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Purification and Some Properties of Lysostaphin, a Glycylglycine Endopeptidase from the Culture Liquid of *Staphylococcus simulans* biovar *staphylolyticus*

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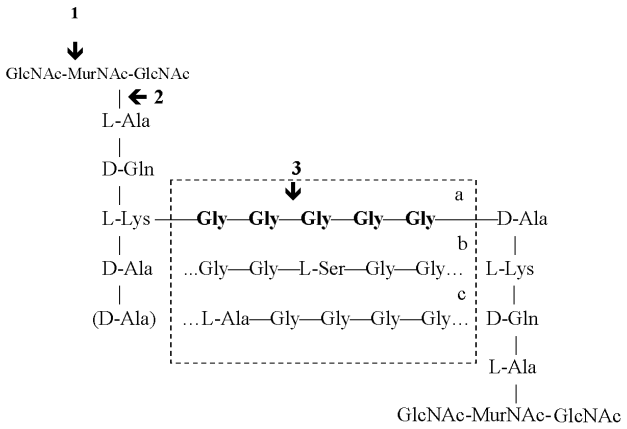
Abstract—This work presents a method for purification of lysostaphin, a glycylglycine endopeptidase, from the culture liquid of *S. simulans* biovar *staphylolyticus* to homogeneity in a few steps. The method includes ultrafiltration and ion-exchange and hydrophobic chromatographies. The enzyme was isolated in preparative amounts with the yield of 51%. Some physical and chemical properties of the enzyme are described.

Key words: lysostaphin, *Staphylococcus simulans* biovar *staphylolyticus*, glycylglycine endopeptidase, purification, properties, hydrophobic chromatography

Lysostaphin is an extracellular zinc-containing endopeptidase cleaving the pentaglycine bridges in peptidoglycans of staphylococci. It was shown that prepro-lysostaphin consisting of 493 amino acid residues is synthesized on ribosomes. While penetrating through the cell wall, 36 residues are split out yielding enzymatically active prolysostaphin. A cysteine proteinase produced by the cells of the producer *S. simulans* biovar *staphylolyticus* performs limited proteolysis of the proenzyme, cleaving the N-terminal fragment after residue 211 to yield lysostaphin that is 4.5-fold more active than pro-lysostaphin [1].

The culture liquid also contains two enzymes—N-acetylglucosaminidase exhibiting the lysozyme-like activity and N-acetylmuramyl-L-alaninamidase [2] (see Scheme).

Within pentaglycine, lysostaphin is supposed to attack the bond between the 3rd and 4th N-terminal residues [3]. Since the Gly–Gly bond is characteristic for the peptidoglycan bridges of staphylococci (in other cases other bonds can be involved in the formation of the bridges), lysostaphin is capable of lysing cells of virtually all strains, including the antibiotic-resistant ones. The Ser/Gly ratio in the bridges differs in different strains and species and can serve as a measure of the resistance towards lysostaphin. The cells of the producer have the highest ratio, 0.62 [1].



Scheme

Fragment of the primary structure of the peptidoglycan of staphylococci. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; a, b, c) three types of interpeptide bridges found in staphylococci; 1, 2, 3) the specific sites for the action of the proteolytic enzymes from the culture liquid of *Staphylococcus simulans* biovar *staphylolyticus*: 1) N-acetylglucosaminidase; 2) 2-acetylmuramyl-L-alaninamidase; 3) glycylglycine endopeptidase (lysostaphin)

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Besides lysostaphin, there are other glycylglycine endopeptidases with similar properties. An enzyme of molecular structure similar to that of prolysostaphin was isolated from the culture liquid of *S. capitis* EPK1. Analysis of the primary structure of glycylglycine endopeptidases led to assigning these enzymes to a new family of zinc-containing peptidases that is characterized by a common sequence of 38 residues [4].

The physicochemical properties of members of this family including lysostaphin are little investigated. The amino acid residues of the active site and the mechanism of its catalytic action have not been determined. To study the properties of glycylglycine proteinases, the purified enzymes must be obtained first.

The goal of the present study was to isolate the necessary amount of highly purified preparation in a few steps and to investigate some properties of lysostaphin.

MATERIALS AND METHODS

In the work we used chemicals from Sigma (USA) without additional purification. Nutrient media based on products of enzymatic and acidic hydrolysis of fish meal were obtained from the State Scientific Center of Applied Microbiology (Russia). Sodium phosphate was a domestic product of analytical grade.

The strain *S. simulans* biovar *staphylolyticus* No. 1030 (Culture Collection of the State Scientific Center of Applied Microbiology) was used as the producer. The strain was stored on a solid nutrient medium based on enzymatic hydrolyzate of fish meal. The strain was cultivated at 33°C in a liquid nutrient medium containing enzymatic fish hydrolyzate (1.2 g/liter) or acidic fish hydrolyzate (1.2 g/liter) with the addition of L-Trp (0.02 g/liter); 50% glycerol solution (6 ml/liter) was used as the source of carbon and energy. The pH of the medium was 7.3-7.4. The culture was grown to the stationary growth phase in different ways: 1) in shaken flasks (120 rpm); 2) in an AKA-210 bioreactor (SKB BP, Pushchino, Moscow Region), working volume of 7 liters, air flow of 5 liters/min, and 200 rpm; 3) in a F-70 bioreactor (GNTs of Applied Microbiology), working volume 50 liters, air flow 30 liters/min, 200 rpm. Concentration of the cells was determined by measuring the absorption at 540 nm.

To purify lysostaphin, the culture liquid (50 liters) was concentrated 8-10-fold by ultrafiltration on hollow fibers retaining substances with molecular weights above 15 kD (an UVA-200 unit, NPO Khimvolokno, Mytishchi, Moscow Region). The cells were separated by centrifugation at 3000*g* for 30 min on an OS-6M centrifuge (Khimvolokno). Then 500 g of cation-exchange resin (Amberlite IRTs-50 in Na^+-form) was added to the supernatant, the pH value was adjusted to 5.5 with 6 M HCl, and the mixture was stirred by a stirrer (60 rpm) for 4-5 h at 4-5°C until no enzymatic activity was detected in the culture liquid. The ion-exchange resin was then placed into a column (6 × 15 cm), and pigments and a part of the extraneous proteins were washed out with 0.2 M phosphate buffer, pH 7.3, until the absorption in the eluate at 280 nm reached values less than 0.01. Then lysostaphin was eluted with the same buffer containing 0.6 M NaCl. The enzyme solution (~550-650 ml) was dialyzed overnight against 0.01 M phosphate buffer, pH 7.0, containing 1 M ammonium sulfate and applied on a column (2 × 6 cm) with phenyl-Sepharose (Pharmacia, Sweden) equilibrated with the same buffer. The column was washed, and then lysostaphin was eluted with the same buffer containing 0.4 M ammonium sulfate and 10% glycerol. The elution profile was recorded measuring the absorption at 280 nm on a UV-1 flow-type spectrophotometer (Pharmacia).

SDS-PAGE was performed as described by Laemmli [5]. A culture of *S. aureus* was grown in the same way as *S. simulans* in a solid nutrient medium containing enzymatic fish hydrolyzate. The biomass grown was washed with 0.01 M phosphate buffer, pH 7.5, containing 0.3 M NaCl. The staphylolytic activity of lysostaphin was determined according to the method described by Schindler and Schuchardt by lysis of live cells of *S. aureus* [6], and the activity of N-acetylglucosaminidase was measured by the hydrolysis of acetone powder of *Micrococcus lysodeikticus* [7]. Protein concentration was determined by the Bradford's method [8]. The concentration of the purified lysostaphin was determined using the value of the absorption coefficient that was calculated according to the Vetlopher equation [9]: $\varepsilon_{280} = (5700 \cdot n_{Trp} + 1300 \cdot n_{Tyr})$, where 5700 and 1300 $M^{-1} \cdot cm^{-1}$ are the values of the molar absorption coefficients for Trp and Tyr, respectively; n_{Trp} and n_{Tyr} are the number of Trp and Tyr residues in the molecule of lysostaphin (8 and 16, respectively). To calculate the specific activity of lysostaphin the molecular weight of the enzyme was taken as 26,900 g/mol [10].

RESULTS

When the culture of *S. simulans* biovar *staphylolyticus* was grown on the acidic fish hydrolyzate, N-acetylglucosaminidase exhibited a lysozyme-like activity, but no activity was detected when the enzymatic hydrolyzate was used. N-Acetylmuramyl-L-alaninamidase is adsorbed on IRTs-50 Amberlite, but it is removed while washing [11] and is not revealed in the purified lysostaphin.

The ion-exchange chromatography on IRTs-50 Amberlite and the subsequent hydrophobic chromatography on phenyl-Sepharose resulted in 157-fold purification of lysostaphin with the yield of 51% (table). The hydrophobic chromatography yielded two fractions (Fig. 1). The first fraction contained some amount of an extraneous high-molecular-weight protein, and the second contained no extrane-

Purification of lysostaphin from the culture liquid of *S. simulans* biovar *staphylolyticus*

Stage of purification	Volume, ml	Activity, U/ml	Total activity, U	Protein, mg	Specific activity, U/mg	Yield, %
Culture liquid	50 000	3.5	175 000	18 000	9.7	100
Ultrafiltration	4500	32.0	144 000	7300	19.7	82
IRTs-50 Amberlite chromatography	630	202.0	127 500	393	325	73
Phenyl-Sepharose chromatography						
1st fraction	7.2	1180.0	8500	15.1	563	5
2nd fraction	27.5	3226	88 730	58.3	1522	51

ous proteins as seen from the data of SDS PAGE (Fig. 2). The value of the absorption coefficient ε_{280} for lysostaphin calculated from the Vetlopher equation is 66,400 $M^{-1} \cdot cm^{-1}$.

The main result of the present work is elaboration of the method based on ion-exchange and hydrophobic chromatographies that allowed purification of lysostaphin to homogeneity with high yield.

DISCUSSION

In the work of Marova and Dadak [2] a method for isolation of lysostaphin is described that includes ultrafiltration, DEAE-cellulose chromatography, and Sephadex G-75 gel filtration. The method yielded a preparation of lysostaphin containing minor impurities with the yield of 22%. It is of interest that the enzyme bound to DEAE-cellulose at a pH

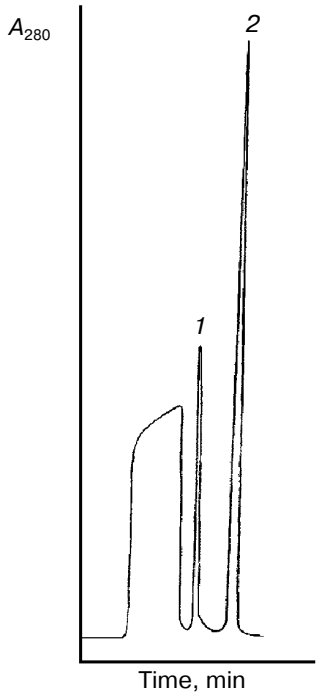

Fig. 1. Hydrophobic chromatography of lysostaphin on phenyl-Sepharose: *1*) fraction of lysostaphin containing extraneous proteins; *2*) purified lysostaphin.

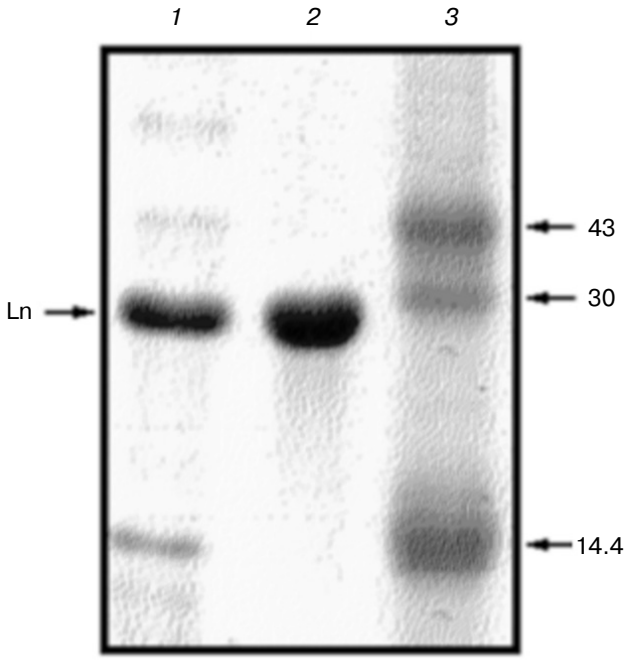

Fig. 2. SDS electrophoresis in 15% polyacrylamide gel: *1*) after ion-exchange chromatography; *2*) after hydrophobic chromatography; *3*) protein markers: ovalbumin, carboanhydrase from bovine erythrocytes, and α-lactalbumin (molecular weights of the markers are shown on the right in kD). Ln, lysostaphin.

value that was almost two units below its isoelectric point (p*I* 9.5 [1]). Such unusual behavior can be explained by the fact that several negatively charged residues are situated close to each other on the surface of the molecule [12]. To isolate lysostaphin, chromatographies on a Cybacron support [13] and on lysostaphin-resistant cells of staphylococci killed by heating with the subsequent elution with 3 M KSCN solution [14] were also used. In the latter case, the total activity constituted 64% of the activity in the culture liquid, and the specific activity increased 12-fold.

A number of globular proteins contain an increased quantity of hydrophobic residues on their surface [14, 15]. The primary structure of lysostaphin includes eight tryptophan residues. If some of them are on the surface of the globule, such a protein can be purified by hydrophobic chromatography. This assumption is supported by the experience.

To purify lysostaphin, we used ultrafiltration, ion-exchange chromatography on an IRTs-50 Amberlite cation-exchange resin, and hydrophobic chromatography on phenyl-Sepharose. The method allowed isolation of enzyme that was homogeneous by the data of SDS PAGE (Fig. 2), the yield being 51%.

It should be noted that for proteins with a high content of aromatic amino acids the values of the absorption coefficient ε_{280} calculated by the Vetlopher equation are usually in agreement with experimental data.

Thus, the use of ion-exchange and hydrophobic chromatographies allows isolation of a highly-purified preparation of lysostaphin applicable for investigation of its properties.

REFERENCES

1. Thumm, G., and Gotz, F. (1997) *Mol. Microbiol.*, **23**, 1251-1265.
2. Marova, I., and Dadak, V. (1993) *Folia Microbiol.*, **38**, 245-252.
3. Schneewind, O., Fowler, A., and Faull, K. F. (1995) *Science*, **268**, 103-106.
4. Sugai, M., Fujirawa, T., Akiyama, T., Ohara, M., Komatsuzawa, H., Inoue, S., and Suginaka, H. (1997) *J. Bacteriol.*, **179**, 1193-1202.
5. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
6. Schindler, C. A., and Schuchardt, V. T. (1965) *Biochim. Biophys. Acta*, **97**, 242-250.
7. Shugar, D. (1952) *Biochim. Biophys. Acta*, **8**, 302-307.
8. Dawson, R., Elliott, D., Elliott, W., and Jones, K. (1991) *Data for Biochemical Research* [Russian translation], Mir, Moscow, pp. 466-467.
9. Kantor, C., and Shimmel, P. (1984) *Biophysical Chemistry* [Russian translation], Mir, Moscow, p. 38.
10. Heinrich, P., Rosenstein, R., Bohmer, M., Sonner, P., and Gotz, F. (1987) *Mol. Gen. Genet.*, **209**, 563-569.
11. Iversen, O. J., and Grov, A. (1973) *Eur. J. Biochem.*, **38**, 293-300.
12. Scopes, R. K. (1985) *Methods of Purification of Proteins* [Russian translation], Mir, Moscow, p. 41.
13. Sakurada, J., Murai, M., Zhijun, L., Usui, A., Seki, K., Kobajashi, K., Sumi, Y., Jitsukawa, H., and Masuda, S. (1993) *Microbiol. Immunol.*, **37**, 29-34.
14. Klotz, J. M. (1970) *Arch. Biochem. Biophys.*, **138**, 704-709.
15. Hofstee, B. H. J. (1975) *Biochem. Biophys. Res. Commun.*, **63**, 618-624.