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Targeted separation of antibacterial peptide from protein hydrolysate of anchovy cooking wastewater by equilibrium dialysis

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ABSTRACT

Anchovy (*Engraulis japonicus*) cooking wastewater (ACWW) is a by-product resulted from the production of boiled–dried anchovies in the seafood processing industry. In this study, the protein hydrolysate of ACWW (ACWWPH) was found to have antimicrobial activity after enzymatic hydrolysis with Protamex. For the targeted screening of antibacterial peptides, liposomes constructed from *Staphylococcus aureus* membrane lipids were used in an equilibrium dialysis system. The hydrolysate was further purified by liposome equilibrium dialysis combined with high performance liquid chromatography. The purified antimicrobial peptide (ACWWP1) was determined to be GLSRLFTALK, with a molecular weight of 1104.6622 Da. The peptide exhibited no haemolytic activity up to a concentration of 512 μg/ml. It displayed a dose-dependent bactericidal effect in reconstituted milk. The change in cell surface hydrophobicity and membrane-permeable action of the purified ACWWP1 may have contributed to the antibacterial effect. This study suggests that liposome equilibrium dialysis can be used for the targeted screening of antimicrobial peptides.

1. Introduction

The increasing problem of multiple drug resistance in bacteria and the potential health risks of chemical food preservatives have lead to a demand for new antimicrobial strategies. With action modes markedly different from that of traditional antibiotics, it has been proposed that antimicrobial peptides (AMPs) might form the foundation for a new class of antimicrobials that are effective against antibiotic-resistant bacteria (Brogden, 2005; Parisien, Allain, Zhang, Mandeville, & Lan, 2008; Reddy, Yedery, & Aranha, 2004). An increasing number of AMPs have been isolated from various different species (Zasloff, 2002). These have helped in the discovery of new antibacterial drugs and in elucidating the exact action mechanism of AMPs. However, the conventional procedure for screening AMPs is an activity-guided chromatographic protocol, in which the multiple-step extraction and isolation of peptides from complex sources are followed by the analysis of the purified individual constituents (Pichu, Ribeiro, & Mather, 2009; Salampessy, Phillips, Seneweera, & Kailasapathy, 2010; Tan, Ayob, & Wan Yaacob, 2013). These methods are arduous and time-consuming. Thus, an effective method for targeted-separation of AMPs is needed.

Equilibrium dialysis, a technique used to characterise the interaction properties of drugs with biological systems (Gunn et al., 2012; Hou, Niu, Song, Liu, & Liu, 2013; Varghese, Johny, Paul, & Ravi, 2011), has been proposed for the screening (Qi et al., 2006) and analysis of the multiple bioactive compounds found in traditional Chinese medicines (Deng et al., 2011). In equilibrium dialysis, each component that is below a certain molecular weight threshold can pass freely through a dialysis membrane. The size of the liposomes used in equilibrium dialysis is larger than the pore size of the dialysis membrane. Components that combine with the liposomes can thus not diffuse out (Ottiger & Wunderli-Allenspach, 1997). As a result, equilibrium dialysis can be used for targeted separation of the active components.

Liposomes have previously been used as model membranes to study the interactions between AMPs and the bacterial cell membrane (Bonev, Chan, Bycroft, Roberts, & Watts, 2000; Epand, Umezawa, Porter, Gellman, & Epand, 2003; Yu et al., 2009). Traditional liposomes were prepared by one or several specific polar lipids at a certain ration. This cannot mimic the highly regulated phospholipid composition and content in real bacterial cellular membranes. Our group constructed liposomes from bacterial membranes which were used in immobilized bacterial membrane liposome chromatography for the separation of AMPs (Tang,

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Zhang, Wang, & Qian, 2014). This kind of liposome was much finer than conventional liposomes and more accurately reflected the interaction between AMPs and the bacterial cell membrane. Liposomes constructed from bacterial membrane lipids have not previously been used for equilibrium dialysis. In addition, equilibrium dialysis has not yet been used for the screening of potential AMPs.

Anchovy cooking wastewater (ACWW) is a by-product resulted from the production of boiled–dried anchovies, a traditional Chinese seafood known as "haiyan". The cooking operation gives rise to approximately 1.5 tons of liquid waste for each ton of canned sardine. Generally, the wastewater generated by fish processing contains valuable amount of protein, peptides and amino acids and lipids (Amado, Vázquez, González, & Murado, 2013; Chowdhury, Viraraghavan, & Srinivasan, 2010; Ferraro et al., 2013). Therefore, the use of this waste for the extraction of valuable compounds has great potentials for the medical, pharmaceutical and food industries. However, ACWW is generally discarded as waste into the sea. The high nutrient content of ACWW means that inappropriate disposal of ACWW could not only waste resources, but also cause eutrophication in coastal waters and environmental pollution. Hydrolysis of fish cooking water has been used to convert waste into value-added forms (Choi, Sung, & Lee, 2012; H-Kittikun, Bourneow, & Benjakul, 2012; Hsu, Lu, & Jao, 2009). To our present knowledge, isolation and characterisation of AMPs from the protein hydrolysate of ACWW (ACWWPH) have been seldom reported.

In this study, a new antibacterial peptide was purified from the protein hydrolysate of ACWW by equilibrium dialysis combined with HPLC. In addition, the antimicrobial spectrum, haemolytic effect, and mode of action of the peptide were studied.

2. Materials and methods

2.1. Materials

Frozen anchovies (*Engraulis japonicus*) were purchased from local aquatic wholesale market (Qingdao, China), and stored at −20 °C until use. Protamex (food-grade) was a *Bacillus* protease complex from Novozymes (Bagsvaerd, Denmark). Full-fat powdered milk was from Inner Mongolia Yili Industrial Group Co., Ltd. (Huhhot, China). Propidium iodide (PI), acetonitrile (ACN), formic acid and trifluoroacetic acid (TFA) for HPLC were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.2. Preparation of protein hydrolysate of anchovy cooking wastewater (ACWWPH)

The ACWW of *E. japonicus* was prepared according to Shiriskar, Khedkar, and Sudhakara (2010). Briefly, frozen anchovies were thawed, washed, and dipped in boiling brine containing 1.0% salt for about 8 min. Then the fish was drained, ACWW was collected and analysed. Total crude protein (N × 6.25) in ACWW was determined using the Kjeldahl method (AOAC, 1990). Amino acid analysis was determined by RP-HPLC (Agilent1100, Palo Alto, CA, USA) equipped with a Zorbax Eclipse XDB C18 analytical column (250 × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, CA) (Zhu, Zhou, & Qian, 2006). Total lipid content was measured by the method of Bligh and Dyer (1959). Ashes were quantified by gravimetric analysis after calcination at 550 °C. ACWW was then concentrated by rotary evaporation until the concentration rate was 10:1. The product was hydrolysed by Protamex to prepare ACWWPH. The enzymic hydrolysis was performed by using 0.02 g of enzyme to 1 g of crude protein at optimal conditions (pH 6.5, 55 ± 2 °C) for Protamex as previously reported (Liaset, Julshamn, & Espe, 2003). After 6 h hydrolysis, the mixture was heated at 100 °C for 10 min to inactivate the enzyme. The mixture was centrifuged (8000*g*, 20 min). The aqueous phase was lyophilized and stored at −20 °C until use.

2.3. Determination of molecular weight distribution of ACWWPH

The molecular weight (MW) distribution of ACWWPH was determined by high performance size-exclusion chromatography (SEC-HPLC) using a Waters 600 HPLC system equipped with a Waters 2487 UV detector and Empower workstation (Waters, Milford, MA, USA). The column was TSKgel 2000 SWXL column (30 mm × 7.8 mm, Tosoh, Tokyo, Japan. ACWWPH was solubilised in ultrapure water, filtered through 0.22-μm membrane filter (Millipore, USA), and then applied to the column, eluted at a flow rate of 0.5 ml/min and monitored at 220 nm at 30 ± 2 °C. A MW calibration curve was prepared using the following standards: cytochrome C (MW: 12500 Da), aprotinin (MW: 6500 Da), bacitracin (MW: 1450 Da), tetrapeptide Gly-Gly-Tyr-Arg (MW: 451 Da) and tripeptide Gly-gly-gly (MW: 189 Da) (Sigma, Louis, MO, USA). The molecular weight was calculated as follows:

$$\log \mathrm{MW} = -0.2381T + 6.7172, R^2 = 0.9887,$$

where MW means molecular weight and T means elution time (Liu et al., 2014; Song et al., 2013).

2.4. Total lipid extraction of Staphylococcus aureus membranes

Total lipid of membranes of *S. aureus* was extracted according to Dennison et al. (2006) with some modifications. The mid-logarithmic cells of *S. aureus* were harvested from culture broth (3 L) by centrifugation (3000*g*, 5 min), and then the pellet (wet weight, 9.108 g) was washed twice with sterile distilled water, centrifuged and re-suspended in Tris buffer (25 mM, pH 7.5). Then a mixture (150 ml) of chloroform–methanol (1:2, v/v) was added to the cell suspension. The whole was subsequently stirred at room temperature for 90 min. After stirring, further 30 ml of distilled water and 30 ml of chloroform were added, and the whole was vortexed for 20 min. The chloroform phase was separated and the aqueous was extracted twice with 50 ml of chloroform. The chloroform phase was collected, and then the solvent was removed by rotary evaporation. The obtained lipid extract was stored at −20 °C until use.

2.5. Preparation of mimic S. aureus membranes liposomes

The *S. aureus* lipid extract was dissolved in chloroform/methanol (2:1, v/v). Then, this mixture was dried to a thin film by rotary evaporation under reduced pressure. The remaining solvent was removed under high vacuum overnight. The film was hydrated with Tris buffer (10 mM, pH 7.2). The lipid suspension was then sonicated with a sonifier cell disruptor (Xinchen, Nanjing, China). The size of the resulting liposomes was measured by a Zetasizer nano ZS instrument (Malvern Instruments, Malvern, UK) to be between 421 and 608 nm. The total lipid concentration was 60 mg/ml.

2.6. Targeted separation of antibacterial peptide by equilibrium dialysis

The obtained *S. aureus* liposomes (60 mg/ml, 1 ml) was first pipetted into a 8-cm length dialysis bag (Spectra/Por Membranes, MW cutoff 25000, Spectrum Laboratory, Rancho Dominguez, CA) and dialyzed against 9 ml of Tris buffer (10 mM, pH 7.2). Phosphorus detection of the dialysate was conducted using the Fiske and