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Isolation and characterisation of two antimicrobial peptides from haemocytes of the American lobster *Homarus americanus*

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Abstract Two antimicrobial peptides from haemocytes of the American lobster, *Homarus americanus* H. Milne Edwards 1837, were isolated and partially characterised — the first such description for this species. CAP-2, an approximately 12 kDa peptide, contained amino acid sequences corresponding to the predicted sequence for Hoa-crustin. Crustins are whey acidic protein (WAP) domain - containing peptides isolated from crustacean haemocytes. CAP-2 did not have any activity towards the Gram positive coccus *Aerococcus viridans* unlike carcinin, a crustin from *Carcinus maenas* haemocytes, which may partially explain the lobster's susceptibility to this bacterium. A second peptide, CAP-1, was a multimer composed of 4—6 kDa subunits with similarities to amphibian temporins. CAP-1 may represent a novel group of antimicrobial peptides for marine invertebrates and has been tentatively named 'homarin'. Homarin had bacteriostatic activity against some Gram negative bacteria and both protozoastatic and protozoacidal activity against two cultured scuticociliate parasites *Mesanophrys chesapeakensis* and *Anophryoides haemophila*, the latter a significant pathogen of *H. americanus*.
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Introduction

Investigation into the antimicrobial properties of the haemolymph of the American lobster, *Homarus americanus* H. Milne Edwards 1837, began in the late 1960s and 1970s [1—3]. Acton et al. [1] demonstrated increasing titres of antibacterial activity in citrated haemolymph of lobsters injected with formalin-killed bacteria. Stewart and Zwicker [3] examined the antimicrobial properties of different components of haemolymph (haemocytes, plasma, and serum) separately and in combination. They determined that serum, representing a combination of plasma and lysed haemocytes, was the most effective component. This

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antibacterial activity was also inducible — injection of lobsters with killed bacteria caused a transient increase in activity of the plasma and serum, but not haemocyte lysates [3]. While haemocytes were integral to the antimicrobial activity of serum, the specific nature of this activity was not determined. It is possible that some of this observed activity could be attributed to antimicrobial peptides in *H. americanus* haemocytes.

Antimicrobial peptides are small proteins, usually less than 10 kDa, present in all types of plants, animals and prokaryotes [4,5]. Antimicrobial peptides are an integral component of the innate immune system. Present in most tissues, particularly in secretions on epithelial surfaces (e.g., respiratory, enteric, genitourinary, and cutaneous) and in high concentrations in white blood cells and haemocytes, they provide an immediate defence against potential pathogens. Production of antimicrobial peptides can be constitutive or inducible [6]. Some peptides are cleavage products of larger molecules, e.g., lactoferricin from lactoferrin, buforin derived from histone H2A, and astacidin from haemocyanin [4]. Originally recognised for their ability to kill bacteria, viruses, protozoa, and some neoplastic cells, they have also been ascribed roles as modulators of the immune system, e.g., enhancement of phagocytosis, chemotactic activity [7,8]. Clearly, the scope of the physiological role, or roles, of these peptides has yet to be determined in many cases.

An appealing aspect of these peptides is that they offer an alternate approach to antimicrobial therapy once economical, large scale production methods are devised [9,5,7]. In contrast to traditional antibiotics, antimicrobial peptides generally destroy the organisms before they have the opportunity to develop resistance, although mechanisms of resistance do exist [7].

Despite their diverse origins and amino acid composition, most recognised antimicrobial peptides are cationic at physiological pH and often adopt an amphipathic structure [4,5]. Antimicrobial peptides can be classified based on their amino acid content, secondary and tertiary structure, length, and presence of internal disulfide bonds [4,5]. The latter group is represented by the defensins found in vertebrates (where they are subdivided into α- and β- defensins), invertebrates (where they are grouped based on their activity), and plants [4]. Cecropins (arthropods), pleurocidin (fish), magainins (amphibians), and cathelicidins (humans) are examples of antimicrobial peptides that adopt an α- helical conformation [4–6].

While knowledge of the location, production, and secondary and tertiary structure of these peptides is increasing, there is still uncertainty regarding their exact mechanism of action in many cases [5,7]. Combining knowledge of physiological membrane composition and function with experimental systems utilising a few well-studied peptides, e.g., magainin and defensin A, mechanistic models have been proposed [5–7]. These include attachment of peptides to target membranes and subsequent destabilisation of the membrane by forming 'pores' or ion channels [5,7]. The peptides may also bind to intracellular proteins, directly leading to disruption of the normal metabolic pathways of the target cell [5,7].

Antimicrobial peptides are well described in the haemolymph of many marine invertebrates [4,9]. These include the tachyplesins and polyphemusins of the Chelicerata, penaeidins, crustins, and callinectin in the Crustacea, defensins and mytilins in Mollusca, and styelin and clavalin in tunicates. Recently, a crustin-like peptide, Hoa-crustin, was predicted for *H. americanus* using a multi-tissue EST library [10]. A sequence closely matching Hoa-crustin has also been identified and studied in the European lobster *Homarus gammarus* [11,12].

In the present study, two antimicrobial peptides were isolated from *H. americanus* haemocytes. The first peptide corresponded to the predicted Hoa-crustin. The second peptide had some sequence similarities to amphibian temporins and may represent a new group of antimicrobial peptides for decapod crustaceans. In addition, the antibacterial and antiprotozoal activity of each peptide, in natural and synthetic forms, was partially characterised.

Materials and methods

Haemolymph ($\sim$15 ml), from either individual lobsters or pooled samples, was centrifuged at 3500 $\times$ *g* for 5 min at 4 °C. Plasma supernatants were removed and the surface of the packed haemocyte pellets washed with TBS (10 mM Tris, 150 mM NaCl, pH 7.4) and 5 µg/ml aprotinin to remove any residual plasma. Washed haemocyte pellets were homogenized in 3 ml of cold 10% acetic acid and 5 µg/ml aprotinin on ice using a glass piston homogeniser and stirred overnight at 5 °C. Lysates were centrifuged at 13,000 $\times$ *g* for 30 min at 4 °C and the supernatants applied to an Oasis® HLB 3 ml reversed phase extraction cartridge (Waters Corporation, Milford, MA) previously conditioned with 3 ml of 100% methanol and washed with 3 ml of ddH$_2$O. The flow-through sample from each cartridge was applied to a second HLB cartridge. Cartridges were washed twice with 3 ml of 5% methanol and eluted with 3 ml of 100% methanol. All flow-through, wash and eluate fractions were retained and the methanol evaporated under nitrogen gas prior to freezing at −80 °C and subsequent lyophilisation (Model 75035 freeze dryer, Labconco Corporation, Kansas City, MO).

Fractions were reconstituted in 500 µl of ddH$_2$O and tested for antimicrobial activity against a variety of bacteria (*Micrococcus luteus* ATCC 4698 (NCIMB 9278), *Aerococcus viridans*, *Vibrio* sp. (lobster intestinal isolates), *Halomonas* sp., *Enterobacter aerogenes*) representing marine and non-marine, Gram negative and Gram positive, recognised pathogenic and non-pathogenic isolates, and commensal and non-commensal agents. Five µl aliquots of the reconstituted fractions were spotted onto Mueller–Hinton agar, supplemented with 3% NaCl, previously coated with a 24-h broth culture (MacFarland standard 0.5) of the test organism. Fractions were also examined by reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [13], using 17% gels, and acid-urea PAGE (AU-PAGE) modified after Wang et al. [14]. Two antimicrobial peptides, NRC-10 and NRC-14, were used as positive controls [15].

For AU-PAGE, continuous separating mini-gels comprised of 19.5% (w/v) acrylamide/*N*,*N*′-methylenebisacrylamide (37.5:1), 0.4% (v/v) *N*,*N*,*N*′,*N*′-tetramethylenediamine, 6.5 M urea, 7% (v/v) acetic acid and 0.2% (w/v) ammonium persulfate were polymerised at 37 °C. Gels were pre-electrophoresed in reverse polarity at 150 V for 2 h

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