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Antioxidant and functional properties of protein hydrolysates obtained from squid pen chitosan extraction effluent

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ABSTRACT

Squid pens were subjected to alkali hydrolysis to extract chitin and chitosan. Proteins present in the alkaline extraction wastewater were recovered at pH 3, 4, 5 and 6, and were subjected to hydrolysis by trypsin, pepsin and a bacterial protease called HT for 1, 2, 4 and 24 h. Hydrolysis of the extracted proteins with either trypsin or HT generated more antioxidant activity than hydrolysis with pepsin. Higher ACE-inhibitory activity was generated in the trypsin and pepsin hydrolysates than in the HT hydrolysate. Squid pen protein recovered from chitosan processing waste alkaline solution can be a potential source of bioactive peptides for addition to foods. The antioxidant and ACE-inhibitory activities of the extracted proteins were initially low and increased upon incubation with the proteases. Pepsin generated significantly lower ($P < 0.05$) antioxidant activities compared to trypsin and HT, while trypsin and pepsin hydrolysates exhibited higher ACE-inhibitory activity than HT ($P < 0.05$).

1. Introduction

Squid pen (SP), a by-product generated by the squid processing industry, is a transparent structure similar in shape to a feather, that is located dorsally within the squid mantle and is almost the same length as the mantle (Yang, Peters, Dies, & Rheinstadter, 2014). The main squid species found in New Zealand coastal waters are the arrow squids, *Nototodarus gouldi* and *Nototodarus sloanii* (Weeber, 2013). Dry SP contains mainly protein (ranging from 52 to 75 g/100 g dry weight) and chitin, with low levels of lipids, lipoproteins, and some minerals (Susana Cortizo, Berghoff, & Alessandrini, 2008). Squid pens are a cheap by-product of squid processing that are normally sold for ~$0.3/kg (Chen, Chiang, Liang, Wang, & Wang, 2012). Interest in SP chitosan, a β-chitosan, is increasing due to its unique properties compared to the α-chitosan form that is obtained from crab and shrimp shells (Shavandi, Bekhit, Bekhit, Sun, & Ali, 2015). Chemical treatment with concentrated alkali solutions (40% NaOH) is the most common method used to extract chitin from SP, which is then deacetylated to generate chitosan (Jung & Zhao, 2014). However, the concentration of the alkali extraction solution and extreme pH are problematic to the environment due to the high organic (protein) and non-organic (minerals and caustic materials) content of the alkali waste water generated the extraction of chitin. As a result of the strong alkali treatment on SP, protein components become available in the wastewater as a by-product of this process.

Previous reports have suggested that the protein remaining in the alkali wastewater is not suitable for use in animal feed due to loss of valuable amino acids (Pacheco et al., 2011). Enzymatic treatments that utilise proteases from various microbial sources have been proposed as being a more environmentally friendly extraction method. Some studies have also reported high antioxidant activities in protein hydrolysates obtained from protease treatments of shrimp shells (Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010a). The antioxidative activity of protein hydrolysates mainly depends on the amino acid sequence of generated peptides. The associated effluent generated from extraction of chitin from SP chitosan, is an environmental consideration. Although the production of chitin from SP has been well studied,

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there have been few reports on the isolation and utilisation of protein derived from the SP chitin processing effluent. The present study aimed to enhance the bioactivity of squid pen protein (SPP) extract obtained at different pH. SPP extracts were treated with different proteases to generate bioactive peptide hydrolysates that were evaluated for antioxidant, angiotensin-I converting enzyme (ACE) inhibitory activity and antibacterial activities. A flow diagram summarising the experimental design is shown in Fig. 1.

2. Materials and methods

2.1. General

Squid pens from arrow squid (*Nototodarus sloanii*) were obtained from Independent Fisheries Corporation, Christchurch, New Zealand. A bacterial metallo endopeptidase referred to as HT, that was sourced from *Bacillus subtilis*, was a gift from Enzyme Solutions Pty Ltd (Croydon South Victoria, Australia). Pepsin, Trypsin, angiotensin I-converting enzyme (ACE), o-phthaldialdehyde (OPA), sodium tetraborate, hippuryl-histidyl-leucine (HHL), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

2.2. Preparation of squid pen protein isolates (SPPI)

The protein isolation was conducted using the method of Chaussard and Domard (2004). Squid pens were first ground into powder using a coffee grinder, then added to 1 M NaOH (1:15 w/v) and shaken for 24 h (Ratek Instruments Pty Ltd, Victoria, Australia). The chitin was separated from the extraction solution by filtration. Aliquots of the extraction solution were adjusted to either pH 3, 4, 5 or 6 using 1 M HCl, which resulted in precipitation of protein from the extraction solution. The precipitated protein was then isolated from the solution by centrifugation at 10,000*g* (Beckman J2-21 centrifuge, IN, USA) for 30 min, the supernatant removed and the protein samples were freeze-dried and stored for use in subsequent experiments.

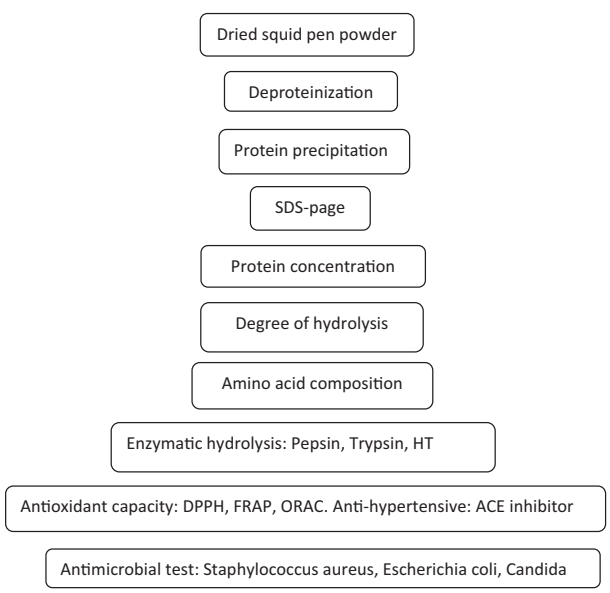

Fig. 1. The experimental design for this study.

2.3. Protease hydrolysis of SPPI

The protease hydrolysis procedure was performed according to the method described by Tang, Wang, and Yang (2009). Aliquots of SPPI (0.2 g) were separately dispersed in 10 ml of deionized water at room temperature, followed by adjustment of the pH and pre-incubation at the optimal pH and temperature of each protease. Each protease preparation was added individually to an aliquot of SPPI to achieve a protease preparation-to-substrate (P/S) ratio of 5% on a weight basis. The protease hydrolysis optimal conditions were selected based on the work of Teh, Bekhit, Carne, and Birch (2016) and were as follows: (1) pepsin: pH 2.0, 37 °C; (2) HT: pH 6.5, 45 °C; (3) trypsin: pH 8.0, 37 °C. The protease hydrolyses were conducted in an incubator shaker (Ratek, Victoria, Australia) at 200 rpm. Aliquots of the squid pen protein hydrolysates (SPPH) were sampled at 0, 1, 2, 4 and 24 h incubation time, and the protease activity in the aliquots was inhibited by heating the samples in a water bath at 90 °C for 20 min. The samples were centrifuged at 10,000*g* for 5 min, and supernatant was transferred to a new tube and stored at −20 °C.

2.4. Determination of total protein

Total protein was determined as described by Karlsson, Ostwald, Kabjorn, and Andersson (1994). An aliquot (150 µl) of each protein containing sample was added to each well of a microtitre plate followed by the addition of 100 µl of trichloroacetic acid (TCA) (60% w/v). The plate was incubated for 20 min at 25 °C and the absorbance was measured at 570 nm in a microplate reader. A range of bovine serum albumin (BSA) concentrations (0–2% w/v in deionized water) were used to construct a standard curve.

2.5. Degree of hydrolysis (DH) determination

The degree of hydrolysis of SPPH was determined using an o-phthaldialdehyde (OPA) assay as described by Zhu et al. (2009). The OPA reagent consisted of 2.5 ml SDS (20% w/v), 25 ml sodium tetraborate (100 mM), OPA (40 mg, dissolved in 1 ml methanol), 100 µl 2-mercaptoethanol and distilled water to make the volume up to 50 ml, in a volumetric flask. Aliquots (20 µl) of the SPPH samples and 150 µl OPA reagent were added to each well of a microtitre plate, and the plate was incubated at 25 °C for 2 min. The absorbance was read in a plate reader at 340 nm. OPA reagent reacts with the free N-termini corresponding to the release of peptides on hydrolysis and therefore the absorbance increases as the degree of hydrolysis increases. Leucine (0–0.4 mol/ml) was used to construct the standard curve. The results are presented as Leu equivalents (mol/ml).

2.6. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity

The DPPH radical scavenging activity assay was performed according to de Ancos, Sgroppo, Plaza, and Cano (2002) using a microtitre plate. A 10 µl sample (4 mg/ml) and 90 µl deionized water were added in each well of the microtitre plate. Then a 100 µl of DPPH methanolic solution (78 mg/l) was added under dim light conditions. The microtitre plate was incubated at 25 °C for 30 min and then the absorbance was read at 517 nm. Methanol (100 µl) and deionized water (100 µl) were added to one well of the microtitre plate and used as a control. The DPPH scavenging activity was calculated using the following Eq. (1):

$$\text{DPPH inhibition} = \left(1 - \frac{\text{test sample absorbance}}{\text{blank sample absorbance}}\right) \times 100\% \quad (1)$$