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Structural Investigations and Identification of the Extracellular Bacteriolytic Endopeptidase L1 from *Lysobacter* sp. XL1

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Abstract—The N-terminal amino acid sequence (23 amino acid residues) and the amino acid composition of the extracellular bacteriolytic enzyme L1 of 21 kD from the bacterium *Lysobacter* sp. XL1 have been determined. The enzyme was hydrolyzed by trypsin, the resulting peptides were isolated, and their primary structures were determined. A high extent of homology (92%) of the N-terminal amino acid sequence and the primary structure of isolated peptides of the enzyme L1 (62 amino acid residues or 31% of protein sequence) to the corresponding sites of α-lytic proteinases (EC 3.4.21.12) of *Lysobacter enzymogenes* and *Achromobacter lyticus* was found. These data allowed identification of the endopeptidase L1 of *Lysobacter* sp. XL1 as α-lytic proteinase EC 3.4.21.12.

Key words: lytic endopeptidase, primary structure, *Lysobacter* sp., α-lytic proteinase

Extracellular bacteriolytic enzymes of microorganisms are widespread in nature and perform different functions. On one hand, they can play a defensive role, and, on the other hand, they can be aggressive factors, being involved in the processes of taking food and occupation of living areals by microorganisms. In any case, these enzymes are involved in relations between microorganisms in their ecological areas, and thus play a significant role.

Practical interest in bacteriolytic enzymes is connected to the possibility of their use in antimicrobial preparations, including those against antibiotic-resistant bacteria [1].

These enzymes hydrolyze a substrate of very complex chemical structure that is multilayer in a number of cases, peptidoglycan of the bacterial cell walls [2]. Based on the substrate specificity towards peptidoglycan of bacterial cell walls, these enzymes are divided into three classes [3]. The first class includes glycosidases destroying the glycan chains of the peptidoglycan molecule. In turn, they are subdivided to endo-N-acetylglucosaminidases (glucosaminidases) and endo-N-acetylmuramidases (muramidases). Muramidases cleave the bond between muramic acid and N-acetylglucosamine yielding peptidoglycan fragments with the muramic acid residue on the reducing ends. Glucosaminidases yield peptidoglycan fragments with the glucosamine residue on the reducing end. The second class of bacteriolytic enzymes includes N-acetyl-muramoyl-L-alanine amidases. They hydrolyze the bond between the lactate residues of muramic acid and the N-terminal residues of peptide subunits. The third class includes endopeptidases hydrolyzing the internal bonds of the polypeptide chains in the peptidoglycan molecule. α-Lytic proteinase (EC 3.4.21.12) from *Lysobacter enzymogenes* and *Achromobacter lyticus* exhibits two activities simultaneously—N-acetyl-muramoyl-L-alanine amidase and endopeptidase activities [4]. The ability of the enzymes to cleave peptidoglycan of the bacterial cell walls depends on the structure of the peptidoglycan molecule, its dimension, the length of the peptide bridges, the content of the peptide subunits, and on the structure of other cell wall components of gram-positive and gram-negative bacteria (in particular teichoic and teichuronic acids) [5, 6].

The complexity of the substrate to be hydrolyzed is likely to determine the structural characteristics of the bacteriolytic enzymes that have been insufficiently studied to date.

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The bacterium *Lysobacter* sp. XL1 (earlier *Xanthomonas campestris* [7]) produces a number of extracellular enzymes including proteolytic ones. The culture liquid of these bacteria was used to obtain bacteriolytic complex lysoamidase, a preparation containing acid polysaccharide and several bacteriolytic enzymes that determine its wide antimicrobial action [1]. Lysoamidase is especially active towards staphylococci.

Three bacteriolytic enzymes, muramidase [8] and two proteinases (L1 [9] and L2 [10]), were isolated from this preparation and characterized. These are proteins of similar molecular weight: 24, 21, and 25 kD, respectively. Determination of the substrate specificity of the enzymes L1 and L2 towards peptidoglycan of *Staphylococcus aureus* showed that enzyme L2 cleaves the amide bond between muramic acid and alanine, thus exhibiting N-acetylmuramoyl-L-alanine amidase activity. The enzyme L1 exhibits two activities: it also cleaves the amide bond between muramic acid and alanine, being N-acetylmuramoyl-L-alanine amidase, and simultaneously hydrolyzes the glycyl–glycine bonds in the peptide bridge of the peptidoglycan, exhibiting endopeptidase activity [11].

The present work is devoted to investigation of the primary structure and identification of the extracellular bacteriolytic endopeptidase L1 from *Lysobacter* sp. XL1.

MATERIALS AND METHODS

Enzyme preparation lysoamidase was obtained from the culture liquid of the bacterium *Lysobacter* sp. XL1 using an experimental technological unit of the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (Pushchino) [12].

The bacteriolytic enzyme L1 was isolated from the preparation of lysoamidase according to the earlier described procedure [10].

Protein composition of the preparations was analyzed by SDS-PAGE as described by Laemmli after precipitation of the proteins with 10% trichloroacetic acid [13].

Protein concentration was determined by the Bradford method [14].

N-Terminal amino acid sequences of the protein and peptides were determined by the automatic Edman degradation procedure using a model 477A protein sequencer (Applied Biosystems, USA) equipped with an one-line analyzer 120A (Applied Biosystems) for automatic identification of the phenylthiohydantoin derivatives of amino acids.

Amino acid analysis was performed using an L5000 amino acid analyzer (Biotronik, Germany) after the complete hydrolysis of the protein in 5.6 M HCl in vacuum ampoules at 110°C for 24 h.

Hydrolysis by trypsin (N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated; Sigma, USA) of the unmodified protein and the protein modified with vinylpyridine was performed in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0, at 37°C for 24 h at the enzyme/substrate ratio of 1 : 50. The hydrolyzate was frozen and lyophilized. Then the hydrolyzate was dissolved in 0.1% trifluoroacetic acid and separated using reversed phase HPLC in 0-80% gradient of acetonitrile on an Ultrasphere-ODS column (Altex, USA) equilibrated with 0.1% trifluoroacetic acid.

The protein was modified at cysteine residues with vinylpyridine (Sigma) as described by Fridman et al. [15]. The modified protein was desalted using gel filtration on a Sephadex G-25 column (Pharmacia, Sweden) equilibrated with 0.1 M N-ethylmorpholine acetate buffer, pH 8.0.

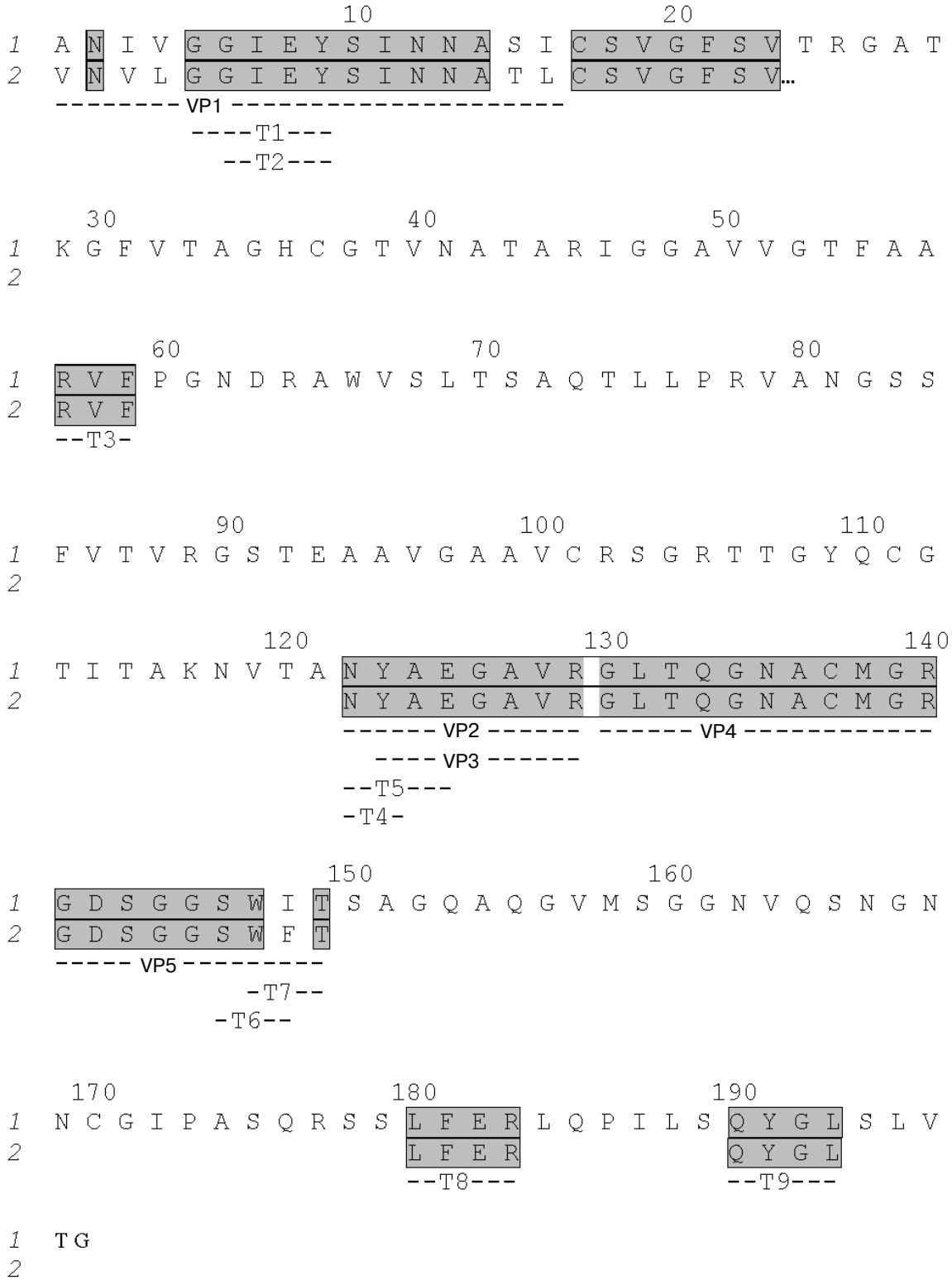
RESULTS AND DISCUSSION

To investigate the structure of the bacteriolytic enzyme L1, it was isolated from the preparation of lysoamidase using the procedure described in [10]. After the terminal stage of isolation, the protein (21 kD) was electrophoretically homogeneous as shown by the data of SDS-PAGE.

Using the automatic Edman degradation procedure, the N-terminal amino acid sequence of the protein was determined (23 amino acid residues), and the purity of the preparation was confirmed (figure). Being compared with the protein sequences presented in the DNASTAR data bank and in the ATLAS system (http://vms.mips.biochem.mpg.de/mips/pnograms/atlas.html), this N-terminal sequence exhibited a high extent of homology with the N-terminal amino acid sequence of α-lytic endopeptidases (EC 3.4.21.12) from *Achromobacter lyticus* and *Lysobacter enzymogenes* [16, 17]. In this sequence, 18 of 23 amino acid residues coincide completely, the substitutions at the 3, 4, 15, and 16 positions being equivalent.

Analysis of the amino acid composition of the investigated enzyme showed that it is close to that of α-lytic proteinase from *Lysobacter enzymogenes*; however, it has an increased content of glycine and histidine (Table 1).

To obtain more complete information on the primary structure of the enzyme, it was hydrolyzed with trypsin under standard conditions. Analysis of the resulting fractions by the automatic Edman degradation procedure detected the presence of a high content of small peptides in the hydrolyzate, this resulting in some difficulties in their separation. We isolated only nine homogeneous peptides (T), whose amino acid sequences were determined by Edman degradation (Table 2). Analysis of the primary structure of the isolated peptides showed that only the peptide T8 was formed due to the hydrolysis of the polypeptide chain of the investigated protein according to the enzymatic specificity of trypsin. Formation of



Position of peptides of bacteriolytic endopeptidase L1 of *Lysobacter* sp. XL1 in the polypeptide chain of the homologous protein of *Lysobacter enzymogenes*: *1*) α-lytic proteinase from the bacterium *Lysobacter enzymogenes* [17]; *2*) amino acid sequence of fragments of bacteriolytic enzyme L1; T) peptides isolated from the tryptic hydrolyzate of the native protein; VP) peptides isolated from the tryptic hydrolyzate of the protein modified with vinylpyridine. Completely homologous sequences are outlined

Table 1. Amino acid composition of bacteriolytic endopeptidase L1 of *Lysobacter* sp. XL1 and α-lytic endopeptidase of *Lysobacter enzymogenes* (mole %) (Pro, Cys, and Trp were not determined)

Amino acid	Endopeptidase L1 of *Lysobacter* sp. XL1	α-Lytic endopeptidase of *Lysobacter enzymogenes*
Asx	9.0	8.1
Thr	8.1	9.7
Ser	10.2	10.8
Glx	6.7	7.0
Gly	20.2	17.2
Ala	12.1	12.9
Val	10.1	10.2
Met	0.0	1.0
Ile	4.9	4.3
Leu	4.0	5.4
Tyr	2.0	2.1
Phe	3.0	3.2
Lys	1.2	1.0
Arg	5.5	6.5
His	3.0	0.5

other peptides could be due to the fact that under the conditions employed (pH 8.0, 37°C) the investigated enzyme itself exhibits a high autolytic proteolytic activity with a wide spectrum of substrate specificity that differs from that of trypsin. To suppress the proteolytic activity, cysteine residues of the enzyme were modified by vinylpyridine so as to destroy the disulfide bridges in the protein molecule. Trypsin hydrolysis of the protein modified with vinylpyridine and separation of the tryptic peptides were performed under conditions analogous to those used for the unmodified protein. The structure of the peptides isolated from the tryptic hydrolyzate of the enzyme modified at cysteine residues (VP) is presented in Table 2. For the presented peptides complete amino acid sequence and for three cases (VP1, VP4, and VP5) partial amino acid sequences were determined.

Comparison of the amino acid sequence of the peptides obtained from the endopeptidase L1 (62 amino aid residues) with the amino acid sequence of α-lytic endopeptidase from *Lysobacter enzymogenes* showed a high extent of homology (92%) of investigated fragment structures with the primary structure of the corresponding sites of the amino acid sequence of the α-lytic endopeptidase (figure). This allowed identification of the extracellular bacteriolytic enzyme L1 of the bacterium *Lysobacter* sp. XL1 as the α-lytic proteinase.

Considering the primary structure of the α-lytic proteinase of *Lysobacter enzymogenes* and assuming a high

Table 2. Amino acid sequences of some tryptic peptides of bacteriolytic endopeptidase L1

Designation of peptide	Amino acid sequence	Number of amino acid residues	Position in the polypeptide chain (figure)
T1	G G I E Y	5	5-9
T2	G I E Y	4	6-9
T3	R V F	3	57-59
T4	N Y	2	122-123
T5	N Y A E	4	122-126
T6	S W F	3	146-148
T7	W F T	3	147-149
T8	L F E R	4	180-183
T9	Q Y G L	4	190-193
VP1	V N V L G G I E Y S I N N A T L ...	>16	1-16
VP2	N Y A E G A V R	8	122-129
VP3	Y A E G A V R	7	123-129
VP4	G L T Q G N A C M G R G D ...	>13	130-142
VP5	G D S G G S W F T ...	>9	141-149

Note: T) peptides isolated from the tryptic hydrolyzate of the unmodified enzyme L1; VP) tryptic peptides obtained after tryptic hydrolysis of the bacteriolytic enzyme L1 modified with vinylpyridine.

extent of homology between the amino acid sequence of this enzyme and the enzyme L1, it can be assumed that the peptides obtained after the autolytic hydrolysis were formed due to the cleavage of the peptide bonds F–T (peptide T6), Y–S (T1 and T2), F–P (T3), Y–A (T4), G–S (T6), G–G (T2 and T6), L–G (T1), L–S (T9), T–S (T7), S–W (T7), S–L (T8), S–Q (T9), S–W (T7), N–Y (T4), A–R (T3), A–N (T4 and T5), and E–G (T5). In lysoamidase, the enzyme L1 is not subjected to autolysis. It can be assumed that the autolytic proteolytic activity of the enzyme is suppressed by the polysaccharide component of lysoamidase.

The homologous α-lytic proteinases of *Lysobacter enzymogenes* and *Achromobacter lyticus* have been well studied. It was shown that these enzymes are serine proteinases and have much in common with mammalian pancreatic serine proteinases [17, 18]. The crystal structure of the free proteinase from *Lysobacter enzymogenes* [19, 20] as well as of its complex with the inhibitor [21] was determined with high resolution. Endopeptidase from *Achromobacter lyticus* cleaves the N-acetylmuramoyl-L-alanine amide bond, as well as D-Ala–Gly and Gly–Gly peptide bonds in peptidoglycan of *S. aureus* [4]. Investigated in this work endopeptidase L1 from *Lysobacter* sp. XL1 has analogous substrate specificity towards the peptidoglycan of *S. aureus* [11]. Thus, the endopeptidase L1 of *Lysobacter* sp. XL1 is likely to demonstrate not only structural homology, but also functional similarity to the α-lytic proteinases of *Lysobacter enzymogenes* and *Achromobacter lyticus*, in particular, the analogous substrate specificity.

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