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Isolation and identification of a novel algicidal peptide from mackerel muscle hydrolysate

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ABSTRACT

To help remedy damage from harmful algal blooms, an attempt was made to isolate an algicidal substance previously observed to be present in mackerel muscle hydrolysate. Crude extract was obtained by cold acetone precipitation, and it dissolved best in water. Through molecular weight cut-off determination and tricine-SDS PAGE, the algicidal substance was determined to be a peptide of < 1 kDa. Based on this result, purification was first performed using size exclusion chromatography and preparative reverse phase high-performance liquid chromatography. Then, the active algicidal fraction was applied to an ultra-performance liquid chromatography-electrospray ionization-mass spectrometry system, followed by MS/MS analysis. The algicidal peptide had linear structure consisting of amino acids with sequence NH-KMNF-COOH. Its calculated properties were: molecular weight 538.66 g/mol; isoelectric point 9.91; net charge +1 at pH 7.0; and 50% hydrophobicity. Algicidal ability of the identified peptide was confirmed using synthesized peptide. The LC_{50} values toward four harmful algal blooming species were 0.69, 0.83, 0.85 and 1.24 mg/ml for *Alexandrium fundyense*, *A. catenella*, *Heterocapsa triquetra*, and *Prorocentrum minimum*, respectively. There was no coincidence in the sequence of the identified peptide with those of known metabolites in the APD, Norine, CAMP, UniProt and METLIN databases. Consequently, this algicidal substance originating from mackerel protein was deduced to be a novel peptide that can usefully be applied to relieve harmful algal blooms.

1. Introduction

Harmful algal blooms (HABs) cause significant damage to finfish, marine mammals, shellfish, and other marine organisms because they both secrete toxins and deplete oxygen and nutrients due to their enormous biomass. The global annual economic loss from damage by HABs has been estimated to be several billion US dollars: a conservative estimate in the USA was approximately US$ 95 million per year (considering inflation) [1]; an estimate in European coastal waters was more than €800 million [2], and that in Japan was more than US$ 1 billion [3]. Many efforts have been made to control HABs, including physical, chemical and biological treatments, but each treatment has some drawbacks. Physical treatment often causes secondary effects on benthic organisms or ecological and environmental impact by the dispersal of clay [4, 5]; chemical treatment causes indiscriminate death of organisms and blooms recur [6]; and biological treatment can cause chaos in ecological systems well after the treatment application stage [7]. Therefore, development of new algicidal substances without any side effects is essential.

The majority of algicidal substances have been derived from bacteria. However, only a few have been characterized in detail, and they have a limited spectrum of activity against microalgae. The reported substances effective against microalgae are: bacillamide against the dinoflagellate *Cochlodinium polykrikoides* [8]; polyunsaturated fatty acids against *Heterosigma akashiwo* [9]; extracellular serine protease against diatoms *Thalassiosira* and *Eucampia zodiacs*, and the raphidophyte *Chattonella antiqua* [10]; and rhamnolipid biosurfactants against *H. akashiwo* [11]. Antimicrobial peptides derived from *Helicobacter pylori* showed algicidal activity against harmful algae [12]. Isolation and identification of natural metabolites from microbes has not been sufficiently studied and it has been challenging [13, 14].

In a previous study, we accidently found that the supernatant of biodegraded mackerel wastewater had some inhibitory activity against dinoflagellates [15]. Thus, in the present study, an attempt was made to isolate and identify an algicidal substance from mackerel muscle hydrolysate. Non-specific hydrolysis of mackerel muscle was conducted, followed by extraction and purification of an algicidal substance. Identification of this substance was performed using mass spectrometry and bioinformatic software. Finally, LC_{50} values of the identified substance toward four HAB species were investigated, along with observation of their morphological change.

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2. Materials and methods

2.1. Hydrolysis of mackerel muscle

The raw material used in this study was mackerel purchased from the market. Cut pieces of mackerel muscle (< 1 × 1 × 0.5 cm) were put in distilled water (DW) and then autoclaved at 121 °C for 15 min. DW was made by μPure RO (Human science, Korea). After autoclaving, the material was squeezed through a fine cloth and the liquid portion was collected. The liquid solution was ready for hydrolysis after its chemical oxygen demand (COD) value was adjusted to 50,000 ± 1000 mg/l using DW. The concentration of COD was measured using COD Test Kit (HS-COD-M, Humas, Korea). Hydrolysis was initiated when two patented bacterial mixtures, Pknufermbacteria (patent No. 10-0822239) and Wormbacteria (patent No. 10-1058245), were inoculated into 400 ml liquid mackerel medium. Pknufermbacteria was composed of seven bacteria: *Bacillus subtilis* (DQ219358), *B. licheniformis* (AY468373), *B. coagulans* (AF466695), *B. circulans* (Y13064), *B. anthracis* (AY138279), *B. fusiformis* (AY548950), and *Brevibacillus agri* (AY319301). Wormbacteria was composed of four bacteria: *B. licheniformis* (EF113324), *B. cereus* (DQ923487), *Brevibacillus agri* (AJ586388), and *Brevibacillus parabrevis* (AB215101). For the hydrolysis, all microbes were previously adapted to the liquid mackerel for 12 h, and the inoculum size was 10% (v/v). After the inoculation, a 1 l-flask containing the liquid mackerel medium was put in a shaking incubator at 45 °C and 130 rpm for hydrolysis for 48 h.

2.2. Extraction

The hydrolyzed mackerel medium solution was centrifuged at 4 °C and 14,000 ×*g* for 15 min. Algicidal substances were extracted from the supernatant. Ten milliliters of the supernatant was vortexed with 40 ml of acetone (1:4 v/v; Guaranteed Reagent (GR) grade, Junsei, Japan) for 15 min, and the mixed solution was placed at −20 °C for 1 h. The resultant mixture was centrifuged at 4 °C and 21,000 ×*g* for 10 min, followed by the measurement of algicidal activity of both the supernatant and pellet fractions. One milligram of the pellet was dissolved in 1 ml tris-HCl buffer (pH 8.0) and then freeze-dried to obtain it as powder for algicidal activity test. The tris-HCl buffer was made from a mixture of Tris(hydroxymethyl)aminomethane (99.9%, BioShop, Canada) and HCl (GR grade, Junsei). After confirmation of its algicidal activity, the pellet portion dissolved in the tris-HCl buffer was extracted and centrifuged at 21,000 ×*g* for 10 min twice more using acetone. The final crude extract was stored at −70 °C until use. The entire extraction step was repeated several times until sufficient amounts of algicidally-active substances were collected.

2.3. Preliminary experiments for purification

For use as a mobile phase in chromatographic methods, commonly used solvents (HPLC-grade acetonitrile, methanol, ethanol, isopropanol, hexane and water, Honeywell, USA; HPLC-grade dichloromethane and tetrahydrofuran, Sigma-Aldrich, USA) were tested to dissolve the crude extract. After the determination of the dissolving capacity of each solvent, usable solvents were mixed and tested as a mobile phase in thin layer chromatography (TLC). The TLC was performed both in a normal phase (NP) using 'Silica 60' (Merck, Germany) and in a reverse phase (RP) using 'Silica gel 60 RP-18' (Merck) with ninhydrin (as coupler) and a mixture of acetonitrile, water, trifluoroacetate and acetic acid (as developer). Ninhydrin (99%, Alfa Aesar, USA) staining was applied to the TLC to trace the algicidal component because algicidal activity was distinctly detected in the 48-h biodegraded mackerel hydrolysate in our previous study [15], and the data suggested it could arise from a low-MW peptide or a fatty acid.

To estimate the molecular weight (MW) of the algicidal substance, tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE), using a 4% stacking gel and 16.5% separating gel, was used with slight modifications of the procedure described by Schägger [16]. The buffer and the gel was run overnight at 90 V and stained with Coomassie Brilliant Blue R (Sigma-Aldrich). Ultra-low Range Molecular Weight Marker (MW 1060–26,600 Da, Sigma-Aldrich) was used to estimate molecular mass.

2.4. Size-exclusion chromatography

The crude extract prepared after acetone evaporation was dissolved in tris-HCl buffer (pH 8.0). For purification of the algicidal substance, 5 ml of the prepared extract solution (1 g/l) was first loaded onto a size exclusion chromatography (SEC) column containing Sephadex G-25 resin (medium, GE Healthcare, USA) for 5 h. The glass column (2 × 49 cm) was packed with 10 g of resin, the mobile phase was tris-HCl buffer (pH 8.0) at a flow rate of 0.5 ml/min, the final eluting solvent was 0.2 M NaOH, and the eluting samples were fractionated every 8 min. After confirmation of algicidal activity, the algicidally-active fractions were freeze-dried. Normal phase TLC was performed to help characterize the active substance and roughly estimate its retention time for the determination of the next purification step. The SEC was performed several times until sufficient amounts of algicidally-active substances were collected.

2.5. Reversed-phase liquid chromatography (RP-HPLC)

The active fractions were applied to preparative reversed-phase liquid chromatography (prep RP-HPLC) for further purification. This step was conducted at the Korea Basic Science Institute (Seoul, Korea). One milliliter of each filtered sample (0.2-μm filter) was injected into an XBridge prep C18 column (19 × 250 mm, 10 μm; Waters, USA) using 0.1% trifluoroacetic acid (solution A; HPLC grade, Honeywell) and 0.1% trifluoroacetic acid/acetonitrile (solution B; HPLC grade, Honeywell) as mobile phases at a flow rate of 7 ml/min. The sample was eluted by a linear gradient of solution B from 60 to 95%, and the resultant peaks were observed at 214 nm. Fractionation was conducted every minute. Algicidally-active fractions were collected and freeze-dried. The degree of purification was confirmed by RP-TLC. The RP-HPLC was performed several times until sufficient amounts of algicidally-active substances were collected.

2.6. Size-exclusion chromatography

SEC using Sephadex G-25 was used again to further purify the algicidally-active substance. Ten grams of resin were packed in the same glass column as above, but this time the solvent used as a mobile phase was 10% (v/v) ethanol/DW (pH 8.0; HPLC grade, Honeywell) at a flow rate of 0.5 ml/min to enhance sample elution. The eluting solvent was 0.2 M NaOH (analytical grade, Sigma-Aldrich), and the eluting samples were fractionated every 2 min. Then, fractions showing algicidal activity were loaded onto an HPLC instrument with a C18 Zorbax SB-phenyl column (4.6 × 250 mm, 5 μm; Agilent, USA). The mobile phase was a mixture of 0.1% trifluoroacetic acid (solution A; HPLC grade, Honeywell) and 0.1% trifluoroacetic acid/acetonitrile (solution B; HPLC grade, Honeywell) at a component ratio of 53:47 (v/v). The flow rate of the mobile phase was 0.3 ml/min and resultant peaks were observed at 214 nm. This step was performed several times until sufficient amounts of algicidally-active substances were collected.

2.7. Ultraperformance liquid chromatography-electrospray ionization tandem mass spectrometry analysis for identification of the algicidal substance

To identify the purified algicidal substance, ultraperformance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) analysis was performed by our request at the Center

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