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In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions

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ABSTRACT

Chicken thigh and breast skin proteins were hydrolysed using alcalase or a combination of pepsin and pancreatin (PP), each at concentrations of 1–4%. The chicken skin protein hydrolysates (CSPHs) were then fractionated by membrane ultrafiltration into different molecular weight peptides (<1, 1–3, 3–5 and 5–10 kDa) and analysed for antioxidant properties. Results showed that the CSPHs had a significantly ($p < 0.05$) lower scavenging activity against DPPH radicals when compared to reduced glutathione. The chicken breast skin hydrolysates had significantly higher DPPH scavenging activity than the chicken thigh skin hydrolysates. DPPH scavenging and metal ion chelation increased significantly ($p < 0.05$) from 29–40% to 86–89%, respectively with increasing proteolytic enzyme concentration. In contrast, the antioxidant properties decreased as peptide size increased. We conclude that CSPHs and their peptide fractions may be used as ingredients in the formulation of functional foods and nutraceuticals for the control and management of oxidative stress-related diseases.

1. Introduction

Erdmann, Cheung, and Schroder (2008) defined biologically active peptides as "food-derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in humans". Bioactive peptides consist of natural amino acid sequences (often 2–20 residues) encrypted in the parent or natural protein molecule, and are usually inactive within the sequence of the protein. They are, however, released during gastrointestinal digestion or *in vitro* protein hydrolysis with proteases and play important roles in the regulation and modulation of metabolism during digestion of food in the intestine. Thus, bioactive peptides have the potential of being metabolic aid supplements, in the form of nutraceuticals and functional food ingredients for the promotion of health and prevention of diseases (Bernardini et al., 2011).

Bioactive peptides have been isolated from various food sources such as milk and whey (Erdmann, Cheung, & Schroder, 2008), meat and fish (Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012; Samanarayaka, Kitts, & Li-Chan, 2010) and quinoa seeds (Aluko & Monu, 2003). The potential metabolic regulatory effects of bioactive peptides relate to nutrient uptake, antihypertensive, antioxidant, anticancer, antithrombotic, opioid or antiproliferative as well as antimicrobial activities (Erdmann et al., 2008; Samanarayaka, Kitts, & Li-Chan, 2010; Udenigwe & Aluko, 2012). Many of the known bioactive peptides exhibit multifunctional properties, are easily absorbed and could be used to reduce symptoms of oxidative stress, hypertension and dyslipidemia, which are all risk factors of coronary heart disease (Erdmann et al., 2008; Lee, Qian, & Kim, 2010; Samanarayaka et al., 2010). Of particular interest to human health is the uncontrolled production of free radicals (superoxide, hydroxyl, singlet oxygen, peroxyl) during cellular metabolism/oxidation, which leads to oxidative stress. Oxidative stress has been implicated in the initiation or progression of many vascular diseases due to extensive damage of critically important biological polymers such as DNA, proteins and lipids (Erdmann et al., 2008). The toxic free radicals can also modify low density lipoprotein (LDL), which may lead to increased atherogenicity of oxidized LDL (Erdmann et al., 2008). This in turn can be a causative factor in many terminal degenerative diseases such as cardiovascular disease, diabetes, cancer, Alzheimer's disease and a host of other conditions (Bernardini et al., 2011; Erdmann et al., 2008; Naqash & Nazeer, 2011; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

Antioxidants play an important role in human health and nutrition as they are known to protect the body against reactive oxygen species (ROS) (Martinez-Maqueda et al., 2012; Ryan et al., 2011). The use of bioactive peptides as antioxidative agents is generating interest not only as natural alternatives to synthetic antioxidants, but for their beneficial effects in terms of health implications,

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non-residual side effects and their functionality in food systems (Bernardini et al., 2011; Erdmann et al., 2008; Girgih, Udenigwe, & Aluko, 2010; Ryan et al., 2011). The ability of endogenous enzymatic antioxidants (catalase, superoxide dismutase and glutathione peroxidase) to regulate this process is often weakened when excess free radicals are produced beyond cellular antioxidant capacity.

Food-derived bioactive peptides with antioxidant properties have been reported in several foods, such as milk and eggs (Erdmann et al., 2008), fish and a few domestic animal muscles (Bernardini et al., 2011; Ryan et al., 2011). They have also been isolated from poultry viscera protein hydrolysate (Jamdar, Rajalakshmi & Sharma, 2012) and flying fish backbone (Naqash & Nazeer, 2011). However, information on the antioxidant properties of chicken skin protein hydrolysates is scanty. Chicken skin is a by-product derived from chicken meat processing which is highly underutilized, constituting huge cost for waste disposal and danger to the environment as well as the loss of nutritional value (Feddern et al., 2010). Several attempts have previously been made at developing novel chicken skin based products in order to diversify the utilisation of chicken skin as well as reduce waste, such as chicken meat balls (Bhat, Kumar, & Kumar, 2011), collagen (Bonifer & Froning, 1996; Cliché, Amiot, & Gariepy, 2003), sausages (Biswas, Chakraborty, Sarkar, Barpuzari, & Barpuzari, 2007) and chicken meat frankfurter (Babji, Chin, Chempaka, & Alina, 1998). However, an area of research that is yet to be explored is the development of chicken skin based products with functional and health promoting values. The high protein content (dry weight basis) could, in addition to contributing to nutrition, also serve as a very active source of value-added products, including bioactive peptide-containing hydrolysates. Therefore, the objective of this study was to determine the effects of muscle source as well as type and level of protease on the *in vitro* antioxidative properties of chicken skin enzymatic hydrolysates and their ultrafiltration membrane peptide fractions.

2. Materials and methods

2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas, EC 232-468-9), alcalase (from fermentation of *Bacillus licheniformis*, 3.4.21.62), trinitrobenzene sulfonic acid (TNBS), Triton X-100, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), potassium ferricyanide, ferrous sulphate, ferrous chloride, 1,10-phenanthroline, reduced glutathione (GSH) and ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2.4-tiazine-4′,4″-disulfide acid sodium salt were purchased from Sigma–Aldrich (St. Louis, MO). All other analytical grade reagents and ultrafiltration membranes (1, 3, 5 and 10 kDa molecular weight cut-offs) were purchased from Fisher Scientific (Oakville, ON, Canada).

2.2. Preparation of chicken skin protein hydrolysates (CSPH)

Fresh thigh or breast chicken skins (approximately 250 g) were packed in freeze drying plates, frozen at −20 °C for 24 h and transferred to −80 °C for 6 h prior to freeze drying. The freeze dried samples were shredded manually and defatted repeatedly by mixing ~1 g of sample with 10 ml acetone (Fisher Scientific, Oakville, ON, Canada). The mixture was stirred in the fume hood for 3 h and decanted manually followed by two additional consecutive extractions of the residue. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Waring blender to produce a fine powder that was stored at −20 °C. For the initial screening test to optimize and select the best enzyme concentration, dried chicken skin powder from the thigh or breast muscles was mixed with water to give 5% (w/v, protein basis) slurries. Two different enzyme treatments (alcalase and pepsin + pancreatin) were separately used for sample hydrolysis. For the alcalase hydrolysis, the slurry was heated to 55 °C, adjusted to pH 8.0 using 2 M NaOH and the hydrolysis initiated by the addition of alcalase enzyme (1–4% w/w, skin protein basis). Each mixture was stirred continuously for 4 h. For the pepsin + pancreatin (PP) hydrolysis, the slurry was heated to 37 °C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of pepsin enzyme (1–4% w/w, skin protein basis). The mixture then stirred continuously for 2 h. After the peptic hydrolysis, the reaction mixture was adjusted to a pH of 7.5 with 2 M NaOH, pancreatin was added (1–4% w/w, skin protein basis) and incubated at 37 °C for 4 h with continuous stirring. At the end of the incubation periods, both the alcalase and PP reactions were terminated by heating at 95 °C for 15 min to ensure complete denaturation of residual enzymes. The mixtures were thereafter centrifuged (7000*g* at 4 °C) for 1 h and the resulting supernatant lyophilized and stored at −20 °C as the chicken thigh skin hydrolysate (CTSH) or chicken breast skin hydrolysate (CBSH) until needed for further analysis. The most active hydrolysate from each enzymatic treatment was subsequently fractionated by sequentially passing the supernatant through ultrafiltration membranes with molecular cut-offs (MWCO) of 1, 3, 5 and 10 kDa in an Amicon stirred ultrafiltration cell. Starting with 1 kDa MWCO, the retentate from each membrane was passed through the next higher MWCO membrane while permeates from each membrane (1, 3, 5 and 10 kDa MWCO) was collected, lyophilized and stored at −20 °C as <1, 1–3, 3–5 and 5–10 kDa fractions respectively, until required for analysis. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell Haas, Biebar, & Tolbert, 1978). The above digestion and fractionation protocols were performed in triplicates and the lyophilized samples combined, analysed for protein content and used for the antioxidative assays.

2.3. Amino acid composition analysis

The amino acid profiles of the defatted chicken thigh skin (CTS), chicken breast skin (CBS) and chicken skin protein hydrolysates from the thigh and breast muscles (CTSH and CBSH) samples were determined using the HPLC system after samples were hydrolysed with 6 M HCl according to the method of Bidlingmeyer, Cohen, and Tarvin (1984). The cysteine and methionine contents were determined after performing acid oxidation according to the method of Gehrke, Wall, Absheer, Kaiser, and Zumwalt (1985). The tryptophan content however, was determined after alkaline hydrolysis by the method of Landryl and Delhaye (1992).

2.4. DPPH radical scavenging assay

The scavenging activity of CSPHs and membrane ultrafiltration fractions against DPPH radical was determined according to the method described by Aluko and Monu (2003) with slight modifications for a 96-well clear flat-bottom plate. Peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 μM. Peptide samples (100 μl) were mixed (final assay concentration of 1 mg/ml) with 100 μl of the DPPH solution in the 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance values of the blank (Ab) and samples (As) were measured at 517 nm. The blank consisted of sodium phosphate buffer in place of the peptide sample while GSH was used as a