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Short communication

Release of antimicrobial peptides through bromelain hydrolysis of leatherjacket (*Meuchenia* sp.) insoluble proteins

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ABSTRACT

Antibacterial peptides have been found to be a natural part of animal and plant defence systems. In some cases, antimicrobial peptides have been found to be released by hydrolysis of food proteins. In the present study, two antibacterial peptide fractions (fractions 9 and 12) had been isolated from bromelain hydrolysate of leatherjacket (*Meuchenia* sp.) insoluble muscle proteins. Assay for antimicrobial activity showed that fraction 12 had a minimum inhibition concentration (MIC) value of 4.3 mg/ml against *Bacillus cereus* and *Staphylococcus aureus*, while fraction 9 only showed some activity against *Bacillus cereus* without a MIC being reached at a 5.35 mg/ml peptide concentration. Further fractionation, on an analytical C-18 column, indicated that the fractions contained many other peptides that could account for the high MIC value relative to the cationic antibiotic polymyxin.

1. Introduction

The discovery and availability of antibiotics have had a profound effect on human health and have contributed to increase average human lifespan by successful treatments of many bacterial infections and improving medical procedures (Hancock, 2001; Hancock & Knowles, 1998). However, this effectiveness seems to be fading, as many pathogenic bacteria have acquired resistance toward antibiotics, while few new drugs are being discovered (Cassell, 1997). Therefore, it is important to seek for possible natural antibiotics, e.g. the antibiotic peptides or cationic peptides (Hancock, 2001; Zasloff, 2002).

Over the past 20 years, more than 700 cationic peptides have been discovered (Zasloff, 2002). These cationic peptides arise from virtually all species, from bacteria to human beings, and possess a broad spectrum of antimicrobial activity, e.g. against bacteria, fungi, viruses and eukaryotic parasites (Hancock, 2001). The amino acid sequences of the peptides are rarely the same from different species, even those that are closely related (Zasloff, 2002). Food proteins, such as the milk protein casein (Meisel, 1997) and egg white lysozyme (Pellegrini et al., 1997) can also yield antimicrobial peptides after enzymic hydrolysis. Casecidins and isracidin, peptides derived from chymosin hydrolysis of casein, showed antimicrobial activity against a variety of Gram-negative and Gram-positive bacteria (Chan & Li-Chan, 2006), while digestion of lysozyme by clostripain produced active peptides against both Gram-negative and Gram-positive bacteria (Pellegrini et al., 1997).

Recently, there have been comparably fewer antimicrobial peptides of marine origin identified than of terrestrial origin (Chan & Li-Chan, 2006). These antimicrobial peptides are mainly from fish milt, e.g. clupeines, salmine and iridine (Ando et al., 1957) and skin secretions such as pleurocidin from winter flounder (Cole, Weis, & Diamond, 1997). Crustacean tissue has also been reported to have antimicrobial peptides (Relf, Chisholm, Kemp, & Smith, 1999; Schnapp, Kemp, & Smith, 1996), and molluscs (Hubert, Noël, & Roch, 1996; Mitta, Vandenbulcke, & Roch, 2000). These active peptides are from hemocytes, tissue, gills and other parts of fish or seafood and there are no reports of active peptides derived from enzymic hydrolysis of fish/seafood proteins. This study focused on the isolation and characterisation of antimicrobial peptides derived from enzymic hydrolysis of fish muscle proteins.

2. Materials and methods

2.1. Material and chemicals

All chemicals were of analytical grade and purchased from Sigma–Aldrich, Castle Hill, NSW, Australia. Milli-Q water (Millipore, Bedford, MA, USA), Molecular Weight Marker for peptides, high range prestained SDS–PAGE Standard (Bio-Rad Laboratories, Gladesville, NSW, Australia), papain (papainase, EC

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3.4.22.2), bromelain (EC 3.4.22.32) and Flavourzyme® 500 L (Novozymes Australia Pty. Ltd., North Rocks, NSW, Australia) were also used.

2.2. Preparation of fish protein hydrolysate (FPH)

Fresh skinned leatherjackets (*Meuchenia* sp.) were purchased from Sydney Fish Market, Pyrmont, NSW, Australia and transported in ice to the preparation laboratory (University of Western Sydney) where they were deboned and minced. Fifty grams of mince were suspended in 100 ml of Milli-Q water and shaken at 5 °C at 200 rpm for 60 min, followed by centrifugation at 5 °C and 5580*g* for 30 min. The supernatant, containing water-soluble proteins, was separated and the pellet containing insoluble protein was resuspended in Milli-Q water with the same ratios (1:2). Papain and bromelain (1% for pellet suspension and 0.5% for supernatant) and Flavourzyme® (2.5% and 1.25% for pellet suspension and supernatant, respectively) were added to both fractions. The mixtures were incubated at 50 °C and 170 rpm for up to 10 h. Aliquots were withdrawn at 2 h intervals, and the hydrolysis was stopped by immersing the flasks in boiling water bath for 15 min.

2.3. Degree of hydrolysis (DH)

The degree of hydrolysis was calculated by determination of free amino groups based on their reaction with 2,4,6-trinitrobenzenesulphonic acid (TNBS) (Adler-Nissen, 1979) with modification of the volume of the final reaction mixture. The number of total amino acid groups was determined from fully hydrolysed samples treated with 6 N HCl at 110 °C for 18 h. The absorbance was measured with a multiwell plate reader (Bio-Rad Benchmark Plus, Bio-Rad Laboratories, Gladesville, NSW, Australia) at 340 nm.

2.4. SDS–PAGE electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 15% gel (Laemmli, 1970). The electrophoresis was carried out using a Bio-Rad Mini Protean 3 System electrophoresis apparatus (Bio-Rad Laboratories, Gladesville, NSW, Australia).

2.5. Culture preparation

Staphylococcus aureus UWS 184, *Escherichia coli* UWS 185, *Bacillus cereus* UWS 106 and UWS *Candida albicans* X26, from the culture collection of the University of Western Sydney, were used throughout this study. The bacteria were transferred into either Luria broth (*E. coli*) or BHI broth (*B. cereus* and *S. aureus*), and incubated at 37 °C for 24 h. The bacterial suspension was spread on nutrient agar plates and incubated at 37 °C for 48 h. Purified colonies were isolated and transferred into their respective broths and incubated at 37 °C for 18–24 h for use in minimum inhibitory concentration (MIC) experiments. The *C. albicans* was transferred into Sabouraud broth and incubated at 37 °C overnight. The suspension was spread on nutrient agar plates and incubated at 37 °C for 48 h. One colony was isolated and inoculated into Sabouraud broth and incubated at 37 °C for 18–24 h. The optical density cultures were measured at 595 nm and adjusted to around 0.1 by addition of the same broth as previously used in the antimicrobial assays.

2.6. Antibacterial susceptibility and MIC assays

Antibacterial susceptibility and MIC tests were performed against *S. aureus*, *B. cereus*, *E. coli* and *C. albicans* in a multiwell sterile plate, based on a colorimetric method (Tunney, Ramage, Field, Moriarty, & Storey, 2004) with some modification. For each susceptibility assay, 50 μl mixtures of hydrolysate and broth of different ratios were mixed with 50 μl of culture suspension. Tetracycline (128 mg/l) was used as positive control while, for a negative control, a 50 μl mixture of tryptone and broth was added instead of hydrolysate. The mixtures were incubated at 37 °C for 4 h. After incubation, 100 μl of 0.5 mg/ml 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were added to each well and further incubated at the same temperature for 1 h and the absorbance was read at 492 nm. The MIC value was determined as the concentration at which no significant change of absorbance was observed.

2.7. Purification of peptides

Active hydrolysate was filtered through 0.20 μm filter before loading into a Shimadzu HPLC system (Shimadzu Scientific Instruments (Oceania) Pty. Ltd., Rydelmere, NSW, Australia), coupled with a PDA detector and fraction collector, for separation on a C-18 Prep-ODS column. HPLC was performed with a gradient solvent of Milli-Q water and acetonitrile containing 0.1% trifluoroacetic acid (TFA). The concentration of acetonitrile was increased up to 60%. Further separation was performed on an analytical C-18 column to examine the purity of fractions separated with the preparative column.

2.8. Statistical analysis

All values are reported as means of at least three observations. Data for degree of hydrolysis and pH during hydrolysis were analysed with ANOVA with three replications, followed by Tukey's test ($P < 0.05$) to evaluate significant difference between treatments.

3. Results and discussion

3.1. Preparation of fish protein hydrolysate (FPH)

Hydrolysis of fish muscle protein fractions, water-soluble and insoluble, was carried out without pH adjustment in order to avoid salt removal from the product. This practice had been reported earlier (Aspmo, Horn, & Eijsink, 2005), as salt removal will increase production cost. However, the pH of hydrolysates, taken at two hour intervals, was measured in order to ensure that the hydrolysis proceeded within the optimum pH range of the enzymes used. During hydrolysis, the pH changed from 5.95 to 6.41 (complete data are not shown) all of which are within the optimum pH range of all enzymes employed. The separation of water-soluble sarcoplasmic proteins and the insoluble myofibrillar and stroma proteins, prior to hydrolysis, is aimed to provide information for a possible use of this method in utilisation of water-soluble and insoluble proteins, separately.

3.2. Degree of hydrolysis (DH)

The extent of hydrolysis was assessed as degree of hydrolysis (DH) and all three proteolytic enzymes were able to hydrolyse the protein, with the DH values ranging from 13.9% to 32.9% (complete data not shown). Results from statistical analysis showed that DH increased significantly ($\alpha = 0.05$).

The SDS–PAGE pattern showed that, as the hydrolysis progressed, smaller peptides were produced. Observation of the electrophoretogram of bromelain hydrolysates of leatherjacket insoluble proteins indicates that most of the peptides fraction is of molecular weight around 10 kDa after hydrolysis, as compared to the peptide marker (Fig. 1).