. mori,* i.e., with 7–12% coverage. A total of 32 peptides were identified within the hydrolysates (14 in Alcalase, 18 in Prolyve, 1 in control, 1 in Flavourzyme and none in the Brewers Clarex hydrolysate) with peptide lengths ranging from 5 to 14 amino acids (Table 2). The low extent of peptide identification for the control, Brewers Clarex and Flavourzyme samples may be due to the poor solubility of these samples in water along with peptide precipitation since a clear pellet was observed following reconstitution of these samples in mobile phase A (0.1% FA) following centrifugation. Two peptide sequences IRLPSGDEM(+15.99)PVRQN and SDKTVDAVVR, were found in both the Alcalase and the Prolyve hydrolysates.

The structure activity relationship of antioxidant peptides is still not fully understood. However, studies using quantitative structure activity (QSAR) techniques have reported that peptides containing hydrophobic amino acids such as V, A, I and L are linked with antioxidant activity (Li & Li, 2013). Therefore, a total of 8 peptides, with a high level of hydrophobic residues (>60%) were selected for synthesis (Table 2, Supplementary Figs. 1–8).

5. Antioxidant activity of silkworm-derived peptides

The antioxidant activity of the synthetic peptides was assessed using the cellular and *in vitro* antioxidant assays. HepG2 cell oxidation (100 %) was induced using the oxidant AAPH (previously optimised in Kleekayai et al. (2020)). Trolox (a synthetic soluble form of Vitamin E) at 100 μM reduced ROS production to 84.40 ± 1.69 % (Fig. 5A). All peptides, with the exception of VISPEHY and EIVGDDAKVI, were able to significantly ($p < 0.05$) reduce ROS production compared to the AAPH control. VIVSPVRT showed the lowest ROS production giving a value of 82.2 ± 6.5 % followed by NDVLFF (83.2 ± 8.0 %) and EVAGLP (83.4 ± 7.0 %). These results are comparable to those reported previously for peptides identified in a soybean Alcalase hydrolysate, i.e., IYVVDLR and IYVFV,

Table 2
Peptides identified (X) in the supernatants of silkworm protein hydrolysates generated using different proteases.

Protein accession number	Protein molecule	Peptide Sequence	Hydrophobic residues (%)	Control	Alcalase	Prolyve	Flavourzyme	Brewers Clarex
P09335	Lipoprotein PBMHP-12	SWFITPF*	71.43		X			
P09338	Lipoprotein PBMHPC-23							
Q00801	Lipoprotein 4	**SWFVTPF**	71.43		**X**			
P09335	Lipoprotein PBMHP-12	**NDVLFF**	66.67		**X**			
Q00801	Lipoprotein 4							
P09338	Lipoprotein PBMHPC-23							
P09179	Sex-specific storage-protein 1	GYPFDRPIDM(+15.99)A	42.11		X			
P20613	Sex-specific storage-protein 2	SNEFVIF	57.14			X		
P20613	Sex-specific storage-protein 2	AQRPDYYNL	55.56			X		
Q00801	Lipoprotein 4	D(-18.01)RVIF	23.08		X			
P09179	Sex-specific storage-protein 1	VIVSPVRTGM(+15.99)LLPT	45.45		X			
P09179	Sex-specific storage-protein 1	M(+15.99)WNEPLETGYWPK	38.10			X		
P09336	Lipoprotein PBMHPC-19	ADSDVPNDIL	50.00			X		
Q00802	Lipoprotein 3							
P20613	Sex-specific storage-protein 2	GKIPEFS	57.14			X		
P20613	Sex-specific storage-protein 2	KEDSVPM(+15.99)TEIM (+15.99)	18.52			X		
P09179	Sex-specific storage-protein 1	IRLPSGDEM(+15.99)PVRQ	33.33		X			
P09179	Sex-specific storage-protein 1	DHILQPT	42.86		X			
P09336	Lipoprotein PBMHPC-19	AENAIKL	57.14			X		
Q00802	Lipoprotein 3							
P09179	Sex-specific storage-protein 1	**VIVSPVRTG**	66.67			**X**		
Q9NL89	Beta-1,3-glucan-binding protein	**ILLEP**	80.00				**X**	
P09179	Sex-specific storage-protein 1	IRLPSGDEM(+15.99)PVRQN	31.82		X	X		
P09179	Sex-specific storage-protein 1	IRLPSGDEMPVRQN	50.00		X			
Q00801	Lipoprotein PBMHP-12	**EVAGLP**	83.33		**X**			
P09335	Lipoprotein PBMHP-12							
P09338	Lipoprotein PBMHPC-23							
P05790	Fibroin heavy chain	**IGVGA**	100.00		**X**			
P20613	Sex-specific storage-protein 2	NTDDEYYKIG	40.00			X		
P09336	Lipoprotein PBMHPC-19	**VIGSPEHY**	62.50			**X**		
Q00802	Lipoprotein 3							
Q20616	Peroxidase skpo-1	NSVMT	40.00	X				
P20613	Sex-specific storage-protein 2	EFDAS	40.00			X		
P09336	Lipoprotein PBMHPC-19	NGRVIGSPEHY	54.55			X		
Q00802	Lipoprotein 3							
P09179	Sex-specific storage-protein 1	KITTDELVTF	40.00			X		
P09179	Sex-specific storage-protein 1	ELQMEEAVKVF	54.55			X		
P09179	Sex-specific storage-protein 1	SDKTVDAVVR	40.00		X	X		
Q00801	Lipoprotein PBMHP-12	TGDYDSA	42.86		X			
P09335	Lipoprotein PBMHP-12							
P09338	Lipoprotein PBMHPC-23							
P09336	Lipoprotein PBMHPC-19	ADSDVPNDILEEQ	38.46			X		
Q00802	Lipoprotein 3							
P09179	Sex-specific storage-protein 1	**EIVGDDAKVI**	60.00			**X**		

Where (+15.99) and (−18.01) corresponds to post-translational modifications associated with oxidation and dehydration, respectively. Peptides listed in bold were selected for further studies. * SWFITPF was synthesised but was not utilised during further studies due to its low solubility in a range of solvents potentially suitable for cell culture (being only partially soluble in formic acid).

which reduced ROS generation to 85.9 and 96.2 %, respectively in H_2O_2-induced Caco-2 cells (Zhang et al., 2019).

Fig. 5B shows the antioxidant activity of the peptides against the superoxide anion expressed as SOD activity. SWFVTPF and NDVLFF were the only peptides displaying significant SOD activity (compared to the control AAPH induced HepG2 cells) with values of 36.96 ± 7.31 and 30.43 ± 7.90 % SO inhibition. All peptide treated cells showed significantly higher GSH levels when compared to the AAPH induced HepG2

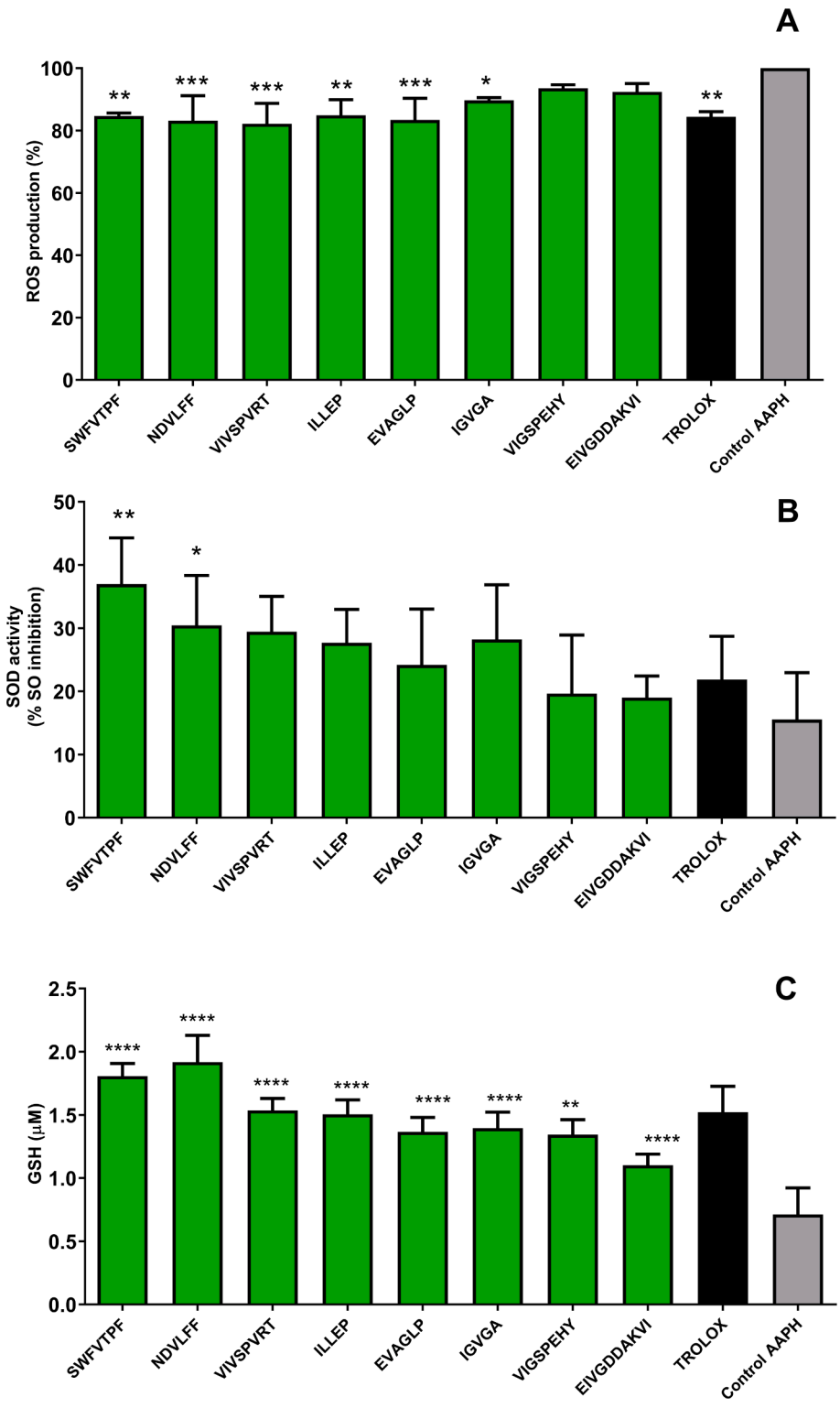

Fig. 5. Impact of treatment with peptides identified in silkworm hydrolysates and on the cellular antioxidant activity of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) induced HepG2 cells where Trolox was used as a positive control. (A) intracellular reactive oxygen species (ROS) generation, (B) superoxide dismutase (SOD) activity and (C) glutathione (GSH) production. *denotes significant differences with the control AAPH treated HepG2 cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

control (0.71 ± 0.21 μM), with values ranging from 1.10 ± 0.08 to 1.91 ± 0.21 μM (Fig. 5C). NDVLFF showed the highest extent of GSH production. Overall, SWFVTPF and NDVLFF were the peptides with highest antioxidant activity when comparing the results obtained from three cell-based assay outcomes.

The synthetic peptides were also tested using the *in vitro* antioxidant assays (FRAP, ORAC and ABTS). Overall, VIGSPEHY and SWFVTPF showed the highest activity values especially in the ORAC and ABTS assays while none of the peptides displayed FRAP activity (Fig. 6). These findings showed the discrepancy (or lack of correlation) between cellular and *in vitro* assays where the former was considered more relevant for human health. Although many peptides from silkworm proteins have been identified as angiotensin converting enzyme (ACE) inhibitors (Jia, Wu, Yan, & Gui, 2015; Li et al., 2014; Tao et al., 2017; Wang et al., 2011; Wu, Jia, Yan, Du, & Gui, 2015), to our knowledge only one previous study has reported on the antioxidant activity of silkworm peptides. Zhang et al. (2021) reported the identification of FKGPACA (from Chorion class high-cysteine HCA protein 12) and

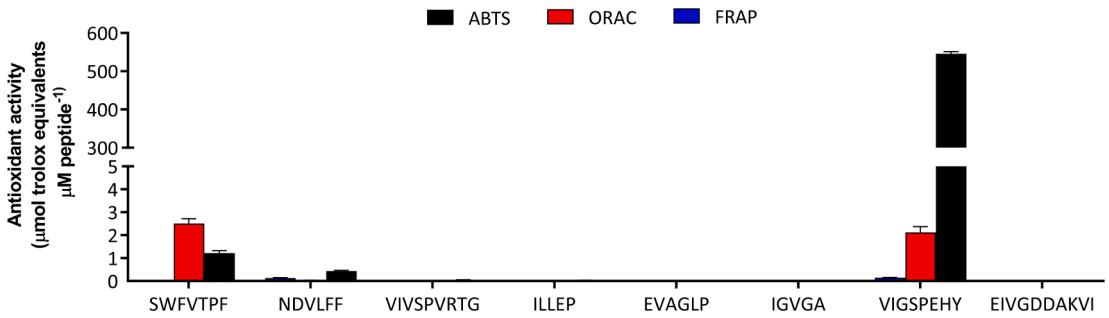

Fig. 6. *In vitro* antioxidant activity of peptides identified in silkworm hydrolysates as determined using the ferric reducing antioxidant power (FRAP), the oxygen radical absorbance capacity (ORAC) and the 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays.

SVLGTGC (from Chorion class high-cysteine HCB protein 13). These peptides demonstrated potent ABTS inhibitory activities with IC_{50} values of 0.312 ± 0.015 and 0.181 ± 0.010 mmol/L for FKGPACA and SVLGTGC, respectively. However, results using other antioxidant assays were not reported therein.

A limited number of studies appear to have identified the sequences of antioxidant peptides derived from insect proteins. A recent study reported the ability of two peptides (YK-10 and LP-10) purified from white grub larvae (*Polyphylla adstpersa*) to decrease ROS production (25.14 and 12.83 % reduction, respectively) with concentrations as low as 25 μM and with the ability to induce the activity of cellular antioxidant enzymes in lung A549 cells, i.e., SOD, catalase and GSH (Khajepour-Zaveh, Asoodeh, & Naderi-Manesh, 2020). Pattarayingsakul et al. (2017) identified CTKKHKPNC from Asian weaver ant with DPPH (IC_{50} = 48.2 ± 2.1 μM) and ABTS (IC_{50} = 38.4 ± 0.2 μM) scavenging activity. In another study, 12 peptides arising from the *in vitro* gastrointestinal digestion of *G. sigillatus, Tenebrio molitor* and *S. gregaria* were identified and tested for their antioxidant capacity (Zielińska, Baraniak, & Karaś, 2018). FDPFPK from *S. gregaria* had the highest ABTS scavenging activity (EC_{50} = 0.08 ± 0.01 mg mL^{-1}). AIGVGAIER, also from *S.gregaria,* had partial similarity to a peptide found herein, IGVGA, and had relatively high ABTS, DPPH and ferric chelating activities (EC_{50} = 1.28 ± 0.02, 0.51 ± 0.02 and 0.101 ± 0.02 mg mL^{-1}, respectively) than the other peptides tested (Zielińska et al., 2018). However, in the study herein IGVGA showed overall lower antioxidant activity than other peptides such as SWFVTPF and NDVLFF in all assays tested (Figs. 5 and 6).

6. Conclusion

The results presented in this study have shown that silkworm pupae are a rich source of antioxidant peptides which can be released by means of enzymatic hydrolysis. However, the chromatographic profiles as well as the antioxidant activity of the resultant silkworm derived hydrolysates are dependent on the enzyme preparation used. For instance, hydrolysates generated with enzymes having endoproteinase activity displayed a higher extent of hydrolysis and a final protein equivalent content than those generated with enzymes which mainly had an *exo*-proteinase activity. Furthermore, hydrolysates generated with Alcalase and Prolyve had greatest radical scavenging activity, possibly due to the higher extent of hydrolysis and therefore the generation of low molecular weight peptides, while Flavourzyme and Brewers Clarex hydrolysates possessed a higher FRAP activity. The *in situ* cellular based assay showed that the Flavourzyme hydrolysate (at 2 mg mL^{-1}) reduced ROS generation by up to 40% in HepG2 cells (compared to untreated AAPH induced cells) and was the only sample showing a significant reduction compared to the control sample (no enzyme) at that same concentration. This would indicate the potential value of *in situ* cellular assays in obtaining more physiologically relevant results and a greater understanding of the *in situ* antioxidant properties of hydrolysates/peptides.

Peptide identification within the silkworm hydrolysates was performed and 9 peptides were identified and synthesised. Overall SWFVTPF and NDVLFF had the highest antioxidant activity in AAPH induced HepG2 cells. In conclusion, the silkworm protein hydrolysates and the peptide sequences identified herein SWFVTPF and NDVLFF have potential to be used as natural antioxidants for the reduction of cellular oxidation. Further studies are required to access the stability of the peptides to gastrointestinal enzymes and their bioavailability.

However, prior to its widespread utilisation as a food ingredient, a detailed safety assessment of the production, processing and storage of silkworm-derived protein ingredients needs to be performed (EFSA, 2015).

7. Ethics statement

This research did not include biological agents of risk, or clinical trials with humans or animal experiments.

Author contributions

MC designed the study, performed the experiments, obtained and analysed the data and wrote the manuscript; CB and MAB performed experiments; MF provided sample, designed experiments and obtained the funding; DF reviewed, edited the manuscript, and secured and provided funding. The authors have read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105052.

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