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Production of bioactive peptide hydrolysates from deer, sheep and pig plasma using plant and fungal protease preparations

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ABSTRACT

Plasma separated from deer, sheep and pig blood, obtained from abattoirs, was hydrolysed using protease preparations from plant (papain and bromelain) and fungal (FP400 and FPII) sources. Antioxidant and antimicrobial activities of the peptide hydrolysates obtained after 1, 2, 4 and 24 h of hydrolysis, were investigated. The release of trichloroacetic acid-soluble peptides over the hydrolysis period was monitored using the o-phthaldialdehyde (OPA) assay, while the hydrolysis profiles were visualised using SDS–PAGE. The major plasma proteins in the animal plasmas were identified using MALDI-TOF–TOF MS. Hydrolysates of plasma generated with fungal proteases exhibited higher DPPH radical-scavenging, oxygen radical-scavenging capacity (ORAC) and ferric reducing antioxidant power (FRAP) than those generated with plant proteases for all three animal plasmas. No antimicrobial activity was detected in the hydrolysates. The results indicated that proteolytic hydrolysis of animal blood plasmas, using fungal protease preparations in particular, produces hydrolysates with high antioxidant properties.

1. Introduction

The meat industry produces substantial amounts of under-utilised by-products, such as blood, tendons and trimmings, which currently contribute negligible economic value to the industry. Moreover, the disposal of such undesirable animal by-products, such as blood, may negatively affect the environment due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (Dàvila Ribot, 2007). Consequently, there is a need for innovative uses and new strategies to add value to under-utilised animal by-products, including blood, for better economic and environmental sustainability. One method for converting these by-products into a broad assortment of valuable ingredients or industrial products with a wider range of applications is achieved by using proteases to produce peptide hydrolysates of the proteins present in the by-products.

The peptides present in protein hydrolysates have attracted increasing attention because of their potential functional properties and health-promoting bioactivities (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). In some cases, the bioactive peptides produced during proteolytic hydrolysis can exert multifunctional effects, such as antioxidative, antimicrobial, antihypertensive, antithrombotic, anticancer, immune-modulatory or opioid activities (Korhonen & Pihlanto, 2003).

The activities of protein hydrolysates are dependent on several factors, such as (i) the type of protease used, (ii) the hydrolysis conditions and (iii) the amino acid composition, sequence and configuration of peptides (Tavano, 2013). It is generally recognised that the type of protease used in the hydrolysis process will have the main impact on the bioactivity, such as antioxidant and antimicrobial activity (Bah, Bekhit, Carne, & McConnell, 2013). Previously, pepsin and trypsin proteases have been used to simulate the proteolysis conditions present in the gastrointestinal digestive system, with the intent of generating peptide profiles that resemble the products of the normal physiological digestion process. However, the peptides generated by these endogenous gastrointestinal proteases are limited by the specific cleavage site specificity of the protease. Therefore, the hydrolytic specificity of the endogenous proteases may not be optimal for the release of bioactive peptides (Udenigwe & Howard, 2013). Exogenous proteases offer the potential to generate different peptides from a protein due to their different and often broader cleavage site specificity, which can maximise the potential for generating peptides of various sizes and functionalities.

Animal blood, collected from abattoirs, is a valuable protein source from which peptide hydrolysates can be produced.

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Compared to the considerable number of studies carried out on the hydrolysis of milk, meat, fish and plant by-products, only a small number of studies have investigated the hydrolysis of animal blood proteins. Among those studies, the majority of hydrolysates exhibiting antioxidant bioactivity have been generated from porcine plasma using either trypsin (Wei & Chiang, 2009), pepsin (Xu, Cao, He, & Yang, 2009), chymotrypsin (Wei & Chiang, 2009), papain (Xu et al., 2009) or Alcalase (Liu, Kong, Xiong, & Xia, 2010).

Conversion of under-utilised blood by-products, into bioactive protein hydrolysates, with potential use in the nutraceutical and pharmaceutical industries, could improve the economics of animal agriculture and add value to a low cost by-product stream. In this study, hydrolysates were generated from deer, sheep and pig plasma, using protease preparations from plant (papain, bromelain) and fungal (FP400, FPII) sources. The first objective of this work was to compare the proteolysis of deer, sheep and pig plasma by the four protease preparations over time, and characterise the yield of soluble peptides. The second objective was to characterise the antioxidant and antimicrobial activities *in vitro* of the peptide hydrolysates obtained.

To our knowledge no previous studies have reported the use of fungal proteases in the hydrolysis of animal plasma and generation of hydrolysates with antioxidative activities. The findings from this work contribute towards the understanding of protease selection and hydrolysis conditions for the production of hydrolysates containing bioactive peptides, and the subsequent identification of bioactive peptide amino acid sequences.

2. Materials and methods

2.1. Blood collection and separation

Fresh deer, sheep and pig blood were obtained from local meat processing facilities. The blood samples collected for each species were pooled samples obtained from the abattoir blood waste stream. The samples were in triplicate. Red deer (*Cervus elaphus*) were of 15–16 months age, sheep (Perendale) were 10–11 months old, and pigs (Duroc) were 5–6 months old. Blood was collected with sodium citrate (10 g/l) added to prevent coagulation and centrifuged at 2060*g*, 4 °C for 15 min to separate the plasma fraction from the blood cell fraction. The plasma fraction was collected, lyophilised and stored at −20 °C until use.

2.2. Chemicals

All chemicals used were of analytical reagent grade, unless otherwise stated. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein sodium salt, ferrous sulphate heptahydrate, sodium citrate, were obtained from Sigma–Aldrich (Missouri, USA). Methanol was obtained from LabServ®, Pronalys Chemicals (Scoresby, Australia). Ferric chloride hexahydrate was from AnalaR®, BDH Chemicals Ltd (Poole, England). 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Cayman Chemicals (Michigan, USA). Trichloroacetic acid (TCA) was from Ajax Finechem (Sydney, Australia). Papain concentrate (a papaya latex powder preparation with 50,000 food chemical codex papain units (FCC PU)/mg), bromelain concentrate (a pineapple fruit stem powder preparation with an activity of NLT 2100 bromelain tyrosine units/g), fungal protease concentrate 400 (FP400 – a peptide peptidohydrolase derived from *Aspergillus oryzae*), and fungal protease II (FPII, an protease preparation derived from *A. oryzae*), were provided by Enzyme Solutions Pty. Ltd. (Melbourne, Australia).

2.3. Enzymatic hydrolysis of deer, sheep and pig plasma

Freeze-dried deer, sheep and pig plasma were each individually reconstituted in phosphate buffer at a protein concentration of 20 mg/ml and pre-incubated at the temperatures stated below, prior to the addition of the protease. The hydrolysis of each animal plasma by various proteases was carried out according to the following reaction conditions as determined by preliminary experiments and manufacturer's guidelines: (i) *papain:* at pH 6.5 and 55 °C; (ii) *bromelain:* at pH 6.5 and 50 °C; (iii) *FP400:* at pH 6.5 and 50 °C; (iv) *FPII:* at pH 6.5 and 50 °C. The protease preparation-to-substrate ratio for all hydrolysis treatments was 1:10 on a weight basis. The animal plasma samples containing protease preparations were incubated in a temperature-controlled incubator shaker. Controls of protease only and plasma only for each protease and animal species were also incubated concurrently. At each hydrolysis sampling time point (1, 2, 4 and 24 h), aliquots of the hydrolysates were subjected to heat treatment at 90 °C for 20 min, then cooled immediately in ice water. Any insoluble material was removed by centrifuging (2800*g*, 20 min) and the clarified supernatants were stored at −20 °C until further analysis. Aliquots of samples were also collected separately for soluble peptide analysis and SDS–PAGE as described in the following sections.

2.4. Measurement of soluble peptides using the OPA assay

Soluble peptides in the hydrolysates were measured using the o-phthaldialdehyde (OPA) assay following the method of Church, Porter, Catignani, and Swaisgood (1985) as described by Nielsen, Petersen, and Dambmann (2001) and Opatha Vithana, Mason, Bekhit, and Morton (2012). The OPA reagent contained 2.5 ml of 20% (w/v) SDS, 25 ml of 100 mM sodium tetraborate, 40 mg OPA (pre-dissolved in 1 ml methanol), 100 µl of 2-mercaptoethanol and distilled water made to a total volume of 50 ml. Hydrolysates were sampled at various time points with immediate addition of 3 parts 0.75 M trichloroacetic acid at 4 °C for 30 min and then centrifuged (4000*g*, 10 min) to obtain the TCA soluble peptide fraction. An aliquot of the supernatant was added to the OPA reagent (ratio 2:15), mixed, and after incubation at room temperature for 2 min, the absorbance at 340 nm was measured. The number of amino groups was determined with reference to a L-serine standard curve (Nielsen et al., 2001) and the proteolytic activity was expressed as µM L-serine equivalent.

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Plasma hydrolysates were displayed by 1D-SDS–PAGE using Bolt™ gradient (4–12%) Bis–Tris gels (Life Technologies, Auckland, NZ). A 2 µl aliquot of fourfold diluted hydrolysate obtained at various hydrolysis time points was mixed with 3.8 µl of Bolt sample buffer, 1.5 µl of Bolt reducing agent and 8 µl of Milli-Q water. The samples were incubated at 90 °C for 5 min prior to being loaded on a gel. Electrophoresis was performed in Bolt running buffer (1×) at 164 V for 34 min at room temperature. Protein standards (Novex sharp pre-stained protein standard, Life Technologies, New Zealand) was included on the gel as a molecular marker. After electrophoresis, the gels were stained using SimplyBlue™ SafeStain (Life Technologies) and then de-stained with Milli-Q water.

2.6. MALDI-TOF/TOF identification of plasma proteins

Selected SDS–PAGE bands as identified in Fig. 2(a–h) were excised from the gel and subjected to in-gel digestion with trypsin. Matrix-assisted laser-desorption ionisation–time-of-flight tandem