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Antibacterial and membrane-damaging activities of mannosylated bovine serum albumin

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ABSTRACT

The aim of this study was to test whether mannosylated BSA (Man-BSA) exerts antibacterial activity on *Escherichia coli* (gram-negative bacteria) and *Staphylococcus aureus* (gram-positive bacteria) via its membrane-damaging effect. Man-BSA caused inhibition of growth of *E. coli* and *S. aureus*. Moreover, bactericidal action of Man-BSA on *E. coli* and *S. aureus* positively correlated with the increase in membrane permeability of the bacterial cells. Morphological examination showed that Man-BSA disrupted bacterial membrane integrity. Destabilization of the lipopolysaccharide (LPS) layer and inhibition of lipoteichoic acid (LTA) biosynthesis in the cell wall increased the bactericidal effect of Man-BSA on *E. coli* and *S. aureus*. Man-BSA also induced leakage and fusion of membrane-mimicking liposomes in *E. coli* and *S. aureus*. Man-BSA showed similar binding affinity for LPS and LTA. LPS and LTA strongly suppressed the membrane-damaging activity of Man-BSA, whereas an increase in the Man-BSA concentration attenuated the inhibitory action of LPS and LTA. Taken together, our data indicate that Man-BSA's bactericidal activity depends strongly on its ability to induce membrane permeability. Moreover, the bactericidal action of Man-BSA proven in this study suggests that Man-BSA may serve as a prototype for the development of anti-infective agents targeting *E. coli* and *S. aureus*.

Introduction

The development of resistance of bacteria to antibiotics is a global problem that underscores the need for new therapeutic agents. Antimicrobial peptides and proteins are currently under consideration as a potential alternative to conventional antibiotics, on account of their widespread occurrence in nature [1,2]. Antimicrobial peptides and proteins display a broad spectrum of activities against a wide range of pathogens including bacteria, fungi and enveloped viruses. A common feature of most of these peptides and proteins is that they are cationic and have amphipathic properties. Because the bacterial membrane consists of abundant negatively charged phospholipids, it is believed that most of the antimicrobial peptides and proteins interact with anionic phospholipids and kill microorganisms by permeabilizing the bacterial membrane, by thinning the membrane or by destabilizing the membrane structure [3]. Nevertheless, antimicrobial peptides and proteins may kill bacteria by inhibiting macromolecular biosynthesis and/or by interacting with specific vital components inside the bacterial cells [3].

Given that membrane composition of bacteria includes abundantly anionic phospholipids, an increase in the positive charge of the antibiotic proteins via blocking of negatively charged carboxylate groups may enhance the interaction of such proteins with the bacterial membrane and thus enhance the potency of the antibacterial effect. Several studies revealed that bovine α-lactoglobulin and bovine lactoferrin have antibacterial properties [4–6]. Pan et al. [7–9] found that amidation of bovine α-lactoglobulin and bovine lactoferrin increases the net positive charge of these proteins, sharply increasing their bactericidal activity. Nevertheless, there are no studies exploring the possibility that novel antimicrobial proteins could be prepared from nonbactericidal proteins after modification of carboxyl groups. Because antimicrobial proteins usually exert their activity by damaging the bacterial membrane, proteins that preferably interact with phospholipids but do not

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show bactericidal activity can be a good candidate for testing of the above possibility. Various studies revealed that bovine serum albumin (BSA)[1] can transfer a lipid amphiphile to lipid bilayer membranes [10], meaning that BSA can interact with lipid bilayers. Moreover, mannosylated BSA (Man-BSA), which is prepared by conjugation of BSA's carboxyl groups with *p*-aminophenyl-α-mannopyranoside is reported to have membrane-damaging effects on zwitterionic lipid vesicles [11]. Thus, the aim of this study was to characterize the antibacterial activity and the mechanism of Man-BSA's action.

Materials and methods

BSA (Fraction V, fatty acid free, catalog number 775835, purity >99%) was obtained from Roche Applied Science. Calcein, rifampin, propidium iodide (PI), cardiolipin, 1-ethyl-3-(diethylaminopropyl) -carbodiimide hydrochloride (EDC), *p*-aminophenyl α-D-mannopyranoside, *p*-aminophenyl α-D-glucopyranoside, rhodamine isothiocyanate, sodium dithionite, egg yolk phosphatidylglycerol (EYPG), egg yolk phosphatidylethanolamine (EYPE), lipopolysaccharide (LPS) 0111:B4 from *Escherichia coli* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were purchased from Sigma–Aldrich Inc., and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine (NBD-PE) and lissamine rhodamine B 1,2-dihexadecanoly-*sn*-glycero-3-phosphoethanolamine (Rh-PE) were the products of Molecular Probes. Unless otherwise specified, all other reagents were analytical grade.

Preparation of Man-BSA and glucosylated-BSA (Glu-BSA)

Man-BSA was prepared by coupling *p*-aminophenyl α-D-mannopyranoside to BSA through water-soluble EDC according to the method described in [11]. BSA contains 41 Asp, 58 Glu and 1 C-terminal carboxyl groups. MALDI-TOF analyses showed that, as compared with BSA, Man-BSA showed an increase in the molecular weight by 24,050 Da [11]. Thus, the conjugation of approximately 95 *p*-aminophenyl α-D-mannopyranoside with the carboxyl groups in Man-BSA was calculated from the increment in the molecular weight. BSA was also modified with *p*-aminophenyl α-D-glucopyranoside according to the procedure described in [11]. MALDI-TOF analyses revealed that approximately 86 of the 100 carboxyl groups in BSA were conjugated with *p*-aminophenyl α-D-glucopyranoside.

Bacterial strains

E. coli (JM109) and *S. aureus* (ATCC 25923) were used in this study. *E. coli* were maintained on Luria–Bertani (LB) agar plate at 37 °C, and *S. aureus* were maintained on tryptic soy agar plate at 37 °C.

Antimicrobial assay

E. coli (JM109) were grown in LB medium and *S. aureus* were grown in tryptic soy broth (TSB) from a single colony with agitation at 37 °C. Bacterial number was then evaluated by measuring the optical density (OD) at 550 nm (OD_{550nm} ~0.3 for *E. coli* and OD_{550nm} ~0.5 for *S. aureus* corresponding to approximately to 10^8 CFU/ml). The bacteria were pelleted, washed with PBS, and then concentrated to 5×10^7 CFU/ml in PBS. One hundred microliters of the bacteria suspension were incubated with Man-BSA at 37 °C for indicated time periods. Then the cells were cultured in 1 ml LB medium for *E. coli* or TSB for *S. aureus* for 6 h with gently shaking. Then antimicrobial activity of Man-BSA was measured using optical density (OD) at 550 nm. Antimicrobial activity of Man-BSA was calculated as (OD_{550} after Man-BSA treatment)/(OD_{550} without Man-BSA treatment) × 100%.

Bacterial membrane permeability assessment

Bacterial suspensions (5×10^7 CFU/ml) were treated with Man-BSA or PBS in a total volume of 100 μl under the same conditions used in antimicrobial assay. Subsequently, the bacterial cells (*E. coli* or *S. aureus*) were incubated with propidium iodide (PI) for 30 min in the dark at room temperature. Then the bacterial cells were washed with PBS, and resuspended in 1 ml PBS. PI fluorescence values were corrected for background (extracellular) dye fluorescence and expressed as a percentage relative to the value obtained after complete permeabilization of the cells by heating at 100 °C for 5 min. Uptake of PI was measured by Beckman Coulter Paradigm™ Detection Platform with excitation at 535 nm and emission at 595 nm.

Competitive replacement of LPS-bound or LTA-bound rhodamine-labeled Man-BSA (Rh-Man-BSA) by unlabeled Man-BSA

Modification of Man-BSA with rhodamine isothiocyanate was prepared according to the procedure described in [12]. Rh-Man-BSA (1 μM) was titrated with increasing concentrations of LPS or LTA until maximal changes in fluorescence intensity of Rh-Man-BSA was achieved. Then increasing concentrations of unlabeled Man-BSA were added to compete for binding of Rh-Man-BSA with LPS or LTA. Competitive binding was monitored at excitation wavelength and emission wavelength at 550 and 580 nm, respectively.

Release of entrapped fluorescent marker from liposomes

Membrane-damaging activity of Man-BSA was determined by measuring the release of the liposome-entrapped, self-quenching fluorescent dye calcein. EYPE/EYPG (75/25, mol/mol) or EYPG/cardiolipin (60/40, mol/mol) were dissolved in chloroform/methanol (v/v, 2:1) and dried by evaporation. Buffer (10 mM Tris–HCl, 100 mM NaCl, pH 7.5) containing 50 mM calcein was added to the film of lipids, and after hydration the suspension was shaken vigorously. The multilamellar vesicles obtained in this way were extruded 10 times, above the transition temperature, through a 100-nm polycarbonate filter and applied to a Sepharose 6B column (2 × 15 cm) to separate the liposome from the free calcein. Leakage was induced by adding aliquots of Man-BSA to a vesicle suspension directly in the cuvette used for fluorescence determination at 30 °C. The kinetics of membrane damage were monitored by the increase in fluorescence with emission at 520 nm and excitation at 490 nm, and the signal was expressed as percentage of total calcein release after the addition of 0.2% Triton X-100.

Fusion assay of phospholipid vesicles

Liposome fusion induced by Man-BSA was measured by increase in fluorescence resonance energy transfer between two lipid probes (NBD-PE and Rh-PE). NBD-PE and Rh-PE were used as donor and acceptor fluorescent lipid, respectively. NBD-PE and

[1] *Abbreviations used:* BSA, bovine serum albumin; Man-BSA, mannosylated BSA; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PI, propidium iodide; EDC, 1-ethyl-3-(diethylaminopropyl)-carbodiimide hydrochloride; EYPG, egg yolk phosphatidylglycerol; EYPE, egg yolk phosphatidylethanolamine; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; Rh-PE, lissamine rhodamine B 1,2-dihexadecanoly-*sn*-glycero-3-phosphoethanolamine; LB, Luria–Bertani; TSB, tryptic soy broth; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.