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Purification and characterization of the antibacterial peptidase lysostaphin from *Staphylococcus simulans*: Adverse influence of Zn^{2+} on bacteriolytic activity

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ABSTRACT

Lysostaphin, a bacteriolytic toxin from *Staphylococcus simulans*, is a Zn^{2+}-dependent endopeptidase that cleaves pentaglycine cross-bridges found in peptidoglycan of certain *Staphylococci*. Here, we have investigated a critical influence of Zn^{2+} ions on lysostaphin-induced bioactivity. Initially, we succeeded in producing a large amount with high purity of the 28-kDa His-tagged mature lysostaphin *via* soluble expression in *Escherichia coli* and subsequent purification *via* immobilized-Ni^{2+} affinity chromatography (IMAC). The purified monomeric bacteriocin exhibited concentration-dependent bioactivity against *S. aureus* and its methicillin-resistant strain through cell-wall hydrolysis rather than membrane perturbation. Following pre-incubation of the purified lysostaphin with exogenous Zn^{2+}, a marked inhibition in staphylolytic activity was observed. When the premixture was exposed to 1,10-phenanthroline (PNT, a Zn^{2+}-chelator), the adverse effect of the exogenous Zn^{2+} on bioactivity was greatly decreased. Conversely, lysostaphin pre-treated with excess PNT retained relatively high bioactivity, indicating ineffective chelation of PNT to detach the catalytic Zn^{2+} from the active-site pocket. Structural analysis of the lysostaphin-catalytic domain together with amino acid sequence alignments of lysostaphin-like endopeptidases revealed a potential extraneous Zn^{2+}-binding site found in close proximity to the Zn^{2+}-coordinating active site. Overall our results provide more insights into an adverse influence of exogenous Zn^{2+} ions on staphylolytic activity of the purified Zn^{2+}-dependent endopeptidase lysostaphin, implicating the presence of an extraneous inhibitory metal-binding site.

1. Introduction

Bacteriocin lysostaphin [Enzyme Commission 3.4.24.75], a member of the M23 metalloprotease family, is a Zn^{2+}-dependent antibacterial endopeptidase produced from the Gram-positive coccus, *Staphylococcus simulans* subsp. *staphylolyticus* and cleaves pentaglycine cross-bridges present in the cell-wall peptidoglycan of certain *Staphylococci* [1]. Unlike chemical antibiotics that may either kill or inhibit the growth of bacteria, lysostaphin is very effective against both actively growing and dormant cells of *Staphylococci* [2]. Although lysostaphin in combination with β-lactam antibiotics could assist in the treatment of oxacillin-resistant *S. epidermidis* infections [3], this bacteriolytic toxin alone not only effectively disrupted both *S. aureus*- and *S. epidermidis*-associated biofilms but also killed *S. aureus* in the biofilms [4]. In other studies, lysostaphin together with LysK-the staphylococcal bacteriophage K endolysin revealed a clear synergistic effect in killing methicillin-resistant *S. aureus* (MRSA) strains [5], indicating the feasibility of lysostaphin to be combined with either antibiotics or other peptidolytic enzymes for improving therapeutic potential in treating both multidrug-resistant and chronic staphylococcal infections.

Antimicrobial peptidase lysostaphin (classified as Class III bacteriocins) is synthesized as a 493-amino acid pre-proenzyme comprising a leader sequence (residues of 1–23), a tandem-repeat region (residues 24–247), a Zn^{2+}-containing catalytic domain (residues 248–384), a flexible linker (residues 385–400) and a cell wall-targeting (CWT) domain (residues 401–493) (*see* Fig. 1A) [6]. Upon maturation *in vivo*, the

Abbreviations: IMAC, immobilized metal ion affinity chromatography; MRSA, methicillin-resistant *Staphylococcus aureus* strain; Lss, the ~28-kDa mature lysostaphin; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; PNT, 1,10-phenanthroline

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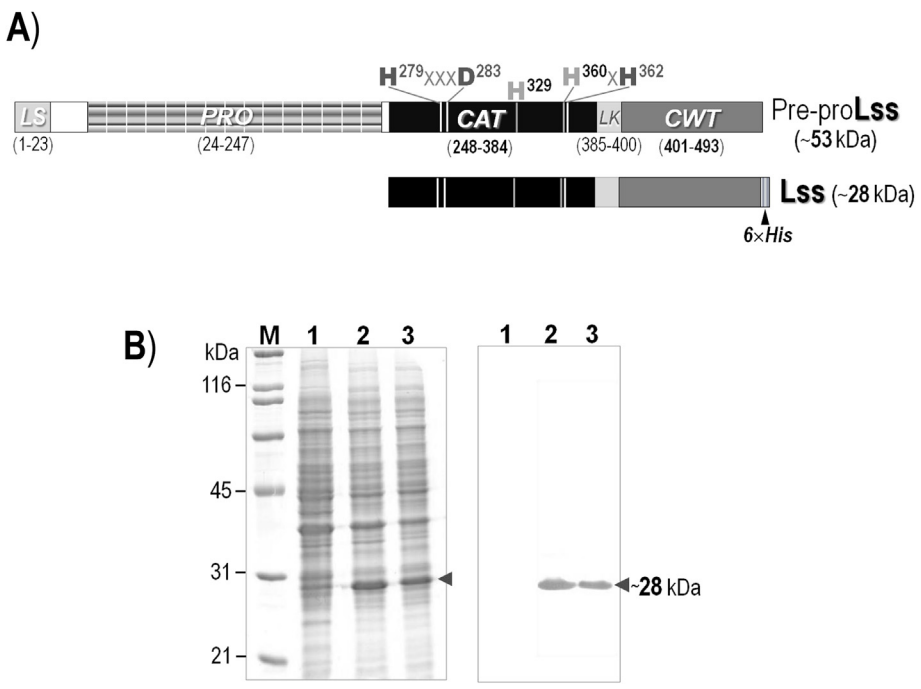

Fig. 1. (**A**) Schematic diagram of the ~53-kDa full length pre-proLss showing leading sequence (LS), proregion (PRO), catalytic domain (CAT), linker (LK) and cell wall-targeting domain (CWT). Stripe boxes within PRO represent individual tandem repeats. Vertical white and gray lines indicate Zn^{2+}-coordinated catalytic residues (His^{279}, Asp^{283} and His^{362}) and two putative exogenous Zn^{2+}-binding residues (His^{329} and His^{360}), respectively. The ~28-kDa Lss was fused at the C terminus with 6 × His tag. (**B**) *Left panel*, SDS-PAGE (Coomassie brilliant blue-stained 12% gel) analysis of crude extracts from *E. coli* cells expressing Lss (lane 2) and soluble fraction of cell lysate from lane 2 after centrifugation (lane 3). Crude extracts from non-induced *E. coli* cells were used as a negative control (lane 1). *Right panel*, the corresponding Western blot probed with anti-His tag antibody followed by ALP-conjugated secondary antibody.

signal sequence and tandem repeats are removed, generating a ~28-kDa active peptidase lysostaphin [7], hereafter termed 'Lss'. This two-domain mature enzyme structurally shares its individual characterized domains [8] with the ~16-kDa catalytic domain of both LytM-autolysin [9] and LytU (another Lss homologue) from *S. aureus* [10] as well as the ~10-kDa CWT domain of ALE-1, a close Lss homologue from *S. capitis* EPK1 [11]. Of particular interest in the Zn^{2+}-containing catalytic domain, the active-site triad residues occur in His-*X*-*X*-*X*-Asp and His-*X*-His characteristics of the M23 endopeptidase family [12], suggesting a general catalytic mechanism of the endopeptidase lysostaphin family that have a preference for cleavage of glycyl bonds, although they might have some divergent aspects.

The role of catalytic Zn^{2+} in Lss-induced lytic activity is primarily due to the activation of a water molecule to serve as a nucleophile in cleaving inter-peptide bridges of the *S. aureus* cell wall [13]. Nevertheless, differences in the Zn^{2+}-coordination state may reflect catalytic variations among the lysostaphin family. Notably, the first X-ray crystal structure of the Lss-catalytic domain showed that the Zn^{2+} cofactor in the active site is hexa-coordinated by three amino acid ligands and three water molecules [8]. Differently, tetra-coordination for the catalytic Zn^{2+} ion by the three corresponding side-chains and only one water molecule was crystallographically described for the LytM-catalytic domain [9]. Very recently, both one- and two-Zn^{2+}-bound catalytic forms of LytU have been observed *via* ^{1}H, ^{13}C and ^{15}N NMR studies [10], implying that this enzyme may possess some other properties that are not shared by any of its homologues.

Besides LytU [10], such dual Zn^{2+} coordination within the enzyme-active site has also been reported for several Zn^{2+}-dependent proteases, including both exoproteases, *e.g.*, bovine carboxypeptidase A [14] and endoproteases, *e.g.*, thermolysin from *B. thermoproteolyticus* [15]. In our present study, we achieved a simple approach for producing a large amount with high purity of the 28-kDa His-tagged Lss *via* IMAC purification of the soluble tagged protein highly expressed in *E. coli*. In addition, we have clearly demonstrated an inhibitory effect of the exogenously added Zn^{2+} ion on Lss-induced staphylolytic activity, similar to that has recently been observed for LytU [10], allowing us to plausibly infer the presence of an extraneous inhibitory metal-binding site.

2. Materials and methods

2.1. Construction of the recombinant plasmid with His-tagged fusion

pPLss-493 recombinant plasmid (see Supplementary Fig. 1, upper) encoding the ~53-kDa pre-proLss (493 residues) was used as a template for gene manipulation. A ~740-bp *Nde*I-*Hin*dIII segment located at the 3′-end of the ~1.5-kb protoxin gene was amplified with an added 6 × His sequence *via* polymerase chain reaction (PCR) using high-fidelity Phusion DNA polymerase (Finnzymes, Vantaa, Finland). The PCR-amplified product was subsequently cloned into the pET-17b vector, giving pLss-246 M/H_6 plasmid (see Supplementary Fig. 1, under) that encodes the ~28-kDa His-tagged Lss peptidase. The resulting plasmid was transformed into *E. coli* strain JM109 for plasmid verification by restriction endonuclease digestion and DNA sequencing before being retransformed into an expression host, protease-deficient *E. coli* strain BL21 (DE3)pLysS.

2.2. Protein expression and characterization

E. coli cells harboring pLss-246 M/H_6 were grown at 37 °C in 500 mL of Luria-Bertani liquid medium containing 100 μg/mL ampicillin until OD_{600} of the culture reached ~0.6 and then protein expression was induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside at a final concentration of 0.1 mM for additional 4 h. Lss-expressing cells were harvested by centrifugation (6000 × *g*, 4 °C, 10 min) and re-suspended in 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. The cell suspension was then subjected to ultra-sonication for cell disruption using VCX 750-Sonics Vibra Cell™ (Sonics & Materials, Inc., Newtown, CT, USA) with the following parameters: 5 cycles of amplitude 60%, 10-s ON, 30-s OFF with a total time ON = 1 min/cycle. After centrifugation (13,000 × *g*, 4 °C, 15 min), the total lysate and supernatant were analyzed by sodiumdodecyl sulfate-(12% w/v) polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Western blotting and mass spectrometry (MS) analysis

Protein samples resolved by SDS–PAGE were electroblotted onto a nitrocellulose membrane. After blocking with 5% skim milk in PBS