JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 94, No. 4, 304–308. 2002

Purification and Characterization of an Extracellular Trypsin-Like Protease of *Fusarium oxysporum* var. *lini*

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Received 23 January 2002/Accepted 3 July 2002

An alkaline serineprotease, capable of hydrolyzing *N*α-benzoyl-DL arginine *p*-nitroanilide, was secreted by *Fusarium oxysporum* var. *lini* grown in the presence of gelatin as the sole nitrogen and carbon source. The protease was purified 65-fold to electrophoretic homogenity from the culture supernatant in a three-step procedure comprising QSepharose chromatography, affinity chromatography, and FPLC on a MonoQ column. SDS–PAGE analysis of the purified protein indicated an estimated molecular mass of 41 kDa. The protease had optimum activity at a reaction temperature of 45°C and showed a rapid decrease of activity at 48°C. The optimum pH was around 8.0. Characterization of the protease showed that Ca^{2+} and Mg^{2+} cations increased the activity, which was not inhibited by EDTA or 1,10-phenanthroline. The enzyme activity on *N*α-benzoyl-DL arginine *p*-nitroanilide was inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, *p*-aminobenzamidine dihydrochloride, aprotinin, 3-4 dichloroisocoumarin, and *N*-tosyl-L-lysine chloromethyl ketone. The enzyme is also inhibited by substrate concentrations higher than 2.5×10^{-4} M. The protease had a Michaelis–Menten constant of 0.16 mM and a V_{max} of 0.60 μmol released product·min^{-1}·mg^{-1} enzyme when assayed in a non-inhibiting substrate concentration. The activity on *N*α-benzoyl-DL arginine *p*-nitroanilide was competitively inhibited by *p*-aminobenzamidine dihydrochoride. A K_i value of 0.04 mM was obtained.

[**Key words:** trypsin-like protease, *Fusarium*, inhibitor effects]

Fusarium is a non-dermatophytic mold, widespread in nature both in the soil and on many plants. The genus includes more than 60 species, of which at least 10 are agents of infection in humans. *F. oxysporum*, *F. moniliforme*, and *F. solani* are the species most frequently isolated from human lesions (1). However, the main interest in these fungi arises because of their ability to cause diseases in economically important plant hosts (2).

It has been suggested that *F. oxysporum* may be a producer of a single-cell protein whose nutritive quality is high (3–6). In Brazil, studies on the nutritive value of the protein produced using vinasse (waste from sugar-cane distillation) as a raw material have indicated that a good quality food might be produced when *F. oxysporum* var. *lini* is grown in this substrate (5, 6). These studies led us to investigate the ability of *F. oxysporum* to grow in glycerol (7), lactic acid (8), and protein (9), since they are the main constituents of vinasse and the main sources of energy and carbon for the production of a single-cell protein in the waste.

Like many heterotrophic organisms, *F. oxysporum* utilizes proteins and peptides as growth substrates and, in addition, produces proteolytic-type enzymes, which are assumed to generate small peptides from the protein-based growth substrates. The effects of different culture conditions on the production of extracellular proteolytic enzymes in microorganisms are well documented (10–12). It has been shown that the activity of extracellular proteases in *F. oxysporum* var. *lini* also involves the well-known induction by exogenous protein balanced by metabolite repression (9, 13). So far, only one trypsin-like protease, which is synthesized as a preproenzyme, has been characterized from *F. oxysporum* (14). Sequence alignment revealed equally close homology to both bovine trypsin and bacterial trypsins.

Microbial proteases are particulary important because they have different substrate specifities, making them useful in several areas of biochemistry and biotechnology (for a review, see Rao *et al.* [15]). In this paper, we report the finding of an extracellular protease produced by *F. oxysporum* var. *lini*, grown in the presence of gelatin as the sole nitrogen and carbon source. It was purified and biochemically characterized.

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Abbreviations: BApNA, *N*α-benzoyl-DL arginine *p*-nitroanilide; BTpNA, *N*-benzoyl-L-tyrosine *p*-nitroanilide; DABB, 4,4′-diazoaminobis-benzamidine; DCI, 3-4 dichloroisocoumarin; E-64, (L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine; pAB, *p*-aminobenzamidine dihydrochoride; PEFABLOC (AEBSF), 4-(2 aminoethyl)-benzenesulfonyl fluoride hydrochloride; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

MATERIALS AND METHODS

Organism and growth conditions *Fusarium oxysporum* var. *lini* ATCC 10960 was maintained on potato dextrose agar at 4°C, after growth for 5 d at 30°C. A spore suspension for inoculation was prepared by adding water to potato dextrose agar in tubes. The fungus was grown in Treschow's medium containing 2% gelatin (16). For growth, 750 ml medium was used in 2-*l* shake flasks, which were placed in an orbital incubator operated at 28°C. Culture supernatants were obtained by filtration on glass fiber membranes (type GF/C; Whatman, UK) after 72-h growth.

Enzyme purification Culture supernatants were used as the enzyme source. (i) Four hundred milliliters of culture supernatant from *F. oxysporum* cell cultures was loaded onto an ion-exchange column of QSepharose (2.5×7.5 cm) previously equilibrated with 0.01 M NH_4HCO_3 buffer, pH 8.0, at a flow rate of 1 ml/min. The fraction that did not interact with the column was able to hydrolyse BApNA (Sigma, St. Louis, MO, USA). The column was then washed with NH_4HCO_3 buffer containing 1 M NaCl to elute bound proteins, fusarin, fusaric acid, and other compounds. (ii) The active material was applied onto an affinity column of Sepharose–*p*-aminobenzamidine (2.0×10.0 cm) equilibrated with 0.05 M NH_4HCO_3 buffer, pH 8.0. Bound fractions were eluted at a flow rate of 1.0 ml/min with 1 mM pAB in bicarbonate buffer. Fractions (2 ml) with activity on BApNA were pooled, dialyzed overnight against 0.01 M NH_4HCO_3 buffer, pH 8.0, and lyophilized. (iii) The lyophilized fraction from the affinity chromatography was resuspended in 1.5 ml of 0.01 M borate buffer, pH 8.5, and further purified by ion-exchange chromatography on a MonoQ HR 5/5 column (Amersham Biosciences, Uppsala, Sweeden) using an FPLC and a linear gradient of NaCl from 0.05 to 1 M in the same borate buffer, at a flow rate of 1 ml/min. The protein content in the column effluent was monitored by determining the absorbance at 280 nm (A_{280}). All the fractions were monitored by enzymatic assay and SDS–PAGE.

Proteolytic assay Proteolytic activity was assayed using BApNA as a substrate. The reaction mixture contained 0.2 ml culture filtrate (or fractions from the columns), 10 mM $MgCl_2$, 2.5×10^{-4} M BApNA, and 0.01 M Tris–HCl buffer, pH 8.0. The mixture was incubated at 45°C for 4 h. The reaction was linear for 6 h. The absorbance at 410 nm (A_{410}) was measured using a spectrophotometer (DU68; Beckman, Fullerton, CA, USA). One unit of enzyme activity was defined as the amount of enzyme that could produce 1 μmol of product·min^{-1} under the described conditions. The specific activity was expressed as the number of units of activity per mg of protein.

Characterization of the enzyme The activity was assayed at different pH values ranging from 4 to 11. The following buffers were used: for pH 4–5, 0.01 M acetate; for pH 6–7, 0.01 M phosphate; for pH 8–9, 0.01 M Tris–HCl; for pHs 10 and 11, bicarbonate. The temperature dependence of the protease was determined in the temperature range 25–70°C, as described above. For thermostability testing, the purified protein was incubated at 55–65°C for different lenghts of time (0–60 min) in Tris–HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and then assayed for 4 h. The effects of different ions (Ca^{2+}, Mg^{2+}, and Zn^{2+}) on protease activity were studied by incubating the purified protein at 45°C in 0.01 M Tris–HCl buffer, pH 8.0, containing 1 and 10.0 mM ion concentrations. Afterwards, the residual activities were assayed with 2.5×10^{-4} M BApNA as the substrate at 45°C for 4 h.

For inhibition studies, pure protease was preincubated with various compounds (1,10-phenanthroline, EDTA, PEFABLOC, PMSF, 3-4 DCI, TPCK, TLCK, pAB, E-64, pepstatin, iodacetamide, and aprotinin) for 60 min at room temperature, in 0.01 M Tris–HCl buffer, pH 8.0 (without $MgCl_2$ in the cases of EDTA and 1,10-phenanthroline), and the BApNA activity was assayed.

Electrophoresis Protein fractions were analysed by SDS–PAGE by the method of Laemmli (17) with a separation gel of 10% polyacrilamide in Tris–glycine buffer, pH 8.2. Gels were stained with silver according to Wray *et al.* (18).

Protein determination The protein content of samples was estimated by the method of Bradford (19) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

In previous work, the extracellular protease production by *F. oxysporum* was analyzed in several media containing different carbon and nitrogen sources (9). Findings suggested that protease production is induced by protein sources and that synthesis of the enzymes is, in part, regulated by catabolite repression.

In the present study, the crude enzyme preparation from the culture supernatant of *F. oxysporum* var. *lini*, cultivated in gelatin as a carbon and nitrogen source, was loaded onto an ion-exchange QSepharose column. In this step of the purification, portions of the contaminating proteins and colored compounds were retained. Further purification of the fraction that did not bind to the QSepharose column (96% of the activity; Table 1) was achieved by an affinity column of Sepharose–*p*-aminobenzamidine (yield, 3%; Table 1). Our results indicated the probability that, more than one enzyme was acting on BApNA, since inhibition by PMSF was observed in the culture broth but not in the active fraction eluted in this step. Only a minor component was recovered using the affinity column. Since increasing the pAB concentration did not result in elution of the major enzyme, most of the protease active on BApNA stayed bound to the Sepharose–*p*-aminobenzamidine column. To adress this problem, we will try to elute the enzyme still bound to the resin with a new inhibitor that has a diglycidyl ether group bound to the *p*-NH_2 group of pAB, simulating the spacer arm of the affinity resin. Only the fractions containing activity, eluted with 1 mM pAB, were pooled, dialyzed, and concentrated. The protein mixture eluted from Sepharose–*p*-aminobenzamidine was separated by FPL chromatography on a MonoQ HR 5/5 column. The first peak, eluted in 0.22 M NaCl, contained a protease that was able to hydrolyse BApNA (Fig. 1). About 87% of the protease activity bound to Sepharose–*p*-aminobenzamidine was eluted in this

TABLE 1. Purification of *F. oxysporum* trypsin-like protease

Purification step	Total protein (mg)	Specific activity (U/mg)×10^2	Total activity (U)×10^2	Purification (fold)	Yield (%)
Culture supernatant	50.4	0.097	4.92	1.0	100.0
QSepharose (fraction not bound)	38.0	0.124	4.72	1.3	95.9
Sepharose–*p*-aminobenzamidine	0.096	1.562	0.15	16.1	3.0
MonoQ	0.018	6.320	0.13	65.1	2.6

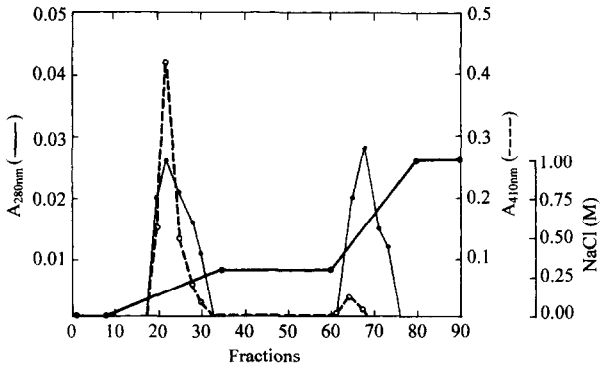

FIG. 1. Representative elution profile on MonoQ HR 5/5 ion exchange chromatography of an enzyme preparation deriving from a Sepharose–*p*-aminobenzamidine column: thin line, $A_{280\,nm}$; dotted line, $A_{410\,nm}$, activity on BapNA; thick line, NaCl gradient.

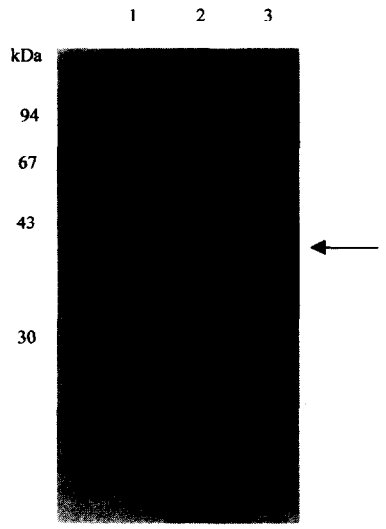

FIG. 2. SDS–PAGE under reducing conditions of fractions eluted at different steps in the purification of the trypsin-like protease from *Fusarium oxysporum* var. *lini*. Lane 1, Molecular weight; lane 2, fraction eluted from *p*-aminobenzamidine chromatography; lane 3, peak 1 from MonoQ column.

peak. Probably, this enzyme, active on BApNA, does not bind to QSepharose (the first step in the purification process) because its isoeletric point is higher than 8.0. Indeed, in the first step of the purification process we observed preferential ligation of other compounds and proteins excreted by *F. oxysporum*.

After the FPLC, the extracellular protease eluted in the first peak was purified 65 fold (Table 1). There was little decrease in the yield in this purification step, which can be explained by the fact that the activity on BApNA is concentrated in the peak I. The enzyme purification was followed by SDS–PAGE, under non-reducing and reducing conditions. The electrophoretic pattern was the same under both conditions. In Fig. 2, the protein composition of the enzyme preparation isolated by Sepharose–*p*-aminobenzamidine chromatography (lane 2) is compared to that obtained by FPLC (lane 3). The material eluted from Sepharose–*p*-aminobenzamidine column (lane 2) gave two outstanding protein bands, with molecular masses of 41 and 23 kDa. After the last purification step, a single band, corresponding to the first peak, was observed after gel electrophoresis of the sample. The molecular mass of the corresponding protein, determined by SDS–PAGE, was 41,000 Da. The *F. oxysporum* trypsin reported by Rypniewski *et al.* (14) is homologous to trypsins from *Streptomyces griseus*, *S. erythraeus* and to bovine trypsin. The enzyme consists of 224 amino acid residues and has a molecular mass of 22,190 Da. The authors mention two other proteinases isolated from *F. oxysporum*: a subtilisin-like enzyme and an aspartic proteinase (14).

Detailed studies were carried out in order to characterize the purified enzyme. The optimum pH within the range from 4.0 to 11.0 was determined using BApNA as a substrate. The enzyme showed maximum activity at pH 8.0, indicating that it is an alkaline protease. The trypsin described by Rypniewski *et al.* (14) had a completely different pH activity profile.

Under the conditions described in Materials and Methods, the purified protease exhibited optimum activity at 45°C. The enzyme remained active in the range from 20°C to 55°C, at which temperatures the relative activity was approximately 29% and 19%, respectively. The enzyme became unstable at about 48°C as deduced from an Arrhenius plot (not shown). Total enzyme activity was lost after incubation of the protein in 0.01 M Tris–HCl buffer, pH 8.0, for 24 h at 55°C. The rate of heat denaturation of the enzyme was 0.62%·min^{-1} at 55°C, 0.65% at 60°C and 1.15% at 65°C (k values: 0.0062, 0.0065, and 0.0115, respectively) as deduced by the equation

$$\ln A/A_0 = e^{-kt}$$

The effects of different protease inhibitors and cations on the enzyme activity were also investigated (Tables 2 and 3). The purified enzyme was strongly inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (PEFABLOC), an irreversible serine protease inhibitor, pAB, and aprotinin. Significant inhibition was also observed with TLCK. Pepstatin, a potent inhibitor of the aspartic proteases, did not affect the activity. The lack of inhibition by iodacetamide and the small inhibitory effect of E-64 excluded the enzyme from the cysteine proteinase class. The protease has trypsin-like characteristics as inferred by the inhibitor effects (Tables 2 and 3), activity on BApNA, and lack of activity on BTpNA. Subtilisins are not inhibited by pAB (Table 4) and DABB (20). The chelating

TABLE 2. Effects of different inhibitors on the activity of the trypsin-like protease from *Fusarium oxysporum* var. *lini*

Compound	Final concentration	Percentage inhibition
None		0
PEFABLOC	1 mM	86.1
Aprotinin	1 μg/ml	79.8
pAB	1 mM	76.1
DCI	1 mM	28.4
TLCK	1 mM	42.8
E-64	1 mM	23.6
Pepstatin	1 μg/ml	0
Iodacetamide	1 mM	0

TABLE 3. Effects of divalent ions and chelating agents on activity of the protease purified from *Fusarium oxysporum* var. *lini*

Compound	Relative activity (%)
None	100
EDTA (1 mM)	100
1,10-Phenanthroline (1 mM)	100
$MgCl_2$ (1 mM)	127
$MgCl_2$ (10 mM)	128
$CaCl_2$ (1 mM)	129
$CaCl_2$ (10 mM)	127
$ZnCl_2$ (1 mM)	107
$ZnCl_2$ (10 mM)	33

TABLE 4. Enzymatic activity ($U \times 10^2$) of subtilisins and *F. oxysporum* purified enzyme

Substrate	Enzymes		
	Subtilisin (BPN)	Subtilisin (Carlsberg)	Purified enzyme (*F. oxysporum*)
BApNA	0.004	0.010	6.501
BTpNA	0.106	0.043	0.108
BApNA+pAB	0.004	0.009	1.544
BTpNA+pAB	0.109	0.042	0.081

The enzymatic activities were determined in 0.01 M Tris–HCl, pH 8.0, 10 mM $MgCl_2$, at 45°C, in the presence of 1 mM BTpNA or BApNA for subtilisins and 1 mM BTpNA or 0.25 mM BApNA for the *F. oxysporum* purified enzyme. The absorbance was measured at 410 nm.

agents EDTA and 1,10-phenanthroline did not affect the proteolytic activity. On the other hand, protease activity was slightly enhanced when $CaCl_2$ or $MgCl_2$ was added to the reaction mixture. On the contrary, $ZnCl_2$ had an inhibitory effect at a high concentration. Most probably, the ions Ca^{2+} and Mg^{2+} are involved in stabilizing the active structure, but they are not needed for the catalytic function or for the protein chain folding process to form the active structure. Calcium ions are known to protect proteinase against denaturation and proteolytic degradation (21, 22). There is no evidence of any divalent cation binding sites in the structure of *F. oxysporum* trypsin as described by Rypniewski *et al.* (14).

Figure 3 shows a plot of $1/V$ against $1/S$. It can be seen that inhibition arose at substrate concentrations higher than 2.5×10^{-4} M. There are a number of causes of this effect. The formation of ineffective complexes with two substrate molecules combined at an active site was discussed by Haldane (23). Our data (Fig. 3, insert) do not fit the model described by Haldane. However, inhibition by a high substrate concentration can be also due to substrate molecules binding to the enzyme in an incorrect orientation or to other factors. Analysis of the kinetic data at lower substrate concentrations (0.25 to 1.0×10^{-4} M) by the graphical method of Lineweaver–Burk indicates a half-saturation constant (K_m) of 0.16 ± 0.02 mM and a V_{max} of 0.60 μmol released product·min^{-1}·mg^{-1} enzyme.

The profile of enzyme inhibition by pAB (Fig. 4) indicates a competitive type of inhibition within the inhibitive concentration range studied. The slopes of reciprocal plots made at a series of different inhibitor concentrations were plotted against the inhibitor concentrations to determine the

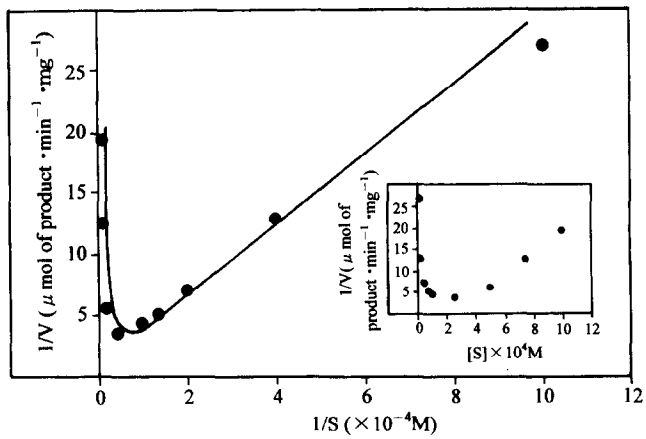

FIG. 3. Effect of substrate concentration on the reaction velocity: Lineweaver–Burk plot of initial velocity of reaction on BApNA. Insert: $1/V$ versus S.

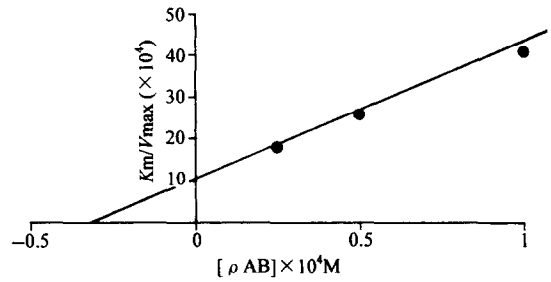

FIG. 4. Determination of K_i.

K_i (23). A K_i value of 0.04 mM was obtained, which is suggestive of strong inhibition.

The stability of the enzyme over wide pH and temperature ranges suggests that it may play an important role in the growth and development of *Fusarium*. It has been proposed that in some fungus–plant interactions, trypsin-like proteases may function as pathogenicity factors (10). In conclusion, our results showed that *F. oxysporum* var. *lini*, secretes a protease that has affinity to *p*-aminobenzamidine. The enzyme can be classified as a trypsin-like serine protease.

ACKNOWLEDGMENT

R. A. Barata was supported by a grant from Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG), Minas Gerais, Brazil.

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