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Production of bioactive hepcidin by recombinant DNA tagging with an elastin-like recombinamer

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ABSTRACT

With the lack of new chemical antibiotics and increasing pathogen resistance to those available, new alternatives are being explored. Antimicrobial peptides (AMPs) with a broad range of effects, including antibacterial, antifungal, and antiviral actions, have emerged as one of the options. They can be produced by recombinant DNA technology, but the chromatographic methods used for peptide purification are expensive and time consuming. Here, we describe the design, production, purification and assessment of the antibacterial activity of the human peptide hepcidin, using an elastin-like recombinamer as fusion partner. The recombinant protein Hep-A200 was produced in *Escherichia coli* and purified by a non-chromatographic procedure, exploiting the thermal properties of the A200 elastin-like recombinamer. Recombinant Hep-A200 was found to retain antibacterial activity against Gram-positive and Gram-negative species.

Introduction

Antimicrobial peptides (AMPs) are of interest as alternatives to chemical antibiotics. These small, ribosomally synthesized peptides are part of the innate immunity of several organisms and display a range of different activities, including antibacterial, antifungal, antiviral, antiparasitic and even antitumor actions [1,2]. In addition, AMPs also play essential roles in the immunity regulation of higher organisms as in chemotaxis, wound healing, pro- and/or anti-inflammatory processes, or as anti-oxidant molecules [2].

Among AMPs, the hepcidin family of peptides plays an important role in the regulation of iron homeostasis in humans [3]. The liver-expressed antimicrobial peptide, also known as Hepc25, is the only member of the hepcidin family that is known to be produced by humans [4,5]. Hepcidin also displays antimicrobial capabilities by inhibiting extracellular iron accumulation as well as by direct lytic activity against several microorganisms. Indeed, hepcidin peptides have demonstrated antibacterial, antifungal and antiviral activities against several organisms [6–9]. These features make them interesting candidates not only as antimicrobials but also for several other biomedical applications [8,10].

AMPs are generally obtained by direct isolation from natural sources, chemical synthesis or recombinant expression [11]. Direct isolation is a high-cost complex strategy as AMPs are present at very low concentrations in the natural organism and implies the use of harsh chemical procedures. Chemical synthesis of AMPs on a large scale is characterized by prohibitive costs and is therefore restricted to small-scale production. Recombinant expression, on the other hand, represents an alternative for large-scale production of the peptides. Once the initial costs of cloning and optimization of production and purification are accounted for, the process can be up-scaled to economically feasible levels. Nevertheless, recombinant production of AMPs can be hampered by factors such as peptide toxicity for the producing host cells or degradation by proteases. Furthermore, purification is often cumbersome and time consuming. Fusion partners are often used to overcome these obstacles; besides serving as purification tags, they can also reduce the peptide´s associated toxicity and inhibit enzymatic degradation [11].

The use of elastin-like recombinamers (ELRs) as fusion partners represents a promising alternative to traditional chromatographic purification methods. ELRs are a class of recombinant protein-based polymer based on the sequence of mammalian elastin. Apart from increasing solubility [12], their most relevant characteristic is their inverse temperature transition behavior [13]. In solution, the polymer acquires a disordered structure, but above its transition temperature (T_t) it self-assembles in a totally reversible process [14,15]. This phenomenon can be used for purification of recombinant proteins by hot

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and cold incubation and centrifugation steps, a process known as inverse transition cycling [16,17].

Here, we describe the production of recombinant human hepcidin Hep25c fused with the ELR A200. A200 is based on 200 repetitions of the pentamer sequence VPAVG and, in addition to the inverse temperature transition behavior, it also displays an acute thermal hysteresis [15,17,18]. In the presence of water, the polymer self-assembles into spherical microparticles at temperatures above a T_t of ~32 °C although the assembled structures only solubilize when cooled to ~10 °C. This is the first report of the production of recombinant human hepcidin using a non-chromatographic method for purification. Hep-A200, the recombinant fusion protein obtained, displays antibacterial properties, supporting its use in microbial infections.

Materials and methods

Biological materials

Escherichia coli XL1-Blue and BL21(DE3) were used for the cloning and production steps. *Pseudomonas aeruginosa* ATCC10145, *E. coli* HB101, *Staphylococcus aureus* ATCC6538 and *Bacillus subtilis* 48,886 were used in the antibacterial activity assays.

Cloning

Hep-A200 construction (MW = 87.9 kDa) was performed using standard molecular genetic techniques by fusing the hepcidin sequence (Supplementary Fig. 1a) in-frame with the N-terminus of A200 (Supplementary Fig. 1b-c). Compatible *Nde*I and *Kpn*I restriction sites were introduced into the chemically synthesized hepcidin (Hep25c) sequence (MW = 2.8 kDa) by polymerase chain reaction (PCR) and fused with the N-terminus of the ELR A200 polymer by ligation reaction. The ELR A200 gene has been previously cloned in a modified pET25b(+) (Novagen) expression plasmid [15]. The ligation reaction was transformed into *E. coli* XL1Blue for plasmid replication and storage. The final expression plasmid was confirmed by DNA sequencing and transformed into *E. coli* BL21(DE3) for recombinant protein production.

Recombinant protein production and purification

E. coli BL21(DE3) cells transformed with the expression plasmid containing the Hep-A200 gene were grown in shake flasks for 22 h at 37 °C, 200 rpms using autoinduction medium (TBlac, yeast extract 24 *g*, tryptone 12 *g*, glycerol 5.04 *g*, K_2HPO_4 12.54 *g*, KH_2PO_4 2.31 *g*, lactose 2 *g* per liter) supplemented with 50 mg/L kanamycin, and a culture volume/flask volume ratio of 1:4 [19]. The protein production levels were assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with copper chloride staining (0.3 M). After production, cells were collected by centrifugation, resuspended in TE buffer (50 mM Tris − HCl, 1 mM EDTA, pH 8.0) and disrupted by sonication in a Sonics VCX 750. The pH was adjusted to 3.5 with 1 M HCl to precipitate endogenous *E. coli* contaminants and remove them by centrifugation [19]. The clear supernatant was then purified by inverse transition cycling (ITC) involving 4 cycles of hot (37 °C) and cold (4 °C) incubation (60 min at each temperature) and centrifugation steps (30 min, 10,000 x g) as previously described for other ELRs [15,17]. The pure polymer fraction was lyophilized and stored at room temperature until use.

Differential scanning calorimetry

The thermal transition events of Hep-A200 were characterized by differential scanning calorimetry (DSC) on a Mettler Toledo DSC 822e with calibration of both enthalpy and temperature achieved with standard indium and zinc samples. Solutions for DSC were prepared at 25 mg/mL in MilliQ water (mQ, Millipore) or PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, per liter, at pH 7.4). For analysis, 20 µL of solutions were placed in hermetically sealed pans. A four-stage

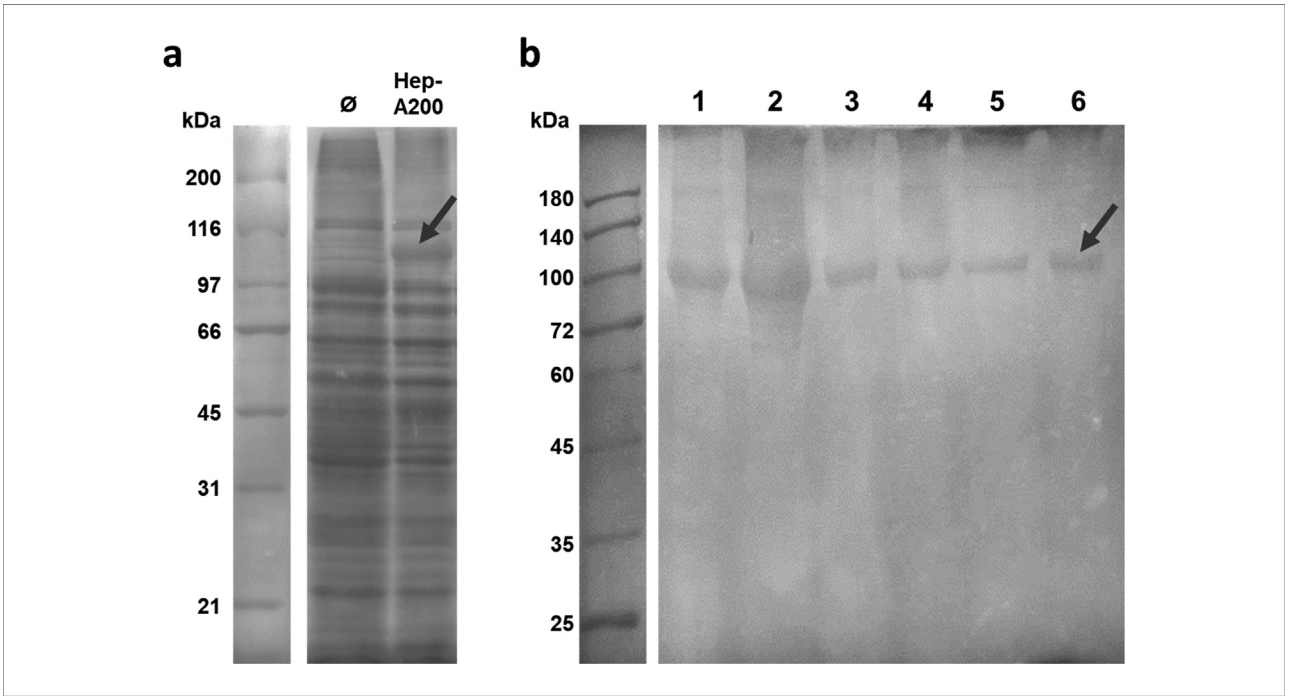

Fig. 1. SDS-PAGE of a) Hep-A200 production in E. coli BL21(DE3), after 22 h at 37 °C, 200 rpm in TBlac medium; Ø corresponds to the empty E. coli BL21(DE3). b) The recombinant polymer Hep-A200 was purified by inverse transition cycling. After sonication the lysate was adjusted to pH 3.5 and centrifuged. Improved purification was obtained after 5 purification cycles using hot and cold centrifugation steps (lanes 2–6). Gels were stained with 0.3 M copper chloride. MWM corresponds to the molecular weight marker. (Note: This Figure was assembled from pictures of different gels; no modifications were made other than cutting, pasting and resizing.).