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Extracellular Yeast-Lytic Enzyme of the Bacterium *Lysobacter* sp. XL 1

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Abstract—An enzyme exhibiting yeast-lytic activity has been isolated from the culture liquid of the bacterium *Lysobacter* sp. XL 1. The optimal conditions for the hydrolysis of *Saccharomyces cerevisiae* cells by the enzyme have been established: 0.15 M sodium acetate buffer, pH 6.0, 50°C. The yeast-lytic activity of the enzyme is inhibited by EDTA, *p*-chloromercuribenzoate, and phenylmethylsulfonyl fluoride. According to the data of SDS-PAGE, the molecular weight of the protein is 36 kD. The enzyme hydrolyzes casein, hemoglobin, and synthetic peptide Abz-Ala-Ala-Phe-pNA, i.e. it exhibits proteolytic activity. The properties of the enzyme and its molecular weight correspond to those of a previously isolated extracellular metalloproteinase. The N-terminal amino acid sequence of the protein exhibits 67% homology with the N-terminal sequence of achromolysine of *Achromobacter lyticus* (EC 3.4.24.-).

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Key words: metalloproteinase, lysoamidase, *Lysobacter* sp., yeast-lytic enzymes

One of the most important problems of contemporary medicine is the fight against pathogenic microorganisms including bacteria and some species of yeast and fungi. A special danger is connected with so-called opportunistic fungi that, being capable of living under natural conditions, cause secondary mycoses in humans with blood diseases and different metabolic disorders after therapy with immunodepressants, corticosteroids, and antibiotics [1]. Secondary mycoses are mainly caused by yeast fungi of the genus *Candida* and mycelial fungi of the genus *Aspergillus*. It is known that the cell walls of yeast and fungi are composed of glucans, mannoproteins, chitin [2], and also of a number of structural proteins [3, 4]. Therefore, enzymes breaking these polymers play the main role in the hydrolysis of the cell walls of yeast and fungi [5-13]. Previously, at the Skryabin Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (Pushchino), the bacteriolytic preparation lysoamidase was obtained from the culture liquid of the bacterium *Lysobacter* sp. XL 1. Lysoamidase efficiently destroys cells of pathogenic bacteria including those resistant to antibiotics [14]. The bacteriolytic action of the preparation is due to a number of lytic enzymes breaking the main structural component of the bacterial cell wall, peptidoglycan [14-19]. Lysoamidase was also shown to efficiently lyse the cells of some species of yeast and fungi, exhibiting the protease, glucanase, and chitinase activities [20]. The goal of the present work was to isolate yeast-lytic enzymes from lysoamidase and to investigate their properties.

Abbreviations: Abz) *o*-aminobenzoate; pNA) *p*-nitroaniline.
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MATERIALS AND METHODS

In the present work, we used the strain *Lysobacter* sp. XL 1 from the All-Russian Collection of Microorganisms of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The cells were grown for 18 h at 29°C and pH 7.0 under constant shaking in a liquid medium containing glucose, peptone, yeast extract, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$, and $FeSO_4 \cdot 7H_2O$.

The yeast-lytic enzyme was obtained from the lysoamidase isolated from the culture liquid of *Lysobacter* sp. XL 1 [14]. The proteins were precipitated by the addi-

tion of ammonium sulfate to 80% saturation; the precipitate was dissolved in 0.05 M Tris-HCl, pH 8.0, and dialyzed against the same buffer. The resulting solution was applied on a DEAE-Sephadex column (5 × 20 cm) equilibrated with the same buffer. The adsorbed proteins were eluted with a 0-0.3 M NaCl gradient. The fraction exhibiting the yeast-lytic activity was purified by gel filtration on a G-75 Sephadex column (2.5 × 70 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0.

Yeast-lytic activity was determined using cells of *Saccharomyces cerevisiae* M660 collected in the beginning of the logarithmic growth stage and incubated for 15 min in boiling water. The enzyme solution in 0.1 M sodium acetate buffer, pH 6.0 (0.5 ml), was supplemented with 0.5 ml of the yeast cell suspension (absorption at 540 nm was 0.7) in the same buffer. The mixture was incubated at 37°C for 7-11 h, and then the absorption of the mixture at 540 nm was measured using a Shimadzu UV 160 A spectrophotometer (Japan). The unit of the yeast-lytic activity was defined as the amount of the enzyme decreasing the absorption of the yeast suspension by 0.01 per hour.

The proteinase activity of protein fractions was determined by the rate of hydrolysis of casein and fluorogenic peptide Abz-Ala-Ala-Phe-pNA. While using casein, 0.2 ml of 1% casein solution in 0.1 M Tris-HCl, pH 8.0, was supplemented with 0.2 ml of an enzyme solution. The reaction was stopped by the addition of 0.8 ml of 5% trichloroacetic acid. After 10 min, the precipitate was removed by centrifugation (10,000 rpm, 5 min) using a Beckman Microfuge^{TM}11 centrifuge (USA), and the absorption of the supernatant at 280 nm was determined. The unit of proteolytic activity was defined as the amount of enzyme providing the increase in the absorption of the supernatant at 280 nm by 1.0 per 10 min at 37°C. While using the peptide Abz-Ala-Ala-Phe-pNA, the reaction was performed at 37°C in 0.01 M Mes, pH 6.5 [21]. The activity of the preparation was estimated from the increase in the fluorescence at 420 nm determined using a Hitachi M 850 spectrofluorimeter (Japan).

β-Glucanase activity of enzyme preparations was determined by the cleavage of the glycoside bonds of low-polymer carboxymethylcellulose or laminarin [22]: 0.05 ml of the substrate (1% solution of low-polymer carboxymethylcellulose or laminarin in 0.1 M sodium acetate buffer, pH 6.0) was supplemented with 0.05 ml of the enzyme preparation and the mixture was incubated at 37°C during 5 h. The reaction was stopped by the addition of 0.1 ml of the Somogyi's solution. After heating in boiling water for 20 min, the reaction mixture was supplemented with 0.1 ml of Nelson's solution and 2.5 ml of water, and the absorption of the resulting solution was measured at 720 nm using a Carl Zeiss JENA Spekol 11 spectrophotometer (Germany). The unit of the activity was defined as the amount of the enzyme liberating 1 μmol of glucose per 1 h at 37°C.

The chitinase activity was determined using as the substrate a colloid solution of chitin azure (3 mg/ml; Sigma, USA) in 0.01 M sodium phosphate buffer, pH 8.0 [20]. The solution of chitin azure (0.25 ml) was supplemented with 0.5 ml of the enzyme solution in the same buffer, and the mixture was incubated at 37°C for 14 h under constant shaking. Then the substrate was removed by centrifugation, and the absorption of the supernatant was measured at 650 nm using a Shimadzu UV 160 A spectrophotometer. The unit of the activity was defined as the amount of the enzyme releasing 0.1 μg azure per h at 37°C.

To determine the optimal pH value for the lysis of the *S. cerevisiae* M 660 cells, we used 0.1 M sodium acetate buffer (pH 5.0-6.5), 0.1 M Tris-HCl (pH 7.0-8.5), or 0.1 M sodium carbonate buffer (pH 9-10).

Thermostability of the enzyme was investigated in 0.1 M sodium acetate buffer, pH 6.5. The enzyme solution (0.1 mg/ml) was incubated for 20 min at different temperatures, cooled on ice, and then its yeast-lytic activity was determined.

The influence of inhibitors on the yeast-lytic activity of the enzyme was investigated in the presence of EDTA, phenylmethylsulfonyl fluoride (PMSF; Sigma), or *p*-chloromercuribenzoate (*p*-CMB; Serva, USA). An enzyme solution (0.2 ml) in 0.1 M sodium acetate buffer, pH 6.0, was supplemented with a solution of an inhibitor (final concentration, 5 mM) and buffer, bringing the final volume to 0.5 ml. EDTA (0.1 M) was dissolved in water and the solution was neutralized with 0.1 M NaOH. PMSF (0.05 M) was dissolved in propanol; *p*-CMB (0.05 M) was dissolved in 0.1 M NaOH. The mixture was incubated at 30°C for 30 min, and then the yeast-lytic activity was determined. The final concentration of the inhibitors in the reaction mixture was 2.5 mM.

SDS-PAGE was performed by the Laemmli method [23] using 12% polyacrylamide gel after precipitation of proteins with 10% trichloroacetic acid. The protein bands were stained with 0.1% Coomassie R-250 (Serva) in an aqueous solution of 50% methanol and 10% acetic acid for 1 h.

The N-terminal amino acid sequence of proteins was determined by automatic Edman degradation using an Applied Biosystems 477A protein sequenator (USA) with subsequent automatic identification of the phenylthiohydantoin derivatives of the amino acids using an Applied Biosystems 120A analyzer.

Protein concentration was determined by the Bradford method [24] or by absorption at 280 nm.

RESULTS AND DISCUSSION

The culture liquid of the bacterium *Lysobacter* sp. XL 1 was assayed for the yeast-lytic, glucanase, chitinase, and proteolytic activities that constituted 9.3, 1.8

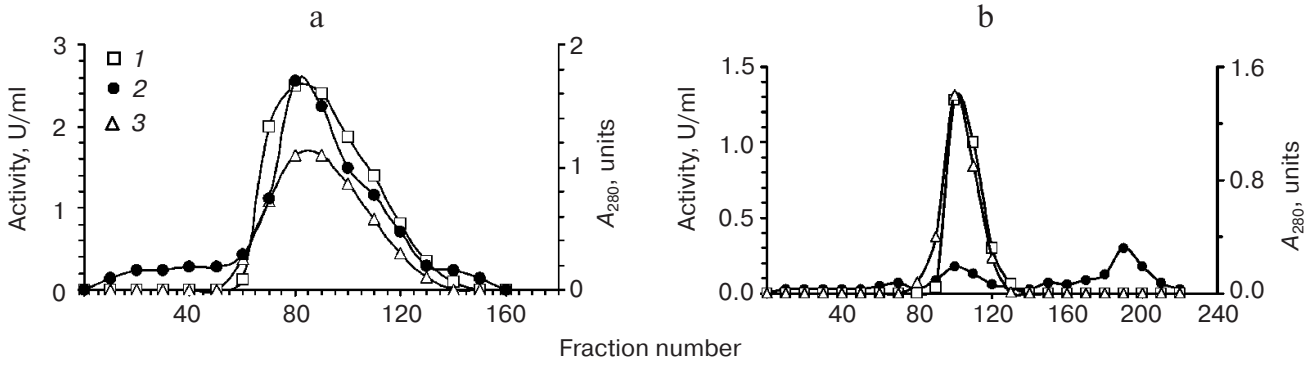

Fig. 1. Chromatography of protein fraction of the culture liquid of *Lysobacter* sp. XL 1: a) elution of proteins with a NaCl gradient from DEAE-Sephadex column; b) gel filtration of the yeast-lytic fraction on a G-75 Sephadex column. *1*) Yeast-lytic activity; *2*) protein; *3*) proteolytic activity.

(towards laminarin), 0.3, and 2 U/ml, respectively. After precipitation of the culture liquid proteins with ammonium sulfate (80% of saturation), all components with the yeast-lytic activity were found in the pellet. The pellet was dissolved in 0.05 M Tris-HCl, pH 8.0, dialyzed against the same buffer, and applied on a DEAE-Sephadex column. While a part of the components with proteolytic and yeast-lytic activities adsorbed to the column, all components with glucanase and chitinase activities and some of the components with the proteolytic activity passed through the column under these conditions. Such a distribution of the yeast-lytic activity indicates that the culture liquid of *Lysobacter* sp. XL 1 contains more than one yeast-lytic enzyme. The adsorbed enzymes were eluted with a NaCl gradient. The yeast-lytic and proteolytic components were eluted from the column at NaCl concentration of 0.1 M as a single symmetric peak coinciding with the protein peak (Fig. 1a). As was shown by SDS-PAGE, the pooled fraction with the yeast-lytic activity contained two main proteins of 36 and 24 kD (Fig. 2). The fraction was purified by gel filtration on a G-75 Sephadex column. The elution profile (Fig. 1b) demonstrates that the protease and the yeast-lytic activities correspond to the first of two protein peaks. According to the results of SDS-PAGE (Fig. 2), this fraction corresponds to the electrophoretically homogeneous protein of 36 kD. This protease was shown to lyse live yeast cells of the early logarithmic growth phase.

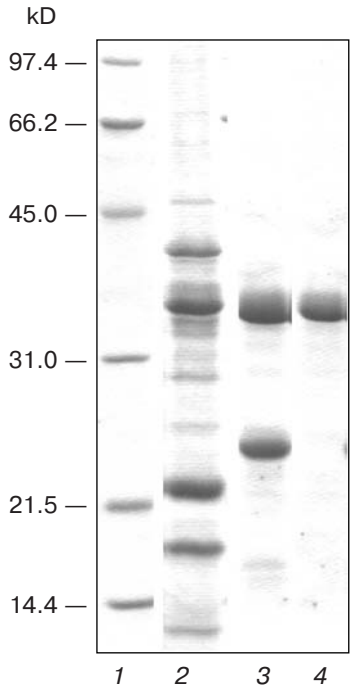

Fig. 2. SDS-PAGE of the yeast-lytic enzymes of *Lysobacter* sp. XL 1 by Laemmli's method: *1*) protein standards: phosphorylase *b* (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carboanhydrase (31 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD); *2*) culture liquid of *Lysobacter* sp. XL 1; *3*) the yeast-lytic fraction obtained after DEAE-Sephadex chromatography (Fig. 1a); *4*) the yeast-lytic fraction obtained after gel-filtration on G-75 Sephadex (Fig. 1b).

The N-terminal amino acid sequence of the isolated enzyme was found to be A-K(L)-V-G-T-G-P-T-G-N-A-x-t-G-Q-. Comparing this sequence with the sequences presented in the PIR and Swiss-Prot databases [http://pir.georgetown.edu] revealed 67% of homology with the N-terminal sequence of achromolysine of *Achromobacter lyticus* (access number P81730 in the UniProt Knowledgebase).

Thus, we obtained a yeast-lytic enzyme exhibiting protease activity. Previously, a metalloproteinase that behaved similarly during chromatography was isolated from the culture liquid of the producer and characterized [21, 25]. For this reason, the enzyme obtained in the present work was investigated using the same scheme as used for the metalloproteinase. As a result, the isolated yeast-lytic enzyme was shown to exhibit properties that are close to the properties of the metalloproteinase. The enzyme cleaves casein and the fluorogenic tripeptide Abz-Ala-Ala-Phe-pNA. The optimal conditions for the

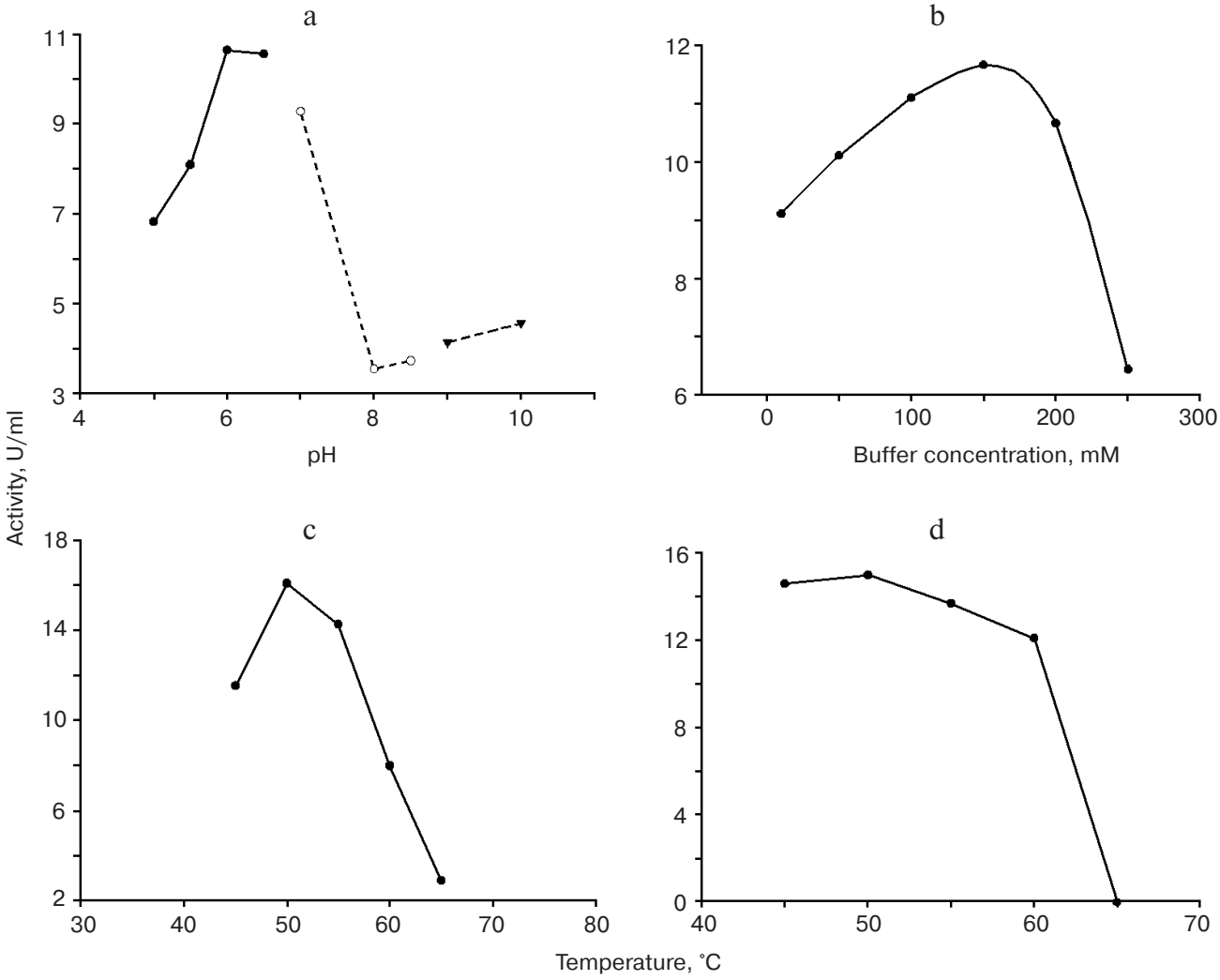

Fig. 3. Dependence of the yeast-lytic activity on pH (a), concentration of sodium acetate buffer, pH 6.0 (b), and temperature (c); thermostability (d).

cleavage of these substrates by the isolated enzyme were the same as for the metalloproteinase. Besides, the caseinolytic activity of the isolated enzyme was completely inhibited by EDTA. Based on these results, it was concluded that the isolated yeast-lytic enzyme and the previously described metalloproteinase is the same enzyme (further called the metalloproteinase).

The optimal conditions for the yeast-lytic activity of the metalloproteinase of *Lysobacter* sp. XL 1 towards *S. cerevisiae* cells have been determined. The pH dependence of the yeast-lytic activity of the enzyme is presented in Fig. 3a. The optimal pH value of the medium for the lysis of yeast cells by the metalloproteinase was found to be 6.0. The dependence of the yeast-lytic activity on the concentration of sodium acetate buffer is a wide peak with a maximum at 0.15 M (Fig. 3b). The dependence of the yeast-lytic activity on temperature exhibits a pronounced maximum at 50°C (Fig. 3c).

Heating of the enzyme for 20 min at 45 and 50°C increased its yeast-lytic activity, heating at 60°C decreased the activity, and heating at 65°C completely inactivated the enzyme. The half-inactivation temperature was 62°C (Fig. 3d).

The effect of inhibitors on the yeast-lytic activity of the metalloproteinase was investigated using EDTA (inhibitor of metalloenzymes), PMSF (inhibitor of serine proteinases), and *p*-CMB (inhibitor of thiol proteinases). As seen from the table, the yeast-lytic activity of the enzyme is very sensitive to the presence of all tested inhibitors. The most effect (100% inhibition) was observed in the presence of *p*-CMB. In the presence of EDTA, the activity of the metalloproteinase decreased by 64%, and PMSF inhibited the yeast-lytic activity by 30%.

The properties exhibited by the metalloproteinase in the presence of the *S. cerevisiae* cells as the substrate were compared to the properties of the enzyme determined in

Effect of inhibitors of the yeast-lytic activity of the metalloproteinase of *Lysobacter* sp. XL 1

Inhibitor	Activity, U/ml	Inhibition, %
Without inhibitor	18.9	0
EDTA	6.9	64
p-CMB	0	100
PMSF	13.2	30

the presence of casein and tripeptide Abz-Ala-Ala-Phe-pNA [21, 25]. The optimal pH values for the yeast-lytic activity of the enzyme and for the hydrolysis of Abz-Ala-Ala-Phe-pNA were shown to be close (6.0 and 6.5, respectively). The optimal pH value for the cleavage of casein is higher and constitutes 8.0 [25]. The optimal temperature for the lysis of yeast cells is 50°C, this exceeding the optimal temperature for the cleavage of the tripeptide Abz-Ala-Ala-Phe-pNA (41°C [21]), but being lower than that for the cleavage of casein (60°C [25]). After heating, the enzyme retains its activity towards both yeast cells and casein. The half-inactivation temperature values obtained with these substrates are approximately the same.

Some differences in the activity of the metalloproteinase towards different substrates were observed in the presence of inhibitors. EDTA strongly inhibited the activity towards casein [25] and towards *S. cerevisiae* cells. While using the tripeptide Abz-Ala-Ala-Phe-pNA as the substrate, the metalloproteinase was completely inactivated after treatment with 0.1 mM EDTA (97% inhibition) [21]. The inhibitors *p*-CMB and PMSF at concentration of 5 mM did not affect the activity of the enzyme towards casein [21], but decreased the activity towards the *S. cerevisiae* cells by 100 and 30%, respectively. After the treatment with these inhibitors (3.3 mM), the enzyme completely retained the activity towards the tripeptide Abz-Ala-Ala-Phe-pNA [21]. Japanese investigators assumed that proteinases with affinity to the polysaccharides of the yeast cell walls could exhibit yeast-lytic action [26]. Presumably, the inhibition of the yeast-lytic activity after the treatment of the metalloproteinase with inhibitors is due to the loss of the ability to bind to the cell wall of yeast.

Thus, the yeast-lytic enzyme isolated from the culture liquid of the bacterium *Lysobacter* sp. XL 1 is a metalloproteinase acting on the proteins of yeast cell walls. Although most authors consider that the main role in the lysis of yeast cells is played by glucanases [13], the results of the present work as well as the works of other investigators [8, 12, 14, 27, 28] indicate the importance of proteolytic enzymes in this process.

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