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Isolation and characterization of a hepcidin peptide from the head kidney of large yellow croaker, *Pseudosciaena crocea*

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ABSTRACT

Large yellow croaker (*Pseudosciaena crocea*) is one of the most important marine cultured fish in China. Acidic extracts of five tissues of large yellow croaker showed strong anti-*Vibrio alginolyticus* activity. Acidic extract of head kidney tissue was subjected to heat-treatment in boiling water, and solid-phase extraction on Sep-Pak C_{18} cartridge. It was found that the antibacterial substances were heat stable, and 20% acetonitrile effluent exhibited strong antibacterial activity. Active extract was further applied to Sephadex G-25 gel permeation chromatography and StableBond C_{18} RP-HPLC. An antibacterial peptide with a single peak was obtained. The results of amino acid sequencing and MALDI-TOF MS suggested that the peptide was RCRFCCRCCPRMRGCGICCRF with an observed molecular mass of 2523.2 Da. BLAST searching suggested that the purified antibacterial peptide was the mature peptide section of the hepcidin preproprotein presumed from cDNA of large yellow croaker, thus designated hepcidin-Pl. Hepcidin-P1 exhibited strong antibacterial activity against four marine vibrios.

1. Introduction

Large yellow croaker, *Pseudosciaena crocea* (Richardson), is mainly distributed between the southern Yellow Sea and the northern Southern China Sea [1,2]. Today, large yellow croaker is one of the most important maricultured fish in China with the highest annual yield. The most significant factor that affects large yellow croaker culture is the incidence of various diseases, especially vibriosis. Some studies on prevention and cure of the disease of large yellow croaker have been carried out [3,4]. However, information on immune mechanisms is sparse and no study on the purification and identification of antibacterial peptides from this fish has been done. The adaptive immune response in teleosts is feebler and short-lived, when compared to that of mammals [5]. Previous studies of our laboratory showed that it took large yellow croaker at least 2 weeks to produce specific antibody [6]. So the innate defenses play important roles in teleosts.

Antibacterial peptides are widely distributed throughout the animal and plant kingdoms [7]. They are regarded as important components of the host innate immune system and play crucial roles in host defense against bacterial invasion [8]. However, only a relatively smaller number of antibacterial peptides has been found so far from teleosts such as pleurocidin [9], paradaxin [10], parasin [11], misgurin [12], piscidins [13], moronecidin [14], hepcidin [15], chrysophsin [16], oncorhyncin [17], and cathelicidin [18].

In this paper, we reported the isolation of a potent antibacterial peptide from large yellow croaker, *P. crocea*. The amino acid sequence and antibacterial activity of the peptide were analyzed. The results indicated that this antibacterial peptide was the mature peptide of large yellow croaker hepcidin.

2. Materials and methods

2.1. Experimental animals and sample collection

Ice-cooled adult large yellow croakers weighing 400–450 g were obtained from Zhongpu aquatic product wholesale market (Xiamen, Fujiang, China). They were just carried from commercial net cage farms (Ningde, Fujiang, China) in approximately 10 h.

Seven kinds of tissue were collected from four individuals on ice-cooled dish. Skin mucus was gently scraped from the dorsolateral surfaces of the fish with a steel spatula and mixed with

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25 mM phosphate buffer (PB), pH 7.0, supplemented with 1.5 μM aprotinin. The excised gills were immersed in the PB solution to prepare gill mucus by scraping the gills. Following excision of gastrointestinal tract, the content of them was washed into PB. The tissue of head kidney, liver and gonad, as well as the gastrointestinal tissue was homogenized in PB solution using a homogenizer.

2.2. Peptide extractions

All of the seven samples in PB were extracted by shaking for 2 h at 0 °C. After centrifugation at 10 000 *g* for 10 min at 4 °C, the supernatants were collected as PB extracts. The precipitates were homogenized in 10% acetic acid supplemented with 1.5 μM aprotinin, stirred and centrifuged in the same manner. The supernatants were collected as acidic extracts. These PB extracts and acidic extracts were lyophilized, resuspended in NH_4Ac solution (10 mM, pH 6.6), then the antibacterial activity and protein concentration were determined.

Head kidney was chosen as the source of antibacterial peptide for further purification. To obtain enough extract and simplify the extraction procedure, the head kidney tissue of 25 individuals was extracted directly with 600 ml of 10% acetic acid supplemented with 1.5 μM aprotinin to prepare the acidic extract of head kidney. The acidic extract was subjected to boiling water bath for 15 min with continuous agitation, and then cooled immediately in ice. After centrifugation, the supernatant was lyophilized, resuspended in 10 mM NH_4Ac solution, pH 6.6, and the antibacterial activity was determined.

2.3. Solid-phase extraction

Acidic extract of head kidney tissue from 25 individuals was acidified with an equal volume of 0.2% TFA in deionized water, then subjected to solid-phase extraction on six Sep-Pak Vac 1g C_{18} cartridges (Waters Associates, USA), which were previously conditioned with 15 ml of methanol and equilibrated with 15 ml of acidified water (0.1% TFA in deionized water), respectively. Following elution with 15 ml of acidified water, each cartridge was eluted by 15 ml of 15, 20, 25, 30 and 50% acetonitrile in acidified water, respectively. All the same fractions from six cartridges were pooled, lyophilized and reconstituted in NH_4Ac solution (10 mM, pH 6.6) for antibacterial activity determination.

2.4. Gel permeation chromatography

Gel permeation chromatography was performed on a Pharmacia ÄKTA Protein Purifier system (Amersham Pharmacia Biotech, Sweden). The 20% acetonitrile effluent was applied to a 1.6 × 60 cm Sephadex G-25 (Amersham Pharmacia Biotech, Sweden) column equilibrated with NH_4Ac solution (10 mM, pH 6.6). Gel column was eluted with the same buffer at a flow rate of 1 ml min^{-1}. Absorbance was measured at 280 nm and 2-ml fractions were collected. The antibacterial activity of each fraction was determined directly.

2.5. RP-HPLC

The active fractions denoted by the bar in Fig. 1 were pooled from each time of G-25 elution, lyophilized and redissolved in 1 ml acidified water. An aliquot (0.1 ml) was subjected to C_{18} reverse-phase high-performance liquid chromatography (RP-HPLC) on an analytical StableBond 300SB column (particle size 5 μm, 4.6 mm × 250 mm, Agilent, USA) equilibrated with acidified water every time. Elution was performed with a linear biphasic gradient elution of ACN (0–15% over 10 min/15–23% over 55 min/23–50% over 5 min) in acidified water at 28 °C at a flow rate of 1 ml min^{-1}.

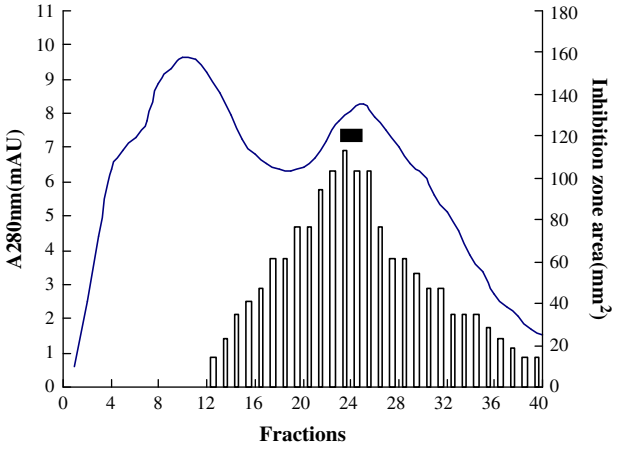

Fig. 1. Purification by gel permeation chromatography on Sephadex G-25 of the 20% acetonitrile effluent of head kidney acidic extract. The histogram represents the inhibition zone areas. The fractions shown by the bar were pooled and subjected to further purification by reverse-phase HPLC.

The absorbance was measured at 280 nm, and 1-ml fractions were collected by hand. Each fraction was lyophilized, reconstituted in 0.2 ml deionized water for antibacterial activity determination.

The active fractions eluted between 58 and 60 min were pooled, lyophilized, reconstituted in acidified water and further purified to homogeneity by a second round of C_{18} RP-HPLC on the same column using a lower linear gradient (0–20% over 5 min/20–23% over 60 min/23–50% over 5 min). Fractions were collected and tested for antibacterial activity. The active fractions eluted between 30 and 32 min were pooled, lyophilized, reconstituted in deionized water for chemical characterization and antibacterial spectra assay.

2.6. Bacterial strains

Six strains of Gram-negative bacteria, *Escherichia coli*, *Aeromonas hydrophila*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio fluvialis*, and *Vibrio parahaemolyticus*, and three strains of Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus lysodeikticus* were obtained from the China General Microbiological Culture Collection Center (Beijing, China) and listed in Table 3. *V. alginolyticus* was used as the main test strain throughout the purification procedure. Other strains were used to determine the antibacterial spectra of the purified peptide.

Marine bacteria were inoculated on marine nutrient agar (2% NaCl) and grown for 18 h at 28 °C. Other strains were grown on normal nutrient agar (0.5% NaCl) for 18 h at 36 °C. After washing of the bacterial cells into sterile 0.85% (w/v) NaCl, the bacterial suspensions were adjusted to a final density of approximately 1×10^6 cfu ml^{-1}.

2.7. Antibacterial assay

Inhibition zone assay was used to determine the antibacterial activity of fractionated samples at each purification step. 20 ml of 1% (w/v) agarose in 1/10 strength nutrient broth was seeded with 1×10^6 bacterial cells and poured into Petri dishes. Aliquots (18 μl) of the samples were added into 3 mm diameter wells punched in the agarose, and incubated at 4 °C for 1 h before a 24 h incubation at 28 °C (marine bacteria) or 37 °C (other bacteria). Negative controls comprised 10 mM NH_4Ac solution, pH 6.6. Antibacterial activity was identified as a clear zone around the well. The diameters of these zones were measured and activity was expressed as clear zone area (in mm^2) minus the area of the well.