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Isolation and identification of novel antioxidant and antimicrobial oligopeptides from enzymatically hydrolyzed anchovy fish meal

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ABSTRACT

Natural Antioxidants and antimicrobials are preferred to prolong food shelf life, therefore we isolated and identified novel natural antioxidant and antimicrobial oligopeptides from anchovy fish meal hydrolysate. Firstly, the oligopeptide mixture was prepared by two-step hydrolysis using alkaline and neutral proteases step by step for enriching oligopeptides. Then, four novel oligopeptide fractions were purified using reversed phase-high performance liquid chromatography, and their sequences were determined using high performance liquid chromatography-time of flight-mass spectrometry. Meanwhile, antioxidant and antimicrobial activities of the fractions were evaluated. Thr-Pro-Ser-Ala-Gly-Lys exhibited the highest hydroxyl scavenging activity, lipid peroxidation inhibition, ferrous ion chelating activity as well as *Escherichia coli* growth inhibition. Thr-Pro-Ser-Asn-Leu-Gly-Gly-Lys showed the highest superoxide scavenging activity, lipid peroxidation inhibition, and *Staphylococcus aureus* growth inhibition. Leu-Glu and Leu-Glu-Glu displayed similarly high inhibition activity on 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid. Almost no suppression effects on *Aspergillus flavus* and *Candida albicans* growth were observed for all isolated fractions. These results indicate that the oligopeptide mixture derived from anchovy fish meal could serve as a suitable source of antioxidant and antimicrobial oligopeptides.

1. Introduction

Food shelf life is limited by food deterioration, which is mainly caused by oxidative rancidity and microbial spoilage during processing and storage [1,2]. Some synthetic antioxidants and antimicrobials such as butylated hydroxyanisole and potassium sorbate have been added to various foodstuffs as functional food ingredients to extend shelf life [3,4]. Nevertheless, use of synthetic compounds is restricted compared to natural molecules, because many are suspected to negatively affect health after long-term consumption [5]. In addition, with the improvement in living standards and food safety consciousness, natural functional food ingredients are increasingly favored by consumers. Hence, there has been a massive motivation for researchers to discover natural antioxidants and antimicrobials to replace or partly replace synthetic compounds used in the food industry.

Antioxidant and antimicrobial oligopeptides are bioactive short peptides composed of 2–10 amino acid residues and less than 1000 Da molecular weight [6–8]. As natural functional food ingredients, antioxidant and antimicrobial oligopeptides derived from natural protein sources, such as pecan meal [9], sea cucumber [10] and Andrias davidianus blood [11], are superior to synthetic compounds, whether in nutrition value or safety, and have, therefore, a good market prospect in the food industry. Enzymatic hydrolysates of marine fish protein were shown to be a promising source of antioxidant and antimicrobial oligopeptides [12,13], and an increasing number of these compounds were recently discovered. For instance, Chi et al. [14] isolated three antioxidant oligopeptide fractions from protein hydrolysates of bluefin leatherjacket heads, which exhibit scavenging activity on DPPH, hydroxyl, and ABTS radical assays. Similarly, Nadia et al. [15] purified four antibacterial oligopeptide fractions from protein hydrolysates of Atlantic mackerel by-products, which are active against *Listeria innocua* and *Escherichia coli*. However, few studies have so far been conducted on antioxidant and antimicrobial oligopeptides generated from anchovy fish meal (AFM).

AFM, the by-product of anchovy fish oil processing, is produced from anchovy fishes that are small, lack edible value, but are abundant in the ocean [16,17]. Due to their high protein content and well-balanced amino acid profile, the overwhelming majority of AFM produced

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from fish meal processing enterprises is used for aquaculture feeds [18]. Nevertheless, the potential utilization of AFM for functional food ingredients is limited. However, discovery of antioxidant and antimicrobial oligopeptides from enzymatically hydrolyzed AFM has not been reported. Therefore, in this study, we isolated and identified novel antioxidant and antimicrobial oligopeptides from anchovy fish meal hydrolysate. Our study will thus provide new potential applications of anchovy fish protein and enhance its added value for fish meal processing enterprises.

2. Material and methods

2.1. Materials

AFM was provided by Zhonghai Ocean Sci-Tech (Qingdao, China). Alkaline protease NS37071 and neutral protease 0.8 L were presented by Novozymes (China) Biotech (Tianjin, China). Acetonitrile (chromatographic grade) was purchased from Sangon Biotech (Shanghai, China). Standards cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da) were purchased from Sigma Chemical (St. Louis, USA). Amino acid mixture standard was purchased from Wako Pure Chemical (Osaka, Japan). Reagents were of analytical grade.

2.2. Preparation of AFM oligopeptide

AFM was mixed with distilled water at the ratio of 1:10 (w/w) and maintained at 55 °C using water bath, then the initial pH was adjusted to 8.0 using sodium hydroxide. Alkaline protease NS37071 was then added to the mixture and stirring was performed for 2 h. Next, neutral protease 0.8 L was added to the mixture and stirring was continued for another 3 h. The enzymatic hydrolysis was ended by heating in boiling water (100 °C) for 10 min, and the supernatant was collected for subsequent desalination of the resulting AFM hydrolysate by centrifugation at 8000 × *g* for 10 min.

Desalination of the AFM hydrolysate supernatant was conducted using a 150-Da molecular weight spiral-wound nanofiltration membrane (General Electric, Fairfield, USA). After a 2 h separation at 0.8 MP and 40 °C, the trapped fluid was lyophilized into powder, referred to as AFM oligopeptide, and stored at −20 °C until use.

2.3. Chemical composition and molecular weight distribution assays

Basic chemical constituents such as protein, lipid, ash, and moisture were determined according to the corresponding methods from the Association of Official Analytical Chemists [19]. Oligopeptide content was determined using the method of Cai et al. [6], which is calculated as total content of trichloroacetic acid-soluble proteins minus free amino acids content. Amino acid composition was measured using the method of Yang et al. [20] with an automatic amino acid analyzer (Hitachi, Tokyo, Japan).

Molecular weight distribution was analyzed according to the method of Tang et al. [21], with some modifications. In brief, the AFM oligopeptide was dissolved in the mobile phase of acetonitrile/water (45:55, v/v) in the presence of 0.1% trifluoroacetic acid and then separated in a gelatin chromatogram column (TSK gel G2000 SWXL, 300 × 7.8 mm, Tokyo, Japan) at a flow rate of 0.5 mL/min and monitored at 220 nm and 30 °C. Cytochrome C, aprotinin, bacitracin, tetrapeptide GGYR, and tripeptide GGG were used as molecular weight standards.

2.4. Isolation of AFM oligopeptide

Isolation of AFM oligopeptide was conducted according to the method of Cheong et al. [22], with some modification. In short, the AFM oligopeptide was dissolved in the mobile phase and injected into a semi-preparative C18 column (9.4 × 250 mm, Agilent Technologies, Santa Clara, CA) for fractionation using reversed phase-high performance liquid chromatography (RP-HPLC, Agilent). The mobile phase was composed of acetonitrile and water (2/98, v/v) with pH was adjusted to 6.0 using trifluoroacetic acid. Four oligopeptide fractions (F1, F2, F3, and F4) were isolated at a flow rate of 8 mL/min at 25 °C, measured at 205 nm, and then collected and lyophilized for subsequent activity and sequence determinations.

2.5. Antioxidant activity assays

Antioxidant activity was determined using the direct radical scavenging (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), superoxide, and hydroxyl), lipid peroxidation inhibition, and ferrous ion chelating activities, according, respectively, to the method of Tang et al. [23], Gu et al. [24], Wang et al. [25], Liu et al. [26], and Zhu et al. [27].

For ABTS scavenging activity assays, the ABTS radical cation was generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate, and the mixture was kept in the dark at 25 °C for 14 h until use. Next, samples were mixed with diluted ABTS radical cation solution at the ratio of 1:4 (v/v), and the absorbance was read at 734 nm after 6 min standing using a microplate spectrophotometer (xMarker, Bio-Rad, Hercules, USA). The scavenging activity was calculated in accordance to the formula $(1 - A_s/A_b) \times 100\%$, where A_p and A_b represent the sample and blank absorbance, respectively.

Pyrogallol autoxidation system was used for superoxide scavenging activity assays. Samples and 3 mM pyrogallol in 10 mM HCl were added to 50 mM Tris–HCl buffer (pH 8.2) and preincubated for 20 min at 25 °C. The rate of superoxide radical-induced polymerization of pyrogallol was then measured as an increase in absorbance at 420 nm for 3 min at room temperature. The scavenging activity was calculated as above.

For hydroxyl radical scavenging activity assays, samples were added to a mixture containing 5 mM 1,10-phenanthroline monohydrate, 0.2 mol/L phosphate buffer (pH 7.4), 50 mM $FeSO_4$, and 15 mM EDTA. Then, 0.1% H_2O_2 was added, and the absorbance was determined at 536 nm after incubation at 37 °C for 1 h. The scavenging activity was calculated as above.

Lipid peroxidation inhibition activity was determined using a linoleic acid emulsion system. Samples were dissolved in 0.05 mol/L sodium phosphate buffer (pH 7.0) and mixed with 20 mM linoleic acid in 99.5% ethanol. After incubation in the dark at 50 °C for 2 h, aliquots of the solution were mixed in sequence with 75% ethanol, 30% ammonium thiocyanate (v/v), and 20 mM ferrous chloride solution in 3.5% HCl (v/v). The absorbance was measured at 480 nm after reaction for 3 min, and the antiperoxidation activity was calculated as above.

For ferrous ion chelating activity assays, samples were mixed with 2 mM $FeCl_2$ and 5 mM ferrozine. The absorbance was then measured at 562 nm after keeping the mixture at room temperature for 10 min, and the ferrous ion chelating activity was calculated as above.

2.6. Antimicrobial activity assays

Antimicrobial activity was assayed against bacteria and fungi as described by Tang [28] and Kim [29], with some modification.

Antibacterial activity was tested by measuring the inhibition of *Escherichia coli* (*E. coli*, ATCC25922) and *Staphylococcus aureus* (*S. aureus*, ATCC25923) cell growth. Samples were added to sterile 96-well plates to final concentrations from 0 to 0.8 mg/mL. *E. coli* and *S. aureus* were then added to the wells (5×10^5 cfu/mL) and incubated at 37 °C until the exponential phase was reached. The inhibitory activity was determined based on the increase in OD_{600} measured with a microplate spectrophotometer (Bio-Rad). The antibacterial activity was calculated based on the formula $(1 - A_s/A_b) \times 100\%$, where A_s and A_b represent the sample and blank absorbance, respectively.