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Characterization of Extracellular Proteinases of the Fungus *Acremonium chrysogenum* 226-A

Karakterizacija izvanstaničnih proteinaza gljive *Acremonium chrysogenum* 226-A

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Summary

Proteolytic enzymes secreted by the fungus ***Acremonium chrysogenum*** *226-A were characterized. Three proteinases were detected in the culture broth, two enzymes were found to be serine proteinases, while the third was a metalloproteinase. Relative molecular masses of the two serine proteinases were 25000 and 16000, respectively, while that of the metalloenzyme was 42000. The 25 kDa proteinase had the isoelectric point at pH = 8.0, while the 16 kDa enzyme focused at pH about 10.0. The study of enzymatic properties of* ***A. chrysogenum*** *proteinases revealed that the preparation was active on casein, collagen, elastin and gelatine, but not on several aminopeptidase substrates. 95 % of the proteolytic activity was due to the two serine-type enzymes. The activity of the preparation was, therefore, strongly inhibited by phenylmethylsulphonylfluoride but also by* Fe^{3+}, Cd^{2+}, Hg^{2+} *and* Ag^{+} *ions. The proteinase pattern of* ***A. chrysogenum*** *production strain 226-A clearly differs from that of other strains of the same species.*

Sažetak

Karakterizirani su proteolitički enzimi koje izlučuje gljiva ***Acremonium chrysogenum****. U podlozi za uzgoj određene su tri proteinaze, od čega dvije serinske i jedna metaloproteinaza. Određene su relativne molekularne mase serinskih proteinaza od 25000, odnosno 16000, te metaloproteinaze od 42000. Proteinaza od 25 kDa imala je izoelektričnu točku pri pH = 8,0, dok se 16 kDa enzim fokusirao pri pH oko 10,0. Studij enzimskih svojstava proteinaza* ***A. chrysogenum*** *pokazao je da je pripravak aktivan prema kazeinu, kolagenu, elastinu i želatini, ali ne i prema nekoliko supstrata aminopeptidaza. Čak 95 % proteolitičke aktivnosti posljedica je djelovanja enzima serinskog tipa. Aktivnost je stoga izrazito inhibirana fenilmetilsulfonilfluoridom, ali i* Fe^{3+}, Cd^{2+}, Hg^{2+} *i* Ag^{+} *ionima. Uzorak proteinaza proizvodnog soja* ***A. chrysogenum*** *226-A jasno se razlikuje od uzoraka drugih sojeva iste vrste.*

Introduction

Extracellular proteinases are produced by many genera of fungi such as *Aspergillus* (1,2), *Thermomyces* (3), *Agaricus* (4) and others. Proteolytic enzymes secreted by *Acremonium chrysogenum* (previously *Cephalosporium chrysogenum*) have first been described in the early seventies and have since then been the subject of extensive work in several laboratories (5–12). However, reports on the number and properties of proteases purified from different *A. chrysogenum* strains indicated differences in enzymatic, as well as physicochemical properties of enzymes from different strains. In five successive papers of Yagi *et al.* (5–9), one proteinase was purified from the ATCC 11550 strain and studied in detail, but no observation about the existence of other proteinases has been published at that time. The described enzyme had the size of 22.5 kDa (estimated by gel filtration), and the isoelectric point at pH = 10.5. It was inactivated by Fe^{3+}, Ag^{+}, Hg^{2+} ions, SDS and DFP, indicating that it was a serine proteinase.

Satoh *et al.* (10) described another proteinase from *A. chrysogenum* MT-62 as a blood coagulating enzyme. Two methods employed to estimate the molecular mass of the enzyme resulted in a pronounced discrepancy, so that the size determined from the sedimentation coefficient was 39000, but that estimated by gel filtration was 124000. The isoelectric point of the enzyme was determined in

the pH region between 3.0 and 4.0, and it was inhibited by EDTA, indicating that it was a metalloproteinase.

Stepanov *et al.* (*11*) first reported the production of two proteinases by the *A. chrysogenum* strain 298-A. According to that report, both enzymes were serine proteinases of the same size (28000), but one being a basic (pI = 10.0) and the other an acidic (pI = 4.0) protein. N-terminal sequences of the two proteinases revealed clear homologies.

The existence of two serine proteinases was corroborated by Tsuchiya *et al.* (*12*) who purified them from another *A. chrysogenum* strain KM388 and, similarly to the enzymes described by Stepanov *et al.* (1986), one of them was a basic (pI = 10.5), while the other was an acidic protein (pI = 3.8). Proteinases were also similar in size, the basic enzyme having the relative molecular mass of 36000 (determined electrophoretically) or 22000 (gel filtration), while the acidic one had 42000 daltons (electrophoretically), or 24000 daltons (gel filtration). Enzymatic properties of these enzymes were comparable to those of the proteases from the strain 298-A.

It is not clear whether discrepancies in the number and properties of proteolytic enzymes secreted by different *A. chrysogenum* strains originate from different genetic backgrounds, or if they are a result of different purification or screening procedures. In this paper we report the isolation and partial characterization of three extracellular proteinases produced by the *A. chrysogenum* strain 226-A. The proteinase pattern clearly differs from those described in other strains.

Materials and Methods

Microorganism and growth conditions

Acremonium chrysogenum cephalosporin production strain 226-A, obtained from the collection of microorganisms of »KRKA« pharmaceutical factory, Novo mesto, Slovenia, was used in this work.

The fermentation was carried in a 130 L Chemap fermenter in a medium containing starch (6 %), soybean flower (7.3 %), malt (0.5 %), $CaCO_3$ (1 %), methionine (1 %) and soybean oil (0.4 %), for 150–200 hours. No significant changes in the proteinase pattern were observed within this period.

Proteinase preparation

The fermentation broth was filtered through Decalite filtration medium in a vacuum rotary filter, and then through Whatmann K300 filter paper to obtain the clear filtrate. The filtrate was passed through an Amberlite XAD-4 column (2.2 × 40 cm) which adsorbed cephalosporin and the brown pigment from the medium, and then through an Amberlite XAD-7 column (4.5 × 30 cm) which bound practically all of the proteolytic activity. Proteinases were eluted with 2 volumes of cold (4 °C) 20 % acetone in 25 mM Tris-HCl buffer pH = 8, at the flow rate of 3.3 mL/min, precipitated by the addition of 3 volumes of cold acetone, centrifuged and lyophilized.

Electrophoresis

SDS electrophoresis of the proteinase preparation was done by the method of Laemmli (*13*). Electrophoresis in slabs with a linear gradient of the polyacrylamide concentration was done as described (*14*). Isoelectric focusing was performed with the Phast System apparatus (Pharmacia) using commercial slabs (pH range 3–10) according to manufacturers instructions.

After electrophoresis, or isoelectric focusing, gels were stained either for proteins overnight in 0.2 % Coomassie brilliant blue R-250 in 40 % methanol, or, since it was shown that the activity of the preparation was not affected by SDS, for the proteolytic activity, as follows: Gel slabs were washed 2 times for 15 min in water and, if required, for 15 min in 5 mM PMSF. Detection gel was prepared by mixing 50 mL of 3 % hot agarose with the same volume of cold (4 °C) substrate solution (10 g casein, 1.83 g Tris in 50 mL water). pH of the solution was 7.5, 60 mL of detection gel (50 °C) was poured over the electrophoresis gel in a Petri dish, allowed to solidify and transferred to 37 °C. After 1–2 hours white bands appeared at the position of proteolytic enzymes. Gels were photographed immediately since bands were not stable and fainted as the degradation of casein proceeded and proteinases diffused within the slab.

Gel filtration

The number and size of *A. chrysogenum* proteinases were determined by gel filtration in an Ultrogel AcA54 column. The sample of proteinase preparation was applied to a column (1.6 × 90 cm) and eluted with 0.02 M Tris-HCl buffer pH = 8.8 with the addition of 0.1 M NaCl at the flow rate 7 mL/h. 2 mL fractions were collected and analyzed for proteins (A_{280}) in the case of molecular weight standards, or for the proteolytic activity, either without any addition, or, in order to determine the type of individual proteinase peaks, with the addition of 1 mM PMSF, or 10 mM EDTA, respectively.

Other methods

Proteinase activity was determined by the method of Kunitz (*15*) using casein (Hammarsten) as the substrate. Proteins were determined by the method of Lowry *et al.* (*16*).

Results

Acremonium chrysogenum 226-A was grown as described in Materials and Methods and the proteolytic activity in the broth was measured. Secretion of proteinases started about 60 hours after the beginning of fermentation, simultaneously with the production of the antibiotic cephalosporin, and reached the maximum in 150 hours. Results corroborate the finding that there is a link between the proteinase biosynthesis in *A. chrysogenum* and the secondary metabolism of cells (*17*). The growth medium was filtered and the filtrate subjected to the procedure described under Materials and Methods. The proteolytic preparation obtained was analyzed by electrophoresis in order to establish the number and size of proteinases secreted by *A. chrysogenum* 226-A. Results, shown in Fig. 1, revealed 3 protein bands which stained for proteinase activity. The upper band appears diffuse since the photograph was taken relatively late in the course of reaction, to allow the appearence of the small

molecular mass band. However, shorter incubations show that it does not overlap any other proteinase activity. The upper two bands migrated with relative molecular masses unusually high for fungal proteinases (130000 and 87000), and their activity was inhibited by PMSF, suggesting that they belonged to the group of serine proteinases (Fig. 1. lane 3). The third enzyme, apparently contributing only with a small fraction of the total proteinase activity, had the relative molecular mass of 36000 and was inhibited by EDTA indicating that it was a metalloproteinase (Fig. 1. lane 4).

To estimate the size of proteinases under nondenaturing conditions (without the SDS treatment) the proteinase preparation was analyzed by electrophoresis in the linear gradient of the polyacrylamide concentration. By this method, however, at least four bands staining for proteinase activity, all inhibited by PMSF, were obtained, ranging in size from 100000 to 500000 Da (not shown). Besides, a smaller (42000), EDTA-sensitive band was obtained. Relatively high number of proteinase bands and large size of enzymes obtained by gradient gel electrophoresis could either be explained by the enzyme oligomerization, or by the anomalous migration of proteinase molecules during the electrophoresis. To check these possibilities the size of proteinases was also determined by gel filtration on Ultrogel AcA54. As it can be seen in Fig. 2, three peaks were obtained and corresponding relative molecular masses were 42000, 25000 and 16000. The 42 kDa enzyme could be completely inhibited by EDTA (not shown) indicating that this was a metalloenzyme. The size of this enzyme was in agreement with the data obtained by SDS- and gradient gel electrophoresis. The two peaks of lower molecular masses were PMSF sensitive and their position indicated that their large size obtained by SDS-, or gradient gel electrophoresis was probably due to an unusual behaviour in the electric field. However, since a number of bands were obtained in a polyacrylamide gradient, some aggregation of molecules could not be excluded. Isoelectric focusing of the proteinase preparation (Fig. 3) revealed two bands, both PMSF sensitive and EDTA resistant, focusing at pH = 8.0 and about 10.0. Metalloproteinase was not recorded, probably due to its low activity. To establish which of the isoelectric points corresponded to each of the proteinases, different gel filtration peaks were analyzed electrophoretically. It was established that the

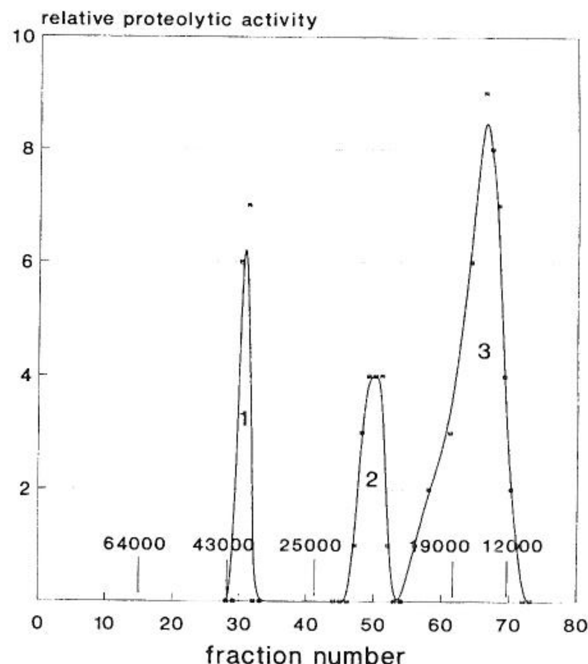

Fig.2. Gel filtration of *A. chrysogenum* proteinases on Ultrogel AcA54. 1. metalloproteinase; 2. and 3. serine proteinases. Position of relative molecular mass markers is indicated.

Slika 2. Gel-filtracija proteinaza *A. chrysogenum* na Ultrogelu AcA54. 1. metaloproteinaza; 2. i 3. serinske proteinaze. Naznačen je položaj standardnih proteina.

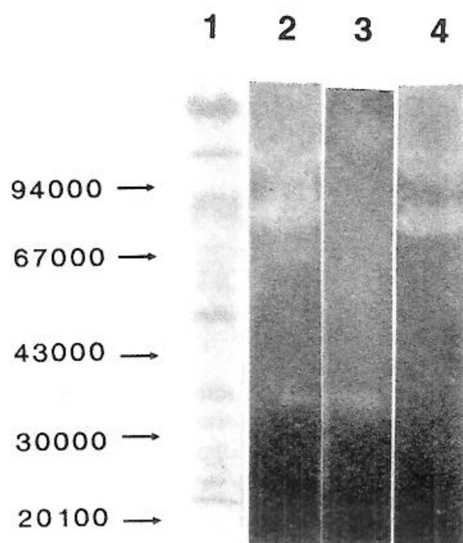

Fig.1. SDS-electrophoresis of the *A.chrysogenum* proteinase preparation. The gel was stained for: 1. proteins; 2. proteolytic activity; 3. proteolytic activity in the presence of PMSF; 4. proteolytic activity in the presence of EDTA. Position of relative molecular mass markers is indicated on the left.

Slika 1. SDS-elektroforeza pripravka proteinaza *A. chrysogenum*. Gel je bojan na: 1. proteine; 2. proteolitičku aktivnost; 3. proteolitičku aktivnost u prisutnosti PMSF-a; 4. proteolitičku aktivnost u prisutnosti EDTA. Položaj standardnih proteina prikazan je s lijeve strane.

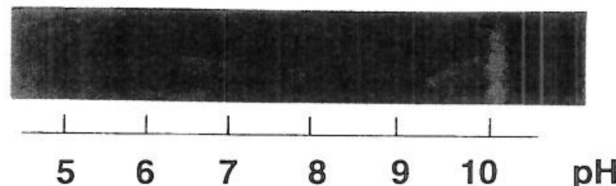

Fig.3. Isoelectric focusing of *A. chrysogenum* proteinases. Proteinase preparation was submitted to isoelectric focusing in Pharmacia Phast System, and the gel was stained for proteolytic activity.

Slika 3. Izoelektrično fokusiranje proteinaza *A. chrysogenum*. Pripravak proteinaza podvrgnut je izoelektričnom fokusiranju u uređaju Pharmacia Phast System, a gel je obojen na proteolitičku aktivnost.

Table 1. Influence of different agents on the proteolytic activity of *Acremonium chrysogenum* proteinases

Tablica 1. Utjecaj različitih agensa na aktivnost proteinaza *Acremonium chrysogenum*

Addition	c/ mM	Activity / %
none		100
PMSF	5	5
iodoacetate	10	100
EDTA	10	95
dithiothreitol	10	100
β-mercaptoethanol	10	100
SDS	70	100
Cu^{2+}	10	100
Fe^{2+}	10	108
Fe^{3+}	10	0
Co^{2+}	10	106
Ni^{2+}	10	81
Cd^{2+}	10	36
Ca^{2+}	10	100
Zn^{2+}	10	95
Ba^{2+}	10	100
Mg^{2+}	10	100
K^{+}	10	116
Mn^{2+}	10	100
Hg^{2+}	10	24
Ag^{+}	10	14
Pb^{2+}	10	75

25 kDa proteinase had the isoelectric point at pH = 8.0, while the 16 kDa enzyme focused at pH about 10.0.

Enzymatic properties of the proteinase preparation were also determined. The preparation had the activity of about 2000 units/mg and it was found that it hydrolysed casein, collagen, elastin and gelatin, but not naphtylamide derivatives of arginine, leucine or proline. The influence of different potential inhibitors and metal ions is presented in Table 1. It can be seen that only about 5 % of the total activity was due to the metalloproteinase (inhibited by EDTA), while the rest was the result of the serine proteinase activity. Besides, Fe^{3+}, Cd^{2+}, Hg^{2+} and Ag^{+} ions were found to inhibit the proteolytic activity significantly.

The optimal temperature for the reaction was found at about 60 °C, while the optimal pH was in the pH range 10–11. The preparation was stable at 4 °C for over 50 days, while at 25 °C a significant loss of activity (80 %) occurred during that period. At 37 °C, 90 % of activity was lost in 6 days, while at 45 °C the same effect was recorded in 12 hours. The relative stability of the preparation was observed in the pH range between 7 and 11, while at pH below 4, or above 12, more than 90 % activity was lost in 1 hour (not shown).

Discussion

Proteolytic enzymes secreted by *Acremonium chrysogenum* have been studied in several laboratories, but different reports contain often contradictory data on the number and characteristics of proteinases secreted by this fungus. The reason could be that different *A. chrysogenum* strains produce different proteinase patterns, since in each report a different strain was used. The early paper of Satoh *et al.* (*10*) described only one, apparently metalloproteinase, while more recent papers, in which production strains 298-A and KM388 were studied, described two enzymes, both of the serine proteinase type, one being an acidic and the other a basic protein. Therefore, it could be speculated that either some strains lack some of the enzymes, or that different reports were due to a smaller contribution of some proteinases in the overall proteolytic activity, so that they remained undetected or neglected. Physicochemical as well as enzymatic properties of serine proteinases described in the two papers of Stepanov *et al.* (*11*) and Tsuchiya *et al.* (*12*) were similar, except the molecular masses of the two enzymes, which could be attributed to the methodology impreciseness. In this work, properties of proteolytic enzymes from another production strain 226-A have been studied. In contrast to previous reports three proteinases have been found, two serine-type enzymes, like in strains KM388 and 298-A, but also one metalloproteinase. Most of the activity was, however, due to the first two enzymes. The difference between the serine proteinases from the first two strains, and those from our strain is that in KM388 and 298-A one of the enzymes was basic (isoelectric point at pH = 10–10.5), while the other was acidic (isoelectric point at pH = 3.8–4.0). In contrast, our strain secretes two basic proteinases with isoelectric points at pH = 8.0 and about 10.0. A very basic proteinase species could be compared to corresponding enzymes from strains KM388 and 298-A although its size seems to be smaller than in the other two strains. Moreover, it has the smallest size reported so far for any *A. chrysogenum* proteinase. The other serine type proteinase described in this work clearly differs from its counterpart in other strains in the isoelectric point, indicating a difference in its amino acid composition. Such differences could result from genetic diversities caused by a number of more or less uncontrolled genetic manipulations directed to the increase of yield or quality of products, frequently observed in industrial microorganisms. Therefore, the genetic background of different strains might vary significantly and often requires a separate investigation for each production strain as in the case of secretory proteolytic enzymes described in this paper. Interestingly, enzymatic properties of enzymes from the strain 226-A do not differ significantly from those from other strains, indicating that these properties might be of an evolutionary importance, and are therefore conserved even when the proteins have changed significantly.

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