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Gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme: Antioxidant activity and its potential in model systems

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ABSTRACT

Antioxidant activities of gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme with different degrees of hydrolysis (DHs: 10%, 20%, 30% and 40%) were evaluated. All antioxidant activity indices of hydrolysates increased with increasing DH ($P < 0.05$). When gelatin hydrolysate with 40%DH was determined for its pH and thermal stability, ORAC and chelating activity remained constant or slightly increased in a wide pH range (1–9) and during heating (100 °C) for 240 min. It was also stable in simulated gastrointestinal tract model system. Moreover, gelatin hydrolysate at a level of 500 and 1000 ppm could inhibit lipid oxidation in both β-carotene linoleate and cooked comminuted pork model systems. Therefore, gelatin hydrolysate from blacktip shark skin (40%DH) can potentially be used as an alternative source of natural antioxidants.

1. Introduction

Blacktip shark (*Carcharhinus limbatus*) has been used for shark fin and fillet production in Thailand. Shark skin is one of the potential sources for gelatin (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). Recently, gelatin hydrolysate from bigeye snapper skin, prepared using fish pyloric caeca, has been reported to possess antioxidant activity including DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (Khantaphant & Benjakul, 2008; Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010). Mendis, Rajapakse, and Kim (2005) reported that Gly and Pro in peptide from gelatin hydrolysate of hoki skin prepared with the aid of trypsin determined the radical scavenging activity. In addition, a peptide isolated from gelatin hydrolysate of Alaska pollack skin prepared using two-step hydrolysis with Alcalase and Pronase E, respectively, which had Gly-Glu-Hyp at N-terminus, showed high antioxidant activity (Kim et al., 2001). Nevertheless, gelaitn hydrolysate generally has a low degree of hydrolysis (DH), possibly due to the molecular constraint associated with its amino acid sequence/composition. Gelatin with a glycine content of around 33% might not be a preferable substrate for proteases. Glycyl endopeptidase is one of the four papaya cysteine proteinases, including papain, glycyl endopeptidase, chymopapain and caricain. It is a major component which constitutes almost 30% of total protein in the latex of *Carica papaya* and has high specificity to cleave only peptide bonds with Gly at P_1 (Buttle, 1994). Thus, papaya latex, which is cheap and available in tropical countries, can serve as a potential protease for production of gelatin hydrolysate with high DH. Nevertheless, no information regarding gelatin hydrolysate from blacktip shark skin with high DH and their antioxidant activity has been reported. Therefore, this investigation aimed to produce gelatin hydrolysate with different DHs and study their antioxidant activity and stability. The use of gelatin hydrolysate in different model systems was also studied.

2. Materials and methods

2.1. Chemicals/enzymes

All chemicals were of analytical grade. 2,4,6-Trinitrobenzenesulphonic acid (TNBS), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), *N*,*N*-dimethyl *p*-nitrosoaniline (DPN), histidine, sodium hypochlorite (NaOCl), taurine,

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potassium iodide (KI), fluorescein, 2,2′-azobis(2-methylpropionamidine (AAPH), trolox, pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas, trypsin from bovine pancreas (EC 3.4.21.4), bile extract porcine, β-carotene and Tween® 40 were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Linoleic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA).

2.2. Preparation of gelatin hydrolysate from blacktip shark skin

2.2.1. Shark skin preparation

Blacktip shark (*C. limbatus*) skin was obtained from Blue Ocean Food Products Co., Ltd. in Samutsakhon province, Thailand. The shark skin was prepared according to the method of Kittiphattanabawon et al. (2010).

2.2.2. Extraction of gelatin

Gelatin from shark skin was extracted with distilled water at 45 °C for 6 h following the method of Kittiphattanabawon et al. (2010). The resulting gelatin was used for the preparation of gelatin hydrolysate.

2.2.3. Preparation of crude enzyme from papaya (Carica papaya) latex

Fresh latex was collected from locally grown papaya in Hat Yai, Thailand. Four to six longitudinal incisions were made on the unripen papaya fruit using a stainless steel knife. The exuded latex was collected using a receiving container. The latex was then transferred to a beaker and stored below 10 °C and used within 3 h.

To prepare crude enzyme, the latex was mixed with cold distilled water (⩽4 °C) at a latex to water ratio of 1:1 (w/v). The mixture was gently stirred at 4 °C for 1 h. Then, the mixture was centrifuged at 9,000*g* at 4 °C for 20 min using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA). The supernatant was filtered using a Whatman No.1 filter paper, followed by freeze-drying. The resulting powder referred to as "crude enzyme from papaya latex" was kept at −40 °C until use. It contained 0.92 g protein/g as determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

The proteolytic activity of prepared crude enzyme was 6.03 U/mg protein. The activity was assayed using casein as a substrate at pH 7.5 and 40 °C according to the method of Khantaphant and Benjakul (2008). After termination of enzymatic reaction, the oligopeptide content in the supernatant was measured as per the Lowry method (Lowry et al., 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 μmol of tyrosine per min (μmol Tyr/min).

2.2.4. Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

Gelatin hydrolysates with different DHs from shark skin were prepared. Gelatin (3 g) was dissolved in 80 ml of distilled water. The pH of mixture was adjusted to 7.5 with 1 M NaOH. The volume of solution was made up to 100 ml by distilled water previously adjusted to pH 7.5 to obtain a protein concentration of 3% (w/v). The hydrolysis reaction was started by the addition of the crude enzyme from papaya latex at the amounts of 0.46, 1.62, 5.18 and 15.23 g/100 g protein, which were calculated from the plot between log (enzyme concentration) and DH to obtain DH of 10%, 20%, 30% and 40%, respectively (Benjakul & Morrissey, 1997). After 1 h of hydrolysis at 40 °C, the enzyme was inactivated by heating at 90 °C for 15 min in a temperature controlled water bath (model W350, Memmert, Schwabach, Germany). The mixture was then centrifuged at 5000*g* at room temperature for 10 min. The supernatant was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained powder referred to as "gelatin hydrolysate" was placed in polyethylene bag and stored at −20 °C until use.

2.3. Determination of antioxidant activities

2.3.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was determined as described by Binsan et al. (2008). A standard curve was prepared using trolox in the range of 0–50 μM. The activity was expressed as μmol trolox equivalents (TE)/g sample.

2.3.2. ABTS radical scavenging activity

The ABTS radical scavenging activity was determined as described by Binsan et al. (2008). A standard curve of trolox ranging from 0 to 500 μM was prepared. The activity was expressed as μmol trolox equivalents (TE)/g sample.

2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined using the electron paramagnetic resonance (EPR) spectrometric method as described by Madhujith and Shahidi (2008) with slight modifications. The samples were dissolved in deionised water to obtain final concentration of 10 mg/ml. The sample (100 μl) was mixed with 10 mM H_2O_2 (300 μl) and 17.6 mM DMPO (200 μl). The reaction was initiated by adding 450 μl of 100 μM $FeSO_4$. The mixture was left at room temperature for 1 min. The mixture was injected to the sample cavity of a Bruker E-scan (Bruker Biospin Co., Billercia, MA, USA) and then the spectrum was recorded by the software of the equipment using the following parameters: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Deionised water was used as the control. The EPR spectra were plotted between the EPR signals and the magnetic field. The amplitude of the second peak of the spectrum represented the amount of DMPO-OH adducts, which related with the amount of hydroxyl radicals scavenged. Trolox (0–50 μM) was used as the standard. The hydroxyl radical scavenging activity was expressed as μmol trolox equivalents (TE)/g sample.

2.3.4. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) was determined using the method of Madhujith and Shahidi (2007) with slight modifications. The samples were dissolved in 75 mM phosphate buffer (pH 7.0) to obtain a final concentration of 0.1 mg/ml. The prepared sample (20 μl) was loaded onto black polystyrene, non-treated 96-well microplate (Costar Corning Inc., Corning, NY, USA). Only the internal wells of the microplate were used. The loaded microplate was inserted to a FLUOstar OPTIMA microplate reader (BMG Labtechnologies GmbH, Offenberg, Germany) equipped with FLUOstar OPTIMA evaluation software version 1.30-0. The samples were equilibrated at 37 °C for 10 min. Then 200 μl of 0.11 μM fluorescein dissolved in 75 mM phosphate buffer (pH 7.0) were automatically injected to the sample at the first cycle. The reaction was started at the second cycle by automatic injection of 75 μl of 60 mM AAPH. The reaction was performed at 37 °C. The fluorescence intensity was measured every 150 s for 62 cycles with excitation and emission filters of 485 and 520 nm, respectively. A gain adjustment was performed by pipetting 200 μl of fluorescein (0.11 μM) onto a designated well before starting the program to optimise signal amplification. The control was prepared in the same manner, except that 75 mM phosphate buffer (pH 7.0) was used instead of the sample. The kinetic curve (AUC) of the samples was plotted between fluorescence intensity and the number of cycles. Area below the curve directly relates