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Specificity of the Action of Lysoamidase on *Staphylococcus aureus* 209P Cell Walls

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Abstract—Specificity of *Staphylococcus aureus* 209P cell wall hydrolysis by the L1 and L2-bacteriolytic enzymes from lysoamidase lytic complex was studied. L1-peptidase was shown to display both glycyl-glycine endopeptidase and N-acetylmuramyl-L-alanine amidase enzymatic activities on the *S. aureus* peptidoglycan molecule, whereas L2-peptidase acts as N-acetylmuramyl-L-alanine amidase.

Key words: lysoamidase, bacteriolytic enzymes, cell wall, substrate specificity

The bacteriolytic complex lysoamidase was earlier extracted from the culture broth filtrate of *Xanthomonas* sp. bacterial strain. The preparation is used for treatment of diseases caused by pathogenic gram-positive microorganisms [1]. It was revealed that the bacteriolytic enzymes in the lysoamidase preparation are capable of degrading *Staphylococcus aureus* peptidoglycan and hydrolyzing bonds between adjacent glycine residues of the cross-linking bridge (similar to glycyl-glycine endopeptidases), bonds between lactyl residues of muramic acid and N-terminal alanine of the peptide subunit (similar to N-acetylmuramyl-L-alanine amidases), and β-1,4-bonds between muramic acid and N-acetylglucosamine (similar to muramidases) [2]. Lysoamidase exhibits N-acetylmuramyl-L-alanine amidase and muramidase activities toward the cell walls of *Micrococcus luteus* (All-Russian Collection of Microorganisms, VKM V-1314) [3]. Cell walls of a penicillin-resistant strain of *Streptococcus pneumoniae* are hydrolyzed by lysoamidase as N-acetylmuramyl-L-alanine amidase and alanyl-alanine endopeptidase [4].

Three bacteriolytic enzymes [5-7] were isolated from the lysoamidase preparation (containing at least 20 proteins with different enzymatic activities) with high purity. One of these enzymes was muramidase; the substrate specificity of the remaining two was not investigated. It was determined that L2 enzyme cleaves the bond between phenylalanine and *p*-nitroanilide in the proteinase-specific substrate (anthraniloyl-alanyl-alanyl-phenylalanyl-*p*-nitroanilide, Abz-Ala-Ala-Phe-pNA) [8], and L1 enzyme also hydrolyzes this substrate [9]. Both enzymes are inactive toward the specific muramidase substrate (3,4-dinitrophenyl-tetra-N-acetyl-β-D-chitotetraoside) [5, 6].

The aim of the presented work was the determination of substrate specificity of lysoamidase L1 and L2 bacteriolytic enzymes with *S. aureus* cell walls as the substrate.

MATERIALS AND METHODS

Staphylococcus aureus 209P cells were grown in yeast peptone medium in 500 ml flasks with 150 ml of the medium on a shaker at 37°C for 24 h. Cultural broth was autoclaved for 30 min at 1 atm. The cells were precipitated by centrifugation at 5000*g* for 20 min, then washed three times with 10 mM Tris-HCl-buffer and lyophilized.

Extraction and purification of enzymes. Microbial preparation of lysoamidase enzyme was obtained from *Xanthomonas* sp. culture broth filtrate cultivated using an experimental device at the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino).

L1 bacteriolytic enzyme was obtained from the lysoamidase preparation by Sephacryl S-200 gel filtration, CM-Sephadex ion-exchange chromatography, and Mono S column FPLC [5].

Purification of L2 bacteriolytic enzyme and muramidase was performed from the culture broth of *Xanthomonas* sp. low-activity strain according to a published procedure [7] that includes CM-Sephadex and DEAE-Toyopearl ion-exchange chromatography and Toyopearl HW-50F gel filtration.

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Preparation of *S. aureus* cell walls. Cell walls were obtained by Sharon's technique modified by Shaw et al. [10]. Accordingly, *S. aureus* cells were rinsed three times with tenfold volume of 0.1 M phosphate buffer, pH 6.8, with centrifugation (5000*g*, 10 min), then frozen at −42°C and broken in a press-disintegrator. DNase and RNase were added to the homogenate (to final concentration 20 μg per ml mixture). Intact cells were removed by centrifugation (1200*g*, 15 min). The cell wall fraction was precipitated (7500*g*, 15 min) and 4 times rinsed with cold 0.1 M Tris-HCl-buffer, pH 7.2 (10,000*g*, 15 min). To inactivate the autolytic enzymes, the cell wall fraction was heated using a water bath for 15 min at 100°C, then suspended in 50 ml of 0.1 M Tris-HCl-buffer, pH 8.0, and incubated with trypsin (final concentration 100 μg per ml mixture) for 3 h at 37°C. The resulting cell walls preparation was rinsed three times with water, suspended in 30 ml of chloroform–methanol (2 : 1 v/v) enabling the extraction of lipids, and dried under vacuum with $CaCl_2$.

Hydrolysis of *S. aureus* cell walls by lysoamidase. Solutions of 2.5 ml of L1 (10 units/ml) and L2 (7 units/ml) enzymes in the working buffer were added to 2.5 ml of *S. aureus* cell wall suspension in 10 mM Tris-HCl-buffer, pH 8.0 (9 mg per ml mixture). The reaction was performed for 4 h at 55°C. Incubation mixture aliquots were taken in the course of the reaction to determine suspension absorbance and amount of free NH_2-groups and reducing groups in sugars.

Determination of reducing sugars. Reducing sugars were determined according to Park and Johnson [11]. Ferricyanide reagent (1 ml, 0.5 g potassium ferricyanide $K_3Fe(CN)_6$ in 1 liter H_2O) and 1 ml carbonate-cyanide reagent (5.3 g of sodium carbonate and 0.65 g KCN per 1 liter of solution) were added to 200 μl of the investigated solution. After agitation, the vials were heated for 15 min in a boiling water bath and cooled. Second, 5 ml of reagent containing ferric ammonium sulfate (1.5 g $NH_4Fe(SO_4)\cdot 12H_2O$ and 1 g SDS in 1 liter 0.025 M H_2SO_4) was added to the sample, and then the mixture was agitated. The blue coloration that developed was determined after 15 min at 690 nm. The number of reducing groups was determined according to a calibration curve plotted for N-acetylglucosamine.

Determination of NH_2 groups. Free NH_2 groups of amino acids released during the cell wall lysis were determined by the technique of Ghuysen and Strominger [12]. Sixty microliters of 2,4-dinitrofluorobenzene solution (13 μl in 1 ml pure ethanol) was added to 0.6 ml of cell wall hydrolyzate. The mixture was heated in a water bath for 30 min at 60°C. Then 2.4 ml of 2 M HCl was added to the samples, and the absorption was measured using an SF-26 spectrophotometer at 420 nm. The content of free NH_2 groups was determined based on a calibration curve plotted for glutamic acid. Cell walls that were not treated by the enzyme were used as a reference.

Determination of N-terminal amino acids in peptides. N-terminal amino acids were determined by the dinitrophenylation technique [12]. Three hundred microliters of 2,4-dinitrofluorobenzene (DNF) solution (13 μl in 1 ml pure ethanol) was added to 3 ml of cell wall hydrolyzate by homogeneous bacteriolytic enzymes. The mixture was held for 30 min in a water bath at 60°C and dried under vacuum. Then the samples were hydrolyzed with 6 M HCl for 18 h at 100°C. After that, DNF-derivatives of amino acids were extracted with chloroform. The resulting samples were analyzed using an LC 6000 E amino acid analyzer (Biotronic, Germany). Cell walls that were not treated by dinitrofluorobenzene were used as a reference.

Thin layer chromatography. DNF-derivatives of amino acids resulting from cell wall hydrolyzate dinitrophenylation were analyzed using TLC on PEI-Cellulose F plates (Schleicher und Schull, Germany) in chloroform–benzalcohol–acetic acid (70 : 30 : 3 v/v).

Reagents used. DEAE-Toyopearl and Toyopearl HW-50 F were from Toyo Soda (Japan), Sephacryl S-200 from Pharmacia (Sweden), and CM-Sephadex and 2,4-dinitrophenol from Wako (Japan). Other reagents were domestic products of analytical grade.

RESULTS AND DISCUSSION

Bacteriolytic enzymes are divided in three classes depending on the type of peptidoglycan bond hydrolyzed [13, 14]. The first class contains glycosidases, which degrade the glycan chains of peptidoglycan. Those are in turn divided into endo-N-acetylmuramidases, hydrolyzing the bonds between Mur (muramic acid) and GlcN (N-acetylglucosamine), and endo-N-acetylglucosaminidases hydrolyzing the bonds between GclN and Mur. The second class of lytic enzymes consists of N-acetylmuramyl-L-alanine amidases. They hydrolyze bonds between muramic acid lactyl residues and N-terminal residues of the peptide subunits. The third enzyme group contains endopeptidases. These enzymes hydrolyze peptide bonds of peptidoglycan cross-linking bridge or a bond between an amino acid located at position 4 of one peptide subunit and that located at position 3 of another peptide subunit [15].

Substrate specificity is an important characteristic of the action of bacteriolytic enzymes. In this study, the substrate specificities of lysoamidase L1 and L2 enzymes toward peptidoglycan from *S. aureus* were determined. For this purpose, the cells walls were prepared from *S. aureus* cells. The cell walls contained the following amino acids: lysine, glutamine, glycine, and alanine in proportion of 8.21 : 8.91 : 39.75 : 18.67 (1 : 1 : 5 : 2). Amino acid analysis after dinitrophenylation revealed the covalent bonds between all amino acids, which indicated the nativity of the cell wall preparation. Teichoic acids were not removed from the cell walls since it was earlier estab-

lished that homogeneous L1 and L2 enzymes do not hydrolyze the native peptidoglycan of *S. aureus* [16].

Degradation of peptidoglycan occurred on treatment of the cell wall with L1 and L2 enzymes. Absorption of the suspension decreased by 67% for L1 and by 6% for L2, the amount of free amino groups increased from 8 to 71 μmol per mg of the cell wall for L1 and to 47 μmol per mg of cell wall for L2 (Figs. 1 and 2). At the same time, the number of reducing sugar groups did not change, which indicates that L1 and L2 enzymes from lysoamidase hydrolyze only the peptide moiety of *S. aureus* peptidoglycan, and they are thus classified as peptidases.

Bacteriolytic peptidases were earlier extracted from the culture broth of a number of other bacteria, including *Myxococcus xanthus* [17], *Cytophaga* [18], *Pseudomonas lytica* [19], etc.

Substrate specificity of L1 and L2 peptidases toward the cell walls of *S. aureus* was determined by the dinitrophenylation technique. Since L1 and L2 enzymes hydrolyzed the peptide moiety of peptidoglycan, the result of their action was the formation of free amino groups capable of interaction with dinitrofluorobenzene. Therefore, the cell wall hydrolyzate was treated by dinitrofluorobenzene, and then total acid hydrolysis of the preparation was performed. Dinitrofluorobenzene derivatives of amino acids were extracted using chloroform; the remaining amino acids were analyzed in the amino acid analyzer. Hydrolyzates of cell walls that were not treated with dinitrofluorobenzene were used as a reference. Based on the difference in amino acid composition between the original *S. aureus* peptidoglycan and *S. aureus* peptidoglycan after dinitrophenylation and subsequent removal of dinitrofluorobenzene derivatives of amino acids, a conclusion was made regarding the bond cleaved by an enzyme. Table 1 illustrates that 0.53 mol of glycine and 0.34 mol of alanine per 1 mol of glutamic acid became accessible to dinitrofluorobenzene reaction after the cell walls hydrolysis by L1 peptidase. These data indicate that in the peptidoglycan molecule treated with L1 peptidase the bonds exposed to hydrolysis were those formed at the N-terminus by Ala and Gly. The analysis showed that the L1 enzyme has both glycyl-glycine endopeptidase and N-acetylmuramyl-L-alanine amidase activities toward *S. aureus* peptidoglycan. Enzymes with similar specificities were found in *Achromobacter lyticus* and *Lysobacter enzymogenes* [20, 21].

As mentioned above, lysoamidase exhibits N-acetylmuramyl-L-alanine amidase and alanyl-alanine endopeptidase activities upon the cell walls of *Streptococcus pneumoniae*, which is resistant to penicillin and has -Ala-Ala-Ser-Lys- interpeptide bridge structure (unlike to pentaglycine bridge of staphylococcus) [4]. The alanyl-alanine endopeptidase activity of lysoamidase on *S. pneumoniae* cell walls is probably also due to L1 peptidase.

The action of L2 peptidase upon staphylococcus cell walls leads to the release of 0.15 mol of N-terminal alanine per 1 mol glutamic acid. These data suggest that the bonds exposed to hydrolysis after peptidoglycan treatment with L2 peptidase were those formed by Ala at the N-terminus; however, the amount obtained was not sufficiently high to allow the unambiguous statement that L2 enzyme is an N-acetylmuramyl-L-alanine amidase.

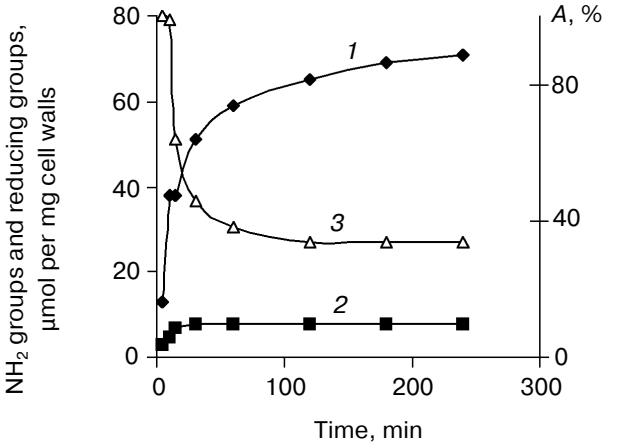


Fig. 1. Release of amino acid NH_2 groups (*1*) and reducing groups of sugars (*2*) in the course of *S. aureus* 209P cell wall hydrolysis by L1 peptidase; *3*) absorption (*A*, %).

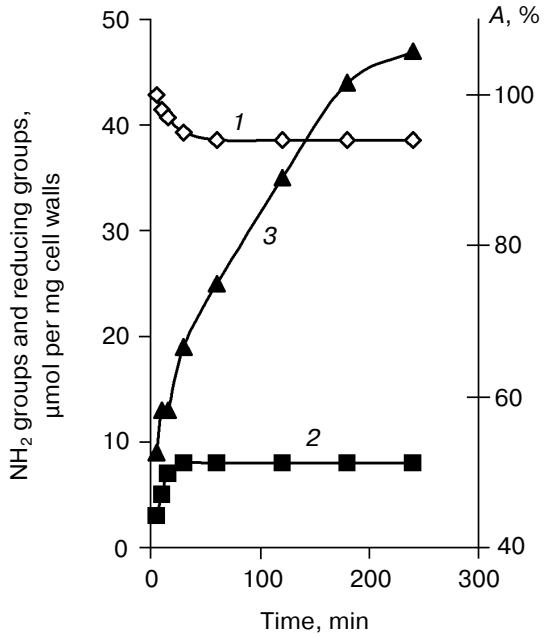


Fig. 2. Release of amino acid NH_2 groups (*1*) and reducing groups of sugars (*2*) in the course of *S. aureus* 209P cell wall hydrolysis by L2 peptidase; *3*) absorption (*A*, %).

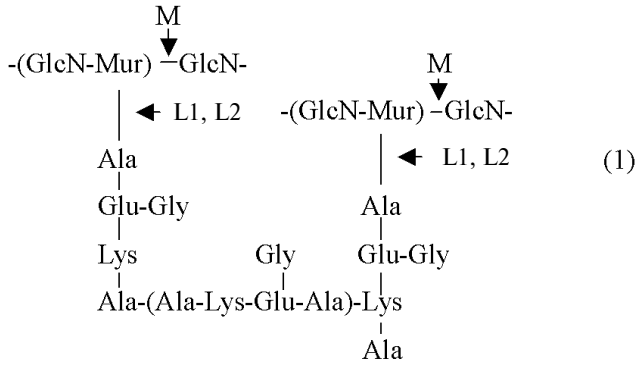
Table 1. Amino acid composition of *S. aureus* 209P cell walls before and after 4 h hydrolysis by lysoamidase, L1 and L2 enzymes

Component	Content, nmol							
	original cell walls		lysoamidase		L1		L2	
Glu	17.85	(1.00)	*23.33*	*(1.00)*	*15.47*	*(1.00)*	*4.05*	*(1.00)*
Gly	80.58	(4.51)	*77.96*	*(3.34)*	*71.21*	*(3.98)*	*19.11*	*(4.71)*
Ala	41.28	(2.31)	*39.63*	*(1.70)*	*35.17*	*(1.97)*	*8.75*	*(2.16)*
Lys	14.09	(0.79)	*19.87*	*(0.85)*	*13.18*	*(0.74)*	*3.61*	*(0.98)*

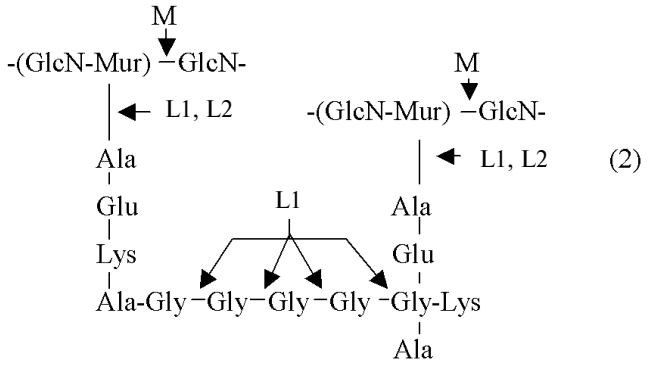
Note: Molar content per 1 mol glutamic acid is shown in brackets; content of amino acids after enzymatic hydrolysis of cell walls and subsequent dinitrophenylation and extraction of dinitrophenol derivatives is shown in italic.

Thin layer chromatography was used to confirm the specificity of L2 peptidase action. DNF-derivatives of Ala were present in the hydrolyzate of cell walls of *S. aureus* subjected to treatment by L2 peptidase, as well as after the treatment with dinitrofluorobenzene, suggesting N-acetylmuramyl-L-alanine amidase activity of L2 peptidase.

A comparative study of lysoamidase enzyme activities on *Micrococcus luteus* and *S. aureus* cells was also performed. The structure of cross-linking bridge of the investigated micrococcus is analogous to the structure of its peptide subunit, and differs from the pentaglycine bridge of *S. aureus*:



(1)



(2)

Arrows show the bonds in the peptidoglycan molecule that are hydrolyzed by bacteriolytic enzymes of lysoamidase (L1, glycylglycine endopeptidase; L1 and L2, N-acetylmuramyl-L-alanine amidase; M, muramidase). It should be noted that cross-linking ratio of *M. luteus* peptidoglycan is lower than in the case of *S. aureus*. Hence, *M. luteus* cell wall lacks glycyl-glycine bond that could be cleaved by the L1 peptidase.

The results are presented in Table 2. Obviously, the values of L2 peptidase bacteriolytic activity toward *S. aureus* and *M. luteus* cells are comparable, and may be defined only by the N-acetylmuramyl-L-alanine amidase activity of the enzyme. The value of the L1 peptidase activity upon *M. luteus* cells is significantly lower than upon *S. aureus* cells, and can be referred to as N-acetylmuramyl-L-alanine amidase activity of the enzyme. Muramidase activity in both cases is low—1.07 and 1.06 units/ml, respectively.

Therefore, the data presented show that *S. aureus* peptidoglycan is hydrolyzed by L1 peptidase as glycylglycine endopeptidase and N-acetylmuramyl-L-alanine amidase, while L2 peptidase displays only acetylmuramyl-L-alanine amidase activity toward this substrate (2).

Table 2. Activity of L1 and L2 peptidases and muramidase from lysoamidase preparation on *S. aureus* 209P and *M. luteus* cells

Cells	Activity, units/ml		
	L1	L2	muramidase
S. aureus	58	6.6	1.07
M. luteus	6	8.5	1.06

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