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Identification of the initial binding sites of α_{s2}-casein f(183–207) and effect on bacterial membranes and cell morphology

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The aim of this work was to identify the initial binding sites to the bacterial membranes of the antimicrobial peptide α_{s2}-casein f(183–207) and also to acquire further insight into membrane permeabilization of this peptide. Furthermore, cell morphology was studied by transmission electron microscopy. In all the experiments, bovine LFcin was employed as a comparison. Results showed that initial binding sites of α_{s2}-casein f(183–207) peptide were lipoteichoic acid in Gram-positive bacteria and lipopolysaccharide in Gram-negative. The peptide was able to permeabilize the outer and inner membranes. Moreover, the α_{s2}-casein peptide f(183–207) generated pores in the outer membrane of Gram-negative bacteria and in the cell wall of Gram-positive bacteria. In the Gram-negative bacteria, f(183–207) originated cytoplasm condensation, and in the Gram-positive bacteria the cytoplasmic content leaked into the extracellular medium. Furthermore, the experiments of inner and outer membrane permeabilization performed with LFcin-B showed that this peptide also has the ability to permeabilize both the inner and outer membranes.

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1. Introduction

Antimicrobial peptides appear abundantly throughout all living nature and presumably form a cornerstone of innate and adaptive immunity [1]. Despite the chemical and structural heterogeneity within the group of antimicrobial peptides, a few common features can be distinguished. Most antimicrobial peptides contain a positively charged domain and are able to adopt an amphipathic conformation allowing their presence at hydrophilic/hydrophobic interfaces. These properties are supposed to be essential for antimicrobial activity, the amphipathic character causing disruption of the negatively charged microbial membrane leading to cell death [2]. Antimicrobial peptides could, also, have intracellular targets such as DNA, RNA or proteins [3], inhibition of macromolecular synthesis [4] and inhibition of bacterial enzymes [5].

The antimicrobial properties of milk have been widely acknowledged for many years. Nowadays, the antimicrobial activity of milk is mainly attributed to immunoglobulins, and to non-immune proteins, such as lactoferrin, lactoperoxidase and lysozyme [6]. Interestingly, over the past 20 years, it has been shown that food proteins can also act as antimicrobial peptide precursors, and in this way, might enhance the organism's natural defences against invading pathogens. Consequently, food proteins can be considered as components of nutritional immunity [7,8]. Two of the most potent antibacterial peptides derived from milk proteins to date are bovine lactoferricin (LFcin), which was released by gastric pepsin cleavage of bovine lactoferrin and bovine α_{s2}-casein f(183–207) [9]. The antibacterial domain of bovine LFcin corresponds to bovine lactoferrin f(17–41) [10]. Lactoferricin has revealed a broad spectrum of activity against Gram-positive and Gram-negative bacteria [11], fungi [12] and parasites [13]. Furthermore, lactoferricin has been shown to have antiviral [14], antitumoral [15,16] and anti-inflammatory properties [17]. Regarding the mode of action of bovine LFcin, it is recognized that the antibacterial activity of LFcin starts by electrostatic interaction with the negatively charged membranes of the bacteria [18]. In this initial binding, lipopolysaccharide and teichoic acid have been identified as binding sites in Gram-negative and Gram-positive bacteria, respectively [19]. However, lactoferricin does not lyse susceptible bacteria but is able to translocate across the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria [20]. It has been demonstrated that once the peptide reaches the cytoplasm, bacterial protein synthesis is inhibited, although the exact mechanism for this inhibition of macromolecule biosynthesis is not known [4].

Fragment (183–207) of bovine α_{s2}-casein was identified together with the f(164–179) of bovine α_{s2}-casein in a peptic hydrolysate of the same protein [21]. Both fragments showed important antibacterial activity against Gram-positive and Gram-negative bacteria with MIC values ranging from 25 to 100 μM in the case of f(164–179), and from 8–16 μM in f(183–207). Recently, a synergistic effect between the f (183–207) and lactoferrin against *Escherichia coli*, *Staphylococcus epidermidis* and *Listeria monocytogenes* was demonstrated [22]. Although the bovine α_{s2}-casein f(183–207) has shown a broad spectrum of activity against Gram-positive and Gram-negative bacteria, to date, its mechanism of action remained unknown.

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The aim of this work was to elucidate the primary binding sites of the antimicrobial peptide α_{s2}-casein f(183–207) and to investigate the permeabilizing capacity of this peptide and Lfcin-B.

2. Material and methods

2.1. Bacterial strains

E. coli ATCC 25922 was from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and *Staphylococcus carnosus* CECT 4491T was from The Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT; Valencia, Spain). *E. coli* ML-35p was kindly donated by Dr. Lehrer (UCLA School of Medicine, Los Angeles, USA). *E. coli* ML-35p was constitutive for a cytoplasmic β-galactosidase, lacked lactose permease, and expressed a plasmic-encoded periplasmic β-lactamase [23]. *E. coli* ML-35p was maintained on lactose broth agar plates (Fluka, Buschs, Germany) containing 100 mg/mL of ampicillin (Sigma, St. Louis, MO, USA). The rest of the bacteria were maintained on trypticase soy agar plates (Scharlau, Barcelona, Spain). To obtain organisms for experiments, 10 mL of trypticase soy broth, or lactose broth containing 100 mg/mL of ampicillin were inoculated with bacteria from a single colony. After overnight incubation at 37 °C, the culture was washed three times with 10 mM sodium phosphate buffer, pH 7.4 as previously described [24], and resuspended in this buffer to the concentration of bacteria required for each experiment.

2.2. Material and peptides

Lactoferricin-B was prepared by using cation exchange chromatography and a second step of reversed phase HPLC as previously described [25]. The primary sequence of the purified peptide as determined by HPLC-mass spectrometry and HPLC-tandem mass spectrometry was: FKC* RRWQWRMKKLGAPSITC*VRRAF, with a disulfide bond between cysteine residues (marked with an asterisk).

Bovine α_{s2}-casein f(183–207) (VYQHQKAMKPWIQPKTKVIPYVRYL) was chemically synthesized by conventional Fmoc solid-phase synthesis method with a 431 A peptide synthesiser (Applied Biosystems Inc. Überlingen, Germany). The peptide was purified after synthesis by semi-preparative RP-HPLC with the conditions described by López-Expósito et al. [24]. Staphylococcal ribitol teichoic acid (TA) and anti-staphylococcal ribitol TA were from Meridian Diagnostics, (Cincinnati, OH, USA); staphylococcal lipoteichoic acid (LTA) and lipopolysacaride (LPS) serotype O55:B5 were from Sigma. 3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid (nitrocefin) was purchased from Calbiochem (San Diego, CA, USA). *o*-nitrophenyl-β-D-galactoside (ONPG) was from Sigma.

2.3. Determination of minimal inhibitory concentrations

Determinations of the minimal inhibitory concentration (MIC) of α_{s2}-casein f(183–207) and Lfcin-B for bacterial strains were performed according to López-Expósito et al. [22].

2.4. Competition assays

Competition assays consisting in checkerboard dilution tests were carried out according to Vorland et al. [19] with some modifications. The antibacterial activity of lactoferricin-B and α_{s2}-casein f(183–207) was performed in the presence of increasing concentrations of LPS when using *E. coli* as bacterial strain, and in the presence of LTA or TA when using *St. carnosus*. LTA and LPS were included in a range between 0 and 100 μg/mL and the final peptide concentrations in the tray were 0–200 μg/mL. The concentration range of TA in the microtiter tray was 0–1 μg/mL, 0–100 μg/mL for α_{s2}-casein f(183–207), and 0–50 μg/mL for Lfcin-B. The bacterial inoculum was 1×10^6 bacteria/mL, except in the competition assay with TA, where a bacterial inoculum of 1×10^4 was used.

2.5. Blocking experiments with antibodies against TA

These assays were performed as previously described [19]. To 100 μL of 10 mM Na-phosphate buffer pH 7.4 with 2% of TSB, 50 μL of *St. carnosus* at a concentration of 1×10^4 bacteria/mL were added. After that, 50 μL of anti-TA in a concentration range from 1:1000–1:10^6 were added and the mixture was incubated at room temperature during 30 min. Later on, α_{s2}-casein f(183–207) or Lfcin-B were added at concentrations in the range of 0–250 μg/mL and 0–30 μg/mL respectively. The mixtures were incubated during 2 h at 37 °C and then plated on TSA. The plates were incubated at 37 °C 24 h before the colonies were counted. The assays were conducted in duplicate.

2.6. Concurrent assessment of inner and outer membrane permeabilization//Nitrocefin outer cell barrier permeabilization assay and inner membrane permeabilization assay

Permeabilization of the inner membrane was assessed by measuring the access of ONPG to the cytoplasm, essentially as described previously [23] using *E. coli* ML-35p cells prepared as described above. Concentration of cells in 10 mM phosphate buffer with 100 mM NaCl was 1×10^6 bacteria/mL. Briefly, 10 μL of 30 mM ONPG were added to 200 μL of bacteria. After that, 10 μL of the test substance dissolved in NaCl at a higher concentration than MIC was added. Standard microplates (Bibby Sterilin, Staffs, UK) were incubated at 37 °C, and *o*-nitrophenol production was monitored at 414 nm during 100 min with a spectrophotometer/fluorometer FLUOstar OPTIMA (BMG Labtech).

Outer membrane permeabilization was measured by the Angus method with some modifications [26]. The assays were performed by using the chromogenic cephalosporin nitrocefin. Briefly, 20 μL of the test substance were added to 200 μL of *E. coli* ML-35p cells prepared as described above. After that, 20 μL of nitrocefin were added to a final concentration of 40 μM. Standard microplates (Bibby Sterilin) were incubated at 37 °C, and nitrocefin cleavage by β-lactamase was monitored at 490 nm during 100 min with a spectrophotometer/fluorometer FLUOstar OPTIMA (BMG Labtech).

2.7. Transmission electron microscopy (TEM)

Cultures of *St. carnosus* CECT 4491T and *E. coli* ATCC 25922 at a concentration of 1×10^8 bacteria/mL were incubated during 2 h at 37 °C with α_{s2}-casein f(183–207) and lactoferricin-B at a concentration of 100 μg/mL for all the samples, except for *E. coli* ATCC 25922 where α_{s2}-casein f(183–207) concentration was 1 mg/mL. The pellet obtained after centrifugation was resuspended in 0.05 M cacodylate buffer containing 2% of glutaraldehyde (SERVA, Heidelberg, Germany) and 4% *para*-formaldehyde (Merck, Darmstadt, Germany). After 1.5 h of incubation at room temperature with shaking, three washes with 0.05 M cacodylate buffer were performed. The pellet was fixed with 1% osmium tetroxide and 1% potassium ferrocyanide during 1 h at 4 °C and treated with 2% uranyl acetate during 1 h before the dehydration in an ethanol series, and embedded in epoxy resin (TAAB, Bershire, UK). Finally, the pellet was sectioned, and examined in a JEM1010 (Jeol, Tokio, Japan) TEM. Microphotographs were taken with a digital camera Bioscan 792 (Gatan Inc, Pleasanton, USA).

3. Results and discussion

3.1. Identification of the initial binding sites of α_{s2}-casein f(183–207) to bacterial membranes

The initial binding step of the peptides to the bacteria is usually the interaction of positively charged peptides with negatively charged

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