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Thiol-Dependent Serine Proteinase from *Paecilomyces lilacinus*: Purification and Catalytic Properties

E. K. Kotlova[1]*, N. M. Ivanova[1], M. P. Yusupova[1], T. L. Voyushina[1], N. E. Ivanushkina[2], and G. G. Chestukhina[1]

[1]*The Scientific Research Institute for Genetics and Selection of Industrial Microorganisms, 1-yi Dorozhny pr. 1, 113545 Moscow, Russia; fax: (495) 315-0501; E-mail: Kotlova@genetika.ru*
[2]*Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia*

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Abstract—An extracellular thiol-dependent serine proteinase was isolated from culture medium filtrate of the microscopic fungus *Paecilomyces lilacinus* with a yield of 33%. The enzyme is inactivated by specific inhibitors of serine proteinases, phenylmethylsulfonyl fluoride, as well as by chloromercuribenzoate and mercury acetate, but is resistant to chelating agents. The proteinase has broad specificity, hydrolyzes proteins and *p*-nitroanilides of N-acylated tripeptides, exhibiting maximal activity in hydrolysis of substrates containing long hydrophobic and aromatic residues (norleucine, leucine, phenylalanine) as well as arginine at the P1 position. The enzyme has a molecular weight of 33 kD. The enzyme is most active at pH 10.0-11.5; it is thermostable and is characterized by broad optimum temperature range (30-60°C), displaying about 25% of maximal activity at 0°C. The N-terminal sequence of the enzyme (Gly-Ala-Thr-Thr-Gln-Gly-Ala-Thr-Gly/Ile-Xxx-Gly) has no distinct homology with known primary structures of serine proteinases from fungi and bacilli. Based on its physicochemical and enzymatic properties, the serine proteinase from *P. lilacinus* can be classified as a thiol-dependent subtilisin-like enzyme.

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Key words: *Paecilomyces lilacinus*, extracellular serine proteinase, subtilisin, substrate specificity

In recent years, microscopic fungi attract increasing attention due to widespread and intensive use of their secretion products, including proteolytic enzymes, in different biotechnological processes [1, 2]. Among these products a considerable number are proteolytic enzymes, in particular, alkaline serine proteinases.

Serine proteinases, belonging to the S8 family of subtilisin-like proteinases, have been isolated from different microorganisms [3-5]. The most studied are the enzymes of bacillary origin, whose representatives are both typical subtilisins, for instance, subtilisin 72 [6] or Carlsberg subtilisin [7], and thiol-dependent subtilisins [8, 9], resembling in their properties the enzyme thermitase isolated from thermoactinomycetes [4]. However, the number of studied fungal proteinases is relatively small. One of the first known representatives of this enzyme group was proteinase K, an alkaline enzyme from *Engyodontium album* (other name *Tritirachium album*) secretion [10-12]. The enzyme resembles subtilisins in molecular weight (28.9 kD), optimal pH (7.5-12.0), thermostability, and dependence on Ca^{2+}. Like subtilisins, the enzyme has low specificity and preferentially hydrolyzes hydrophobic and aromatic residues at the P1 position. The main difference from proteinase K is that it contains five cysteine residues, whereas typical bacillary subtilisins do not contain cysteine residues at all, and thiol-dependent subtilisins belonging to the subgroup of thermitase contain only one cysteine residue [3, 4, 13]. In the case of both proteinase K and thermitase one cysteine residue, not involved in the formation of disulfide bond, is located in the vicinity of histidine of the catalytic triad, resulting in inhibition of these enzymes by mercury ions [4, 8, 11].

By inhibitory analysis, i.e. sensitivity to Hg^{2+} or *p*-chloromercuribenzoate (*p*-CMB), known subtilisin-like serine proteinases from fungi *Trichoderma viride* (synonym *T. lignorum*) and *T. koningii* [14] as well as

Abbreviations: *p*-CMB) *para*-chloromercuribenzoate; DMFA) dimethylformamide.

* To whom correspondence should be addressed.

Acremonium chrysogenum [15] more resemble proteinase K than typical bacillary subtilisins. However, fungal serine proteinase from *Aspergillus oryzae* [16] displays all features characteristic for bacillary subtilisins, and is not inhibited by *p*-CMB. The ability to hydrolyze collagen and elastin is exhibited by serine proteinase from *A. fumigatus* [17], also belonging to subtilisin family. Another interesting example of this family is extracellular enzymes from *Arthrobotrys oligospora* [18], *Verticillium chlamidosporium* [19], and *Paecilomyces lilacinus* [20] involved in nematode infection.

We have studied an extracellular serine proteinase from the fungus *P. lilacinus*, isolated from buried soil (in a group of burial mounds Tary, Volgograd Region, Russia). This species is known as nematode egg parasite, capable of degrading the outer eggshell. In connection with this feature, *P. lilacinus* is used in a number of countries as a biological insecticide. The biological effect of the fungus is associated with its ability to secrete chitinase and serine proteinase, hydrolyzing chitin and a protein (vitelline) contained in the eggshell. Bonants et al. partially purified and characterized extracellular subtilisin-like proteinase from *P. lilacinus*, the secretion of which was induced by addition of colloidal chitin and vitelline in the fermentative medium [20]. In a number of features, the enzyme isolated by us resembles the enzyme characterized by Bonants et al. [20], but its N-terminal sequence (12 residues) has no homology with any of the known subtilisin-like enzymes.

MATERIALS AND METHODS

In this work we used *Paecilomyces lilacinus* (Thom) Samson VKM F-3891 strain (obtained from the All-Russia Collection of Microorganisms (VKM) as *Aspergillus niveus* Blochwitz) of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The strain was isolated from a soil sample from a burial mound with dated construction time of about three thousand years B.C.E. (burial hills Tary).

Isolation of proteinase from *P. lilacinus*. The fungus was cultivated at 26°C for seven days using medium containing 1.5% tryptone, 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, and 1% glucose. The *P. lilacinus* proteinase was isolated from secretion of the fungus at 5-7°C.

Protein was precipitated by slow addition of ammonium sulfate to the culture broth up to 85% saturation. The resulting suspension was incubated for 12 h at 0°C and then centrifuged. The pellet was resuspended in 0.05 M Tris-HCl buffer, pH 7.6 (buffer A) and dialyzed against the same buffer.

Affinity chromatography. The dialyzate was applied to a column with bacitracin-Silochrome equilibrated with buffer A. The column was washed with the initial buffer and then with 1 M NaCl in the same buffer. The enzyme was eluted with 25% isopropyl alcohol in buffer A containing 1 M NaCl. The eluate was ultrafiltered on a UM 10 membrane (Millipore, USA). The sample was chromatographed on bacitracin-Sepharose under the same conditions as the chromatography on bacitracin-Silochrome.

Gel-permeation chromatography. The concentrate obtained after ultracentrifugation on a UM 10 membrane (Millipore) was applied to a column with Superdex 75 (Amersham Bioscience, Sweden) equilibrated with 0.02 M Tris-HCl buffer, pH 7.6, containing 0.4 M NaCl. The protein was eluted with the same buffer at a flow rate of 0.5 ml/min. Protein concentration was determined by the Bradford method [21].

Activity of proteinase *P. lilacinus* towards protein substrates was determined as follows: 250 µl of Z-Ala-Ala-Leu-*p*NA substrate (0.5 mg in 1 ml dimethylformamide (DMFA)) and 20 µl of enzyme were added to 1 ml of 0.05 M Tris-HCl buffer, pH 8.0. The mixture was incubated at 37°C for 10 min. The reaction was stopped by addition of 1 ml of 1 M citrate buffer, pH 3.0, containing 20% DMFA. The reaction was followed with a UV-240 spectrophotometer (Shimadzu, Japan) at 410 nm. One unit of proteolytic activity was defined as the amount of enzyme that is required for formation of 1 µmol of *p*-nitroaniline during 1 min under the standard conditions.

Electrophoresis under denaturing conditions was performed according to Laemmli's method [22] in 12.5% gel. The following proteins (Sigma, USA) were used as molecular weight markers: lactalbumin (14.2 kD), soybean trypsin inhibitor (20.1 kD), chymotrypsinogen (24 kD), carboanhydrase (29 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), ovalbumin (45 kD), and BSA (66 kD).

Amino acid composition was determined on a LC 2000 analyzer (Biotronic, Germany) after oxidation of the protein with performic acid [23] and hydrolysis with 6 M HCl at 105°C for 24 h.

N-Terminal amino acid sequence was determined using a 470A automatic gas phase sequencer (Applied Biosystems, USA). The protein was previously subjected to SDS-PAGE followed by electrotransfer onto an Immobilon-P^{SQ} membrane (Millipore). The search for sequences orthologous to those determined experimentally was carried out using a BLAST Search for short, nearly exact match algorithm and the NCBI GenBank database.

Temperature optimum of proteolytic activity of the purified enzyme was determined by measuring the rate of enzymatic reaction at 0-70°C during a 4 min incubation. To investigate the thermostability, the isolated enzyme was incubated at 37, 50, 60, or 70°C for 20, 60, and 180 min and then cooled rapidly. After that, the enzymatic activity was determined under standard conditions.

Effect of calcium chloride on thermostability of *P. lilacinus* proteinase was studied by incubation of the enzyme at 60°C for 60 min in the presence of 1 and 10 mM $CaCl_2$. Then the residual enzyme activity was determined.

The pH-dependence of the enzymatic activity of the proteinase was determined using the following buffer systems (0.1 M): sodium acetate (pH 4.0-5.5), Mes (4-morpholinoethanesulfonic acid) (pH 5.5-6.5), Tris-HCl (pH 7.5-8.9), and Capso (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) (pH 8.9-11.5). To determine the pH stability the enzyme preparation was incubated at 20°C for 1 h in the indicated buffers. Then the residual enzymatic activity was determined.

Enzyme activity and stability in the presence of SDS was studied by the incubation of the enzyme at 37°C for 60 min in the presence of 0.1, 0.5, or 1% SDS solution. Then the residual enzymatic activity was determined.

Substrate specificity of *P. lilacinus* proteinase was determined under standard conditions using *p*-nitroanilides of N-acylated di-, tri-, and tetrapeptides as substrates.

Proteolytic activity towards protein preparations was determined by incubation of an aliquot (10-50 μg) of the enzyme in 500 μl of 0.1 M Tris-HCl buffer, pH 7.5, containing 2-5 mg of substrate. After incubation of the mixture for 24 h at 28°C, the reaction was stopped by addition of 200 μl of 5% TFA, the remaining uncleaved protein was removed by centrifugation at 17,000*g*, and the absorbance of the resulting solution was measured at 280 nm. The following protein preparations were used: BSA, Hammersten casein, soluble rat collagen type I (ImTek, Russia), food gelatin, and denatured collagen type I from bovine Achilles tendon (Sigma) preliminarily pulverized at pH 7.5 and subjected to thermal treatment on a boiling water bath during 15 min.

Kinetic characteristics were determined using Z-Ala-Ala-Leu-*p*NA and Z-Ala-Ala-Arg-*p*NA as substrates at 37°C in a thermostatted cuvette with the UV-240 spectrophotometer. The substrates used were synthesized in our laboratory.

Inhibitory analysis was performed upon the incubation of the enzyme for 1 h at 20°C in the presence of bivalent metal ion salts or inhibitors (10^{-2}-10^{-5} M). Then the residual enzymatic activity was determined.

RESULTS AND DISCUSSION

Isolation of serine proteinase from *P. lilacinus*. The results of the isolation of *P. lilacinus* proteinase are presented in Table 1. The enzyme yield was 33%. According to SDS-PAGE data, the preparation was characterized by the presence of a single band, corresponding to a protein with molecular weight of 33 kD. The same molecular weight value was determined for a serine proteinase from *A. fumigatus* [17] and fungal chymoelastase-like proteinase from *V. chlamydosporium* [19]. Similar molecular weight values are typical for proteinases from *P. lilacinus* (33.5 kD) [20], *A. oligospora* (35 kD) [18], and *A. oryzae* (30 kD) [16]. In molecular weight, the enzyme is similar to many microbial alkaline proteinases, in particular, to bacillary subtilisins (28 kD) [4].

Dependence of stability and proteolytic activity of *P. lilacinus* proteinase on pH. The isolated proteinase is stable at pH 5.0-11.5 (data not shown), which is typical for subtilisins; however, in some cases, for instance in the case of serine proteinase of fungus *A. chrysogenium* [15], enzyme stability in the alkaline range is lower (pH 4-10).

The *P. lilacinus* proteinase displays maximal activity towards hydrolysis of tripeptide substrate Z-Ala-Ala-Leu-*p*NA at pH 10-12. The optimal pH for the cleavage of a

Table 1. Isolation of proteinase from culture medium of *P. lilacinus*

Purification step	Total activity, μmol/min	Protein, mg	Specific activity, μmol/min per mg protein	Yield, %	Purification degree
Culture medium	142.0	2350	0.06	100	
Precipitation with ammonium sulfate	118.0	1108	0.1	83	1.7
Chromatography on bacitracin-Silochrome	100.0	95	1.05	70	17.5
Ultrafiltration	95.0	80	1.2	67	20
Chromatography on bacitracin-Sepharose	85.0	16	5.7	60	95
Gel-permeation chromatography on Superdex 75	47.3	2.4	19.7	33	328

protein substrate, azocasein, is also shifted into the alkaline range (Fig. 1). Such pH dependence is also typical for another *P. lilacinus* proteinase, isolated from the same fungus earlier (pH optimum 10.3) [20], as well as for the enzyme from *A. chrysogenium* [15], displaying maximal activity at pH 11.0-11.5 and 10.0 in hydrolysis of protein and peptide substrates, respectively. However, fungal enzymes from *A. oligospora* [18] and *A. oryzae* [16] are most active at pH 7-9. A fungal proteinase of *A. fumigatus* [17] is also characterized by the maximum of proteolytic activity at pH 9.0 in the case of azocollagen hydrolysis. Therefore, *P. lilacinus* proteinase belongs to the subgroup of subtilisin-like enzymes whose optimum of proteolytic activity is located in the alkaline range (pH 10-12).

Dependence of thermostability and proteolytic activity of *P. lilacinus* proteinase on temperature. The studied enzyme is relatively stable, preserving 40% of its activity after a 3-h incubation at 60°C (Fig. 2). Relatively high thermostability is inherent to virtually all representatives of subtilisin-like proteinase family including bacillary subtilisins, for instance, subtilisin 72 [24] and thermitase [4]. It should be noted that at higher temperatures (70°C) the stability of the proteinase from *P. lilacinus* is approximately 2 times lower than the stability of subtilisin 72 (Fig. 2).

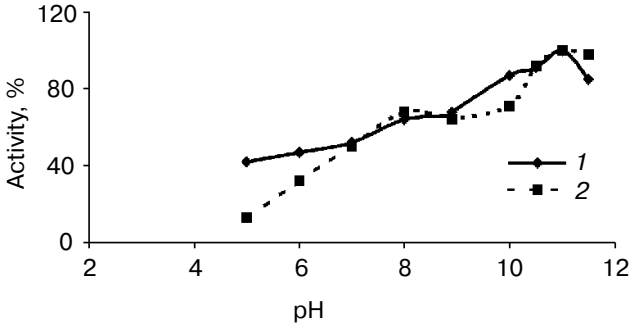

Fig. 1. The pH optimum of activity of *P. lilacinus* proteinase in hydrolysis of azocasein (*1*) and Z-Ala-Ala-Leu-*p*NA (*2*).

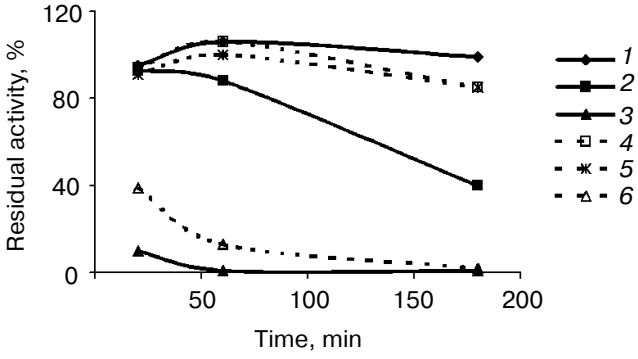

Fig. 2. Thermostability of the serine proteinase from *P. lilacinus* (*1-3*) and subtilisin 72 (*4-6*) at 50, 60, and 70°C, respectively.

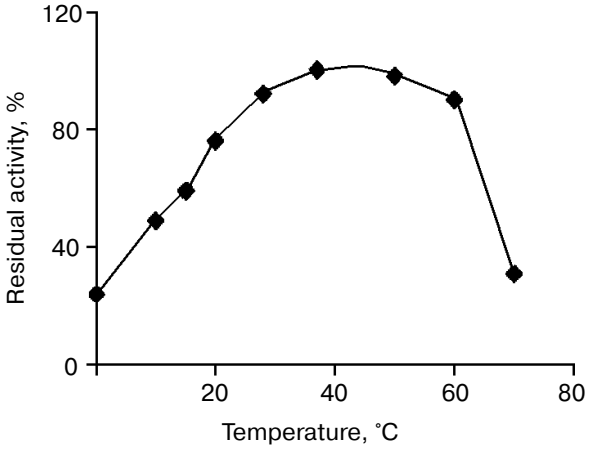

Fig. 3. Temperature optimum for activity of the *P. lilacinus* proteinase.

The proteinase from *P. lilacinus* exhibits the maximum of its activity at 30-60°C and has no distinct temperature optimum (Fig. 3). Such temperature dependence of activity is also characteristic for subtilisin 72. The alkaline serine proteinase from *P. lilacinus* [20] and the *A. chrysogenium* enzyme [15] have temperature optima of 60 and 55°C, respectively.

However, a proteinase from the closely related fungus *A. fumigatus* [25] is more thermolabile and has a temperature optimum at 37-42°C. Let us mention that the authors [25] classify the enzyme as a chymotrypsin-like serine proteinase based on the specificity of the enzyme.

An interesting feature of the *P. lilacinus* proteinase isolated by us is the ability to effectively hydrolyze proteins at lowered temperature, retaining about 25% of activity even at 0°C (Fig. 3).

Effect of Ca^{2+} and SDS on the stability of the *P. lilacinus* proteinase. The above data on the thermostability were obtained in the presence of 10 mM $CaCl_2$. However, in the absence of Ca^{2+} the enzyme is not that stable and retains only 29% of activity after 1 h incubation at 60°C, whereas in the presence of 1 and 10 mM $CaCl_2$ the residual activity is 77 and 79%, respectively. The absence of Ca^{2+} leads to destabilization, especially thermal, of the majority of proteinases belonging to the subtilisin family [26], in particular, proteinase K [10].

In a number of cases, the presence of Ca^{2+} is necessary for exhibiting maximal activity of the enzyme [4, 10]. Crystallographic studies of subtilisin-like enzymes demonstrated that their molecules contain two subsites for Ca^{2+} binding [4]. However, there are several serine proteinases described, for example, a subtilisin-like enzyme from *A. oryzae* [16], which are not sensitive to the presence of Ca^{2+}.

One of the features of the isolated enzyme that attracts most attention is its ability, like proteinase K, to maintain structure and activity in the presence of deter-

Table 2. Protein substrate specificity

Substrate	Activity, μmol/min per mg protein		
	subtilisin Carlsberg	proteinase from *P. lilacinus*	subtilisin 72
ZAlaAla**Leu**-*p*NA	55.5	24.0	38.2
ZAlaAla**Phe**-*p*NA	6.1	16.7	3.4
ForAlaAla**Phe**-*p*NA	–	2.2	1.3
ZAlaAla**Nle**-*p*NA	20.3	46.1	7.2
ZAlaAla**Val**-*p*NA	0	0	0
ZAlaAla**Ala**-*p*NA	2.6	11.0	0.54
ZAlaAla**Gly**-*p*NA	0	0	0
ZAlaAla**Pro**-*p*NA	0	0	0
ZAlaAla**Arg**-*p*NA	3.7	8.2	2.9
ZAlaPhe**Arg**-*p*NA	–	0.2	–
ZAlaAla**Lys**-*p*NA	0.64	2.0	0.6
ZThr**Leu**-*p*NA	–	0.1	0.3
ZAlaAlaAla**Leu**-*p*NA	–	10.5	24.8
ZGlyAlaPro**Leu**-*p*NA	–	0	0
ZAlaAlaVal**Leu**-*p*NA	–	0	1.2
ZAlaAlaLeu**Arg**-*p*NA	–	0.1	0
ZAlaAlaPhe**Arg**-*p*NA	–	0.1	0
ZAlaAlaMet**Arg**-*p*NA	–	0.1	0
ZAlaAlaTyr**Arg**-*p*NA	–	0.2	0
ZAlaAlaAla**Arg**-*p*NA	–	0.1	0
ZAlaAlaTrp**Arg**-*p*NA	–	0.2	–

gent. Thus, after incubation in 0.1 and 0.5% SDS for 1 h, the enzyme retains 91 and 52% of activity, respectively.

Substrate specificity. The substrate specificities of the *P. lilacinus* proteinase, subtilisin 72, and subtilisin Carlsberg are presented in Table 2. All these enzymes hydrolyze substrate containing leucine at the P1 position with high rate. Relatively high activity towards this substrate is also exhibited by other bacillary serine proteinases: e.g. *Bacillus licheniformis* enzyme and subtilisin BPN′ [27], as well as a fungal proteinase from *A. chrysogenium* [15], although the specific activity of the latter is approximately 50 times lower. However, contrary to the above enzymes, the *P. lilacinus* proteinase isolated by us preferentially hydrolyzes substrate with norleucine at this position. Moreover, the isolated enzyme removes *p*-nitroaniline from phenylalanine, alanine, and arginine in tripeptide substrates with relatively high rate, whereas the true subtilisins hydrolyze these substrates at a much slower rate (Table 2). These data indicate broader substrate specificity of *P. lilacinus* proteinase, preferring extensive, linear, aromatic, or positively charged residues in the S1 binding pocket.

The studied enzyme virtually does not hydrolyze protected dipeptide substrates Z-Xaa-Leu-*p*NA and For-Yaa-Phe-*p*NA, where Xaa is Ala, Val, Pro and Yaa is Phe, Ala, and also Bz-Arg-*p*NA. Similar specificity is exhibited by a fungal proteinase from *A. oligospora* [18], having an extremely low activity towards short substrates. Enzymes from *A. fumigatus* [25] and *V. chlamydosporium* [19] preferentially hydrolyze substrates containing a phenylalanine residue at the P1 position.

The studied enzyme cleaves native BSA and casein, but has no collagenolytic, gelatinase, or elastolytic activities typical for some bacillary subtilisin-like proteinases [28] and fungal enzymes, for instance, proteinases from *A. oligospora* [18], *V. chlamydosporium* [19], and *A. fumigatus* [17, 29].

Kinetic studies. The efficiency of hydrolysis of Z-Ala-Ala-Leu-*p*NA substrate by the *P. lilacinus* proteinase is 2 times lower than by subtilisin 72, first of all due to weakened binding (Table 3). The values are similar to K_m and k_{cat} values reported for hydrolysis of Glp-Ala-Ala-Leu-*p*NA substrate by serine proteinase from the archaebacterium *Halobacterium mediterranei* [30, 31] and equal to 0.14 mM and 36.9 sec^{-1}, respectively. Values of similar order are also typical for kinetics of hydrolysis of ternary *p*-nitroanilide substrates Suc-Ala-Ala-Ala-*p*NA by subtilisin Carlsberg ($K_m = 0.22$ mM and $k_{cat} \geq 97$ sec^{-1}) [32], as well as serine proteinase from *Bacillus* sp. ($K_m = 17$ mM and $k_{cat} = 3700$ sec^{-1}) [33].

One of the features of the isolated enzyme, which makes it distinct from typical subtilisins (Table 3), is its ability to hydrolyze substrates containing an arginine residue at the P1 position. The binding of leucine and arginine substrates occurs similarly, whereas lower efficiency of catalysis (~15 times) is determined by lowered rate of hydrolysis (~6 times) of the arginine substrate.

Inhibitory analysis. The effects of different compounds on the activity of the *P. lilacinus* proteinase are

Table 3. Kinetic characteristics of enzymes

Enzyme	ZAALpNA			ZAARpNA		
	$K_m \times 10^3$, M	k_{cat}, sec^{-1}	k_{cat}/K_m	$K_m \times 10^3$, M	k_{cat}, sec^{-1}	k_{cat}/K_m
P. lilacinus proteinase	1.6	121	75,625	4.2	20	4762
Subtilisin 72	0.7	95	135,714	–	–	–

Table 4. Effect of inhibitors and metal ion salts on the *P. lilacinus* proteinase

Reagent	Concentration, M	Residual activity, %
Initial preparation	–	100
$CoCl_2$	10^{-3}	100
$CuSO_4$	10^{-3}	63
Iodoacetamide	$2 \cdot 10^{-3}$	96
	$2 \cdot 10^{-4}$	93
p-CMB	$3 \cdot 10^{-5}$	7
PMSF	10^{-3}	6
EDTA	$5 \cdot 10^{-3}$	78
EGTA	10^{-3}	100
$MgCl_2$	$5 \cdot 10^{-3}$	100
$Hg(CH_3COO)_2$	10^{-4}	0
$Pb(CH_3COO)_2$	10^{-4}	97

given in Table 4. The enzyme is inhibited by phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteinases, in particular subtilisins [3], and also of fungal serine proteinases, for instance *A. fumigatus* proteinase [17]. The effect of such reagents as mercury acetate and *p*-CMB causes a complete enzyme inactivation, which is typical for proteinases belonging to the thermitase [4] and proteinase K [11] subgroups. This feature makes the *P. lilacinus* proteinase significantly different from the true bacillary subtilisins, as well as from serine proteinase from *P. lilacinus* isolated by Bonants et al. [20]. Indeed, contrary to typical subtilisins, which do not contain cysteine residues, the *P. lilacinus* proteinase contains (according to the data of amino acid analysis), like proteinase K, five half-cystine residues, whereas at least one of them is significant for exhibiting proteolytic activity of the enzyme. In the case of proteinase K, it was demonstrated [11] that the inhibition of activity by *p*-CMB is explained by the existence of two mercury-binding sites, one of which is located in the vicinity from the enzyme active site. The serine proteinase from *P. lilacinus* characterized by Bonants et al. [20] contains six cysteine residues, which probably form disulfide bonds in the protein molecule. Based on the presence of functionally important sulfhydryl groups, the proteinase from *P. lilacinus* can be classified as a member of the subfamily of thiol-dependent subtilisin found in taxonomically different microorganisms [15].

The chelating agents EDTA and EGTA had no effect on the activity of the enzyme.

Therefore, the proteinase from *P. lilacinus* is more likely to resemble proteinase K and bacillary thiol-dependent subtilisins than other fungal alkaline enzymes.

Structural studies. The N-terminal sequence of *P. lilacinus* proteinase has been determined (Gly-Ala-Thr-Thr-Gln-Gly-Ala-Thr-Gly/Ile-Xxx-Gly). It does not match the N-terminal sequence of subtilisin-like *P. lilacinus* proteinase (Ala-Tyr-Thr-Gln-Gln-Pro-Gly-Ala-Pro-Trp-Gly-Leu-Gly) isolated from the same fungus earlier [20]. No clear homology was revealed between the sequence of the *P. lilacinus* proteinase determined by us and known primary structures of bacillary and fungal serine proteinases. However, it should be noted that based on determination of only a short region of N-terminal sequence it is difficult to judge the difference in primary structure of the compared proteins, especially because the terminal regions of protein molecules are most variable.

Therefore, the our results allow classifying the isolated enzyme as a member of thiol-dependent subtilisin-like proteinases. This subfamily is believed to be the most ancient, since this kind of enzymes is found both in lower and higher organisms. The present work studied the enzyme produced by the *P. lilacinus* strain, isolated from soil buried under the earth mound over 3000 years ago; therefore, it could be assumed with a small degree of probability that the difference in structures of the two characterized subtilisin-like proteinases reflects certain evolutionary changes. However, it is more likely that such difference indicates the presence of at least two genes in the fungal genome, encoding for the two enzymes, one of which is induced by vitelline [20]. Nevertheless, it should be emphasized that this is one of the few studies aimed at biochemical and enzymatic comparison of modern strains and the strains of microorganisms that existed on earth thousands of years ago.

The discovered unique ability of the isolated proteinase to efficiently hydrolyze substrates both at low (down to 0°C) and high temperatures, as well as the ability to maintain activity in the presence of a detergent can facilitate the successful use of the enzyme in industry, for instance in production of washing agents.

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