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Novel Trypsin Inhibitors from the White Rot Fungus *Abortiporus biennis*. Partial Purification and Characterization

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Abstract—Novel trypsin inhibitors from the white rot fungus *Abortiporus biennis* were isolated, partially purified, and characterized. The inhibitors were purified by heat treatment, anion-exchange chromatography, and gel filtration. SDS-PAGE of the purified preparation demonstrated the presence of two proteins with molecular masses of 20 and 21.5 kDa. The *A. biennis* inhibitors were most active against trypsin, while chymotrypsin α, proteinase K, and Carlsberg subtilisin were inhibited to a smaller extent. The inhibitors are acidic proteins with remarkably high heat stability.

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Proteases are encoded by ~2% of all known genes, which can be taken as a measure of their importance for living organisms [1]. However, despite their physiological necessity, these enzymes are potentially hazardous to cells, and their activity must be precisely regulated. This control can be achieved through regulation of gene expression, limited proteolysis of inactive protease precursors (zymogens), specific degradation of mature enzymes, pH of the surroundings, and by protease inhibitors [2, 3].

The majority of natural protease inhibitors are proteins or peptides, and they are important factors in controlling proteolysis. This is indicated by their abundance in many cells, tissues, and biological liquids, as well as by the variety of their molecular forms characterized from all groups of organisms, especially from animals and plants. These proteins function as regulators of endogenous proteolytic enzymes or as a part of the defense against pathogens and pests. They can be grouped into aspartic, cysteine, serine, and metalloprotease inhibitors. The majority of the inhibitors characterized so far are directed towards serine proteases (reviews [1-5]).

Abbreviations: AMC, 7-amino-4-methylcoumarin.

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While the number of these proteins isolated from bacteria, plants, and animals is very large, little is known about their fungal equivalents. Most of them are very different from those from other groups of organisms. This paper describes partial purification and characterization of novel serine protease inhibitors from the white rot basidiomycete *Abortiporus biennis* (Bull. ex Fr.) Sing. White rot fungi are known as the most efficient lignin degraders. These organisms produce ligninolytic enzymes: laccase and peroxidases, potentially applicable in biotechnology (e.g. bleaching and pulping processes, as well as in removal of environmental pollutants) [6, 7].

MATERIALS AND METHODS

Strain and growth conditions. *Abortiporus biennis* (Bull. ex Fr.) Sing was obtained from the culture collection of the Department of Biochemistry, M. Curie-Sklodowska University in Lublin, Poland (FCL 123). The collection cultures are maintained on 2% (w/v) malt agar slants. Stock and experimental cultures of the fungus were cultivated at 26°C under stationary conditions in Erlenmeyer flasks containing 100 ml of liquid medium prepared according to Lindeberg and Holm [8], with glu-

cose (10 g/liter) as a carbon source and L-asparagine (2.5 g/liter) as a nitrogen source. The stock culture was inoculated with a section of the agar culture (~5 mm diameter) and grown until the mycelium covered the whole surface of the liquid. The experimental flasks were inoculated with sections of mycelium (~5 mm diameter), cut aseptically from the stock culture, and cultivated for 14 days, until the beginning of idiophase when the activity of protease inhibitors was the highest.

Isolation and purification of protease inhibitors. Fourteen-day-old mycelia were harvested, washed with distilled water, and homogenized in 0.1 M Na-phosphate buffer (pH 7.0), in an ice-chilled motor-driven Potter homogenizer. The homogenate was centrifuged for 15 min at 15,000*g*. The supernatant was collected, heated in a water bath at 80°C for 15 min, and centrifuged (15,000*g*, 10 min) to remove precipitated proteins. The supernatant was desalted and lyophilized. Then, the sample was dissolved in distillated water and loaded onto a DEAE-Sepharose (Sigma, USA) column (50 ml) equilibrated with 0.05 M Tris-HCl buffer (pH 7.0), and eluted with a linear gradient of NaCl (0-1.0 M). Fraction size was 10 ml in the washing stage and 5 ml in salt gradient elution. The flow rate was 2 ml/min. Fractions were analyzed for protein content (A_{280}) and trypsin inhibitory activity. Gradient fractions showing protease inhibitory activity were pooled, desalted, and lyophilized. In the next stage of purification, the sample was dissolved in distilled water and applied to a Sephadex G-75 (Pharmacia, Sweden) gel filtration column (100 ml) equilibrated with 30 mM phosphate buffer (pH 7.0). Fractions (5 ml) were collected at a flow rate of 1 ml/min and analyzed as described above.

Determination of inhibitory activity. Inhibitory activity was measured using the modified method described by Lee and Lin [9]. Samples (0.25 ml) were preincubated with the same volume of trypsin (50 μg/ml), chymotrypsin α (50 μg/ml), Carlsberg subtilisin (8 μg/ml), or proteinase K (8 μg/ml) solution in 0.1 M glycine-NaOH buffer (pH 9.5) at 37°C for 20 min (trypsin was from ICN Biomedicals (USA) and all other enzymes from Sigma). After the preincubation, 0.5 ml of 1% (w/v) solution of hemoglobin (Sigma) dissolved in the same buffer was added, and the mixture was incubated for 40 min. The reaction was stopped by addition of 2 ml of 5% (w/v) trichloroacetic acid. Samples were centrifuged at 15,000*g* for 10 min, and the absorbance of the supernatant was measured at 280 nm (Varian Inc. Scientific Instruments, USA). In controls, 0.25 ml of sample was replaced by distilled water. The chosen enzyme concentrations gave an increase in A_{280} of approximately 0.005 absorbance unit/min. One inhibitor unit (U) was defined as the amount of the inhibitor that caused inhibition of 1 mg of protease in 40 min.

To confirm specificity of the protease inhibitors, inhibitory activity was additionally measured using specific fluorogenic peptide substrates: Bz-Arg-AMC·HCl (Bachem, Switzerland) for trypsin, Glut-Phe-AMC (Sigma) for chymotrypsin, and Z-Gly-Gly-Leu-AMC (Bachem) for subtilisin and proteinase K (AMC, 7-amino-4-methylcoumarin). The reaction mixture contained 12.5 μl (0.25 μM) of substrate, 375 μl of 0.1 M Tris-HCl buffer, pH 8.5, 25 μl of enzyme solution (2 mg/ml), and 50 μl of inhibitor solution. The enzymes were preincubated with inhibitors (in the case of the control samples, with the buffer) at 37°C for 15 min. Then the buffer and substrates were added and reaction mixtures were incubated for another 15 min at 37°C. The cleavage of substrates was quantified using FluoroMax-2 spectrofluorimeter (Instruments SA, USA), with excitation at 345 nm and emission at 440 nm. The level of released AMC was determined from a standard curve. Inhibitory activities were calculated by the comparison of the amount of AMC released in the presence and absence of inhibitor, and presented as the percentage of inhibition.

Native polyacrylamide gel electrophoresis. Native PAGE was performed in 10% slab gels according to Laemmli [10] without the addition of SDS. Samples were mixed (1 : 1) with 0.02% bromophenol blue in saturated sucrose solution and applied to stacking gels. Separations were performed at 10 mA for the stacking gel and 15 mA for the separating gel. After electrophoresis, the gels were incubated with trypsin (16 μg/ml) dissolved in 0.1 M phosphate buffer (pH 7.4) for 15 min at 37°C. Gels were then washed with distilled water and stained for 45-60 min as described by Uriel and Berges [11]. The reaction was stopped with 2% acetic acid.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was carried out in 15% gels according to Laemmli [10]. Separations were performed at 10 mA for the stacking gel and 15 mA for the separating gel. Protein bands were silver stained [12].

Isoelectric focusing (IEF). Isoelectric focusing was performed on a slab apparatus (16 × 16 cm). The samples of partially purified inhibitors were concentrated using a Centricon membrane system (Millipore, USA). Proteins were separated in the pH range of 3.5-9.8. Electrode solutions were 0.01 M iminodiacetic acid and 0.01 M ethanolamine (Aldrich, Switzerland). Electric voltages supplied during the protein separation procedure were 600 V for the first 4 h and 700 V during the final hour of electrofocusing. The gels were stained for protein content with Violet 17 (Serva, Germany) as well as for protease inhibitor activity as described above.

Protein determination. Protein concentration was determined by the modified method of Lowry et al. [13] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Detailed study on protease inhibitors in the class of Basidiomycetes is limited to only several proteins. Serine

protease inhibitors are represented by proteins isolated from three white rot species—*Pleurotus ostreatus*, *Lentinus edodes*, and *Trametes versicolor* [14-16]. Another wood-degrading fungus, *Ganoderma lucidum*, has been shown to produce an aspartic protease inhibitor [17]. To our knowledge, the only known fungal proteinaceous cysteine protease inhibitor, clitocypin, has been purified from *Clitocybe nebularis* [18]. Additionally, there are a number of more general reports. Trypsin inhibitors were detected by Gzogyan et al. [19] in extracts from fruiting bodies of 18 species of Basidiomycetes belonging to families Bolatecae, Agaricaceae, Pleurotaceae, Russulaceae, Tricholomataceae, Paxilaceae, Polyporaceae, and Hericiaceae. The presence of serine and cysteine protease inhibitors has also been shown in extracts (50% methanol) from many species of Basidiomycetes; however, their chemical nature has not been described [20, 21]. In the present study, we partially purified and characterized novel protease inhibitors from liquid-cultured mycelia of the white rot fungus *A. biennis*.

Isolation and purification of protease inhibitors. In order to purify protease inhibitors from mycelia, a three-step procedure was used. The first stage of the process was heat treatment (80°C, 15 min) of the crude cell extract. After centrifugation, the supernatant was desalted, lyophilized, and chromatographed on a DEAE-Sepharose column. As illustrated in Fig. 1a, a single peak of inhibitory activity was detected in the fractions eluted with a 0-1.0 M NaCl linear gradient. The active fractions were lyophilized and applied to a Sephadex G-75 gel column. Similarly, a single inhibitory peak was observed (Fig. 1b). The presence of protease inhibitors was simultaneously monitored by native electrophoresis in polyacrylamide gels. In the crude homogenate, three inhibitor bands (a, b, and c) were found (Fig. 2). A similar electrophoretic pattern was found after heat treatment and DEAE-Sepharose chromatography. However, after the gel filtration, the inhibitor band of the lowest electrophoretic mobility was hardly visible.

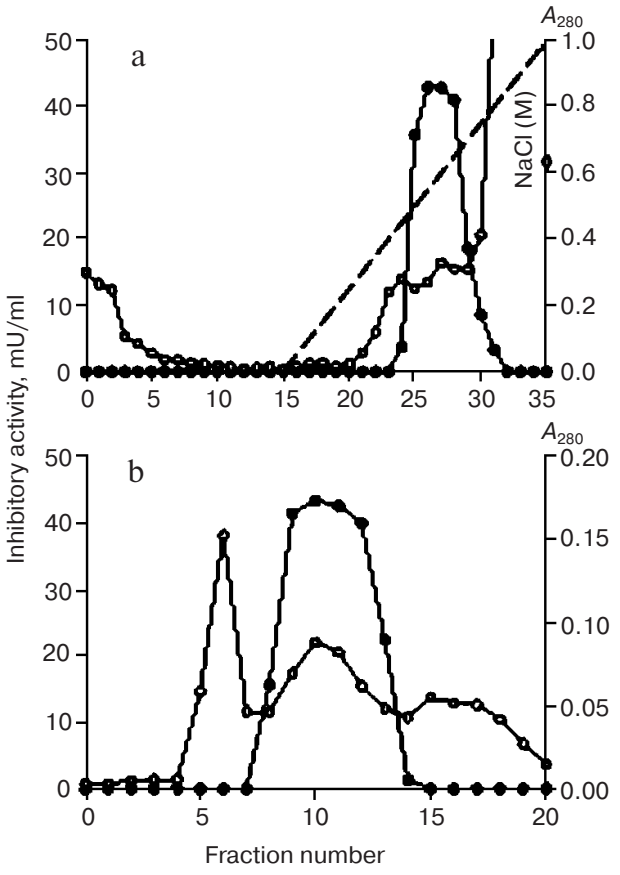

Fig. 1. Chromatographic purification of *A. biennis* protease inhibitors: a) anion-exchange chromatography; b) gel filtration. After elution from DEAE-Sepharose, fractions containing inhibitors were desalted and lyophilized. Closed circles, inhibitory activity; open circles, A_{280}.

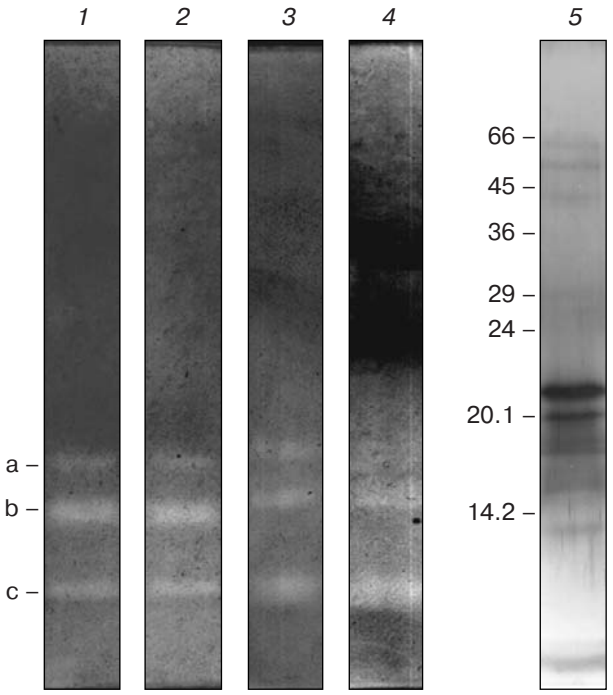

Fig. 2. Electrophoretic patterns at different stages of the purification of the protease inhibitors. Aliquots (20 µl) of protein samples were analyzed by native PAGE: *1*) crude homogenate; *2*) after heat treatment; *3*) eluate from DEAE-Sepharose column; *4*) eluate from Sephadex G-75 column. Lane *5* (silver stained) is after SDS-PAGE of 5 µg of the Sephadex G-75 fraction. The numbers show molecular masses of standard proteins for the SDS-PAGE.

Properties of the protease inhibitors. The analysis of protein composition of the three-step-purified sample by SDS-PAGE revealed the presence of two major protein bands with molecular masses of 20 and 21.5 kDa (Fig. 2). These masses are not much different from the masses of the trypsin inhibitor from *L. edodes* fruiting bodies (16 kDa) and the cysteine protease inhibitor from *L. nebularis* (16.5 kDa) [15, 18].

To further characterize the purified *A. biennis* inhibitor preparation, IEF was performed. Two protein bands with inhibitory activity, with p*I* of 3.8 and 4.0, were detected in the IEF gel (data not shown). Low isoelectric points of the described proteins are not surprising when we consider their strong binding to DEAE-Sepharose during the ion-exchange chromatography. Low values of p*I* are not rare among protease inhibitors, including fungal ones. For example, protease inhibitors from *C. nebularis*, *P. ostreatus*, and *T. versicolor* and proteinase B inhibitor from *Schizosaccharomyces pombe* are also acidic proteins [14, 16, 18, 22].

As demonstrated by the isoelectrofocusing and SDS-PAGE, *A. biennis* produces at least two trypsin inhibitors. We speculate that they are isoforms. However, these findings do not explain the presence of as many as three well-separated inhibitory activity bands observed in 10% native gels. Because molecular masses and isoelectric points of the inhibitors are quite similar, a good separation of these two proteins in low density native gels may be difficult to achieve. The described phenomenon may be rather explained by oligomerization of the inhibitors. The inhibitory band with the highest electrophoretic mobility could consist of monomers. The other bands would represent oligomeric forms. It is known that some protease inhibitors occur as noncovalent oligomers, like SSI, the subtilisin inhibitor from *Streptomyces* (dimer) or chymotrypsin (PI-1, tetramer) and proteinase K (PLPKI, heptamer) inhibitors from potato [23, 24]. Among fungal inhibitors, clitocypin from *L. nebularis* was isolated as a dimeric protein [18].

To test for specificity, inhibitory activities against various commercially available proteases (trypsin, chymotrypsin α, proteinase K, and Carlsberg subtilisin) were compared using hemoglobin as a substrate (table). Protease inhibitors from *A. biennis* were found to be active against all the tested enzymes. Among them, trypsin was the most effectively inhibited. Interactions of the inhibitors with chymotrypsin α and particularly with proteinase K and Carlsberg subtilisin were distinctly weaker. The results were additionally confirmed by spectrofluorimetric measurements of inhibitory activities using fluorogenic substrates for trypsin, chymotrypsin, and subtilisin. The data suggest that the protease inhibitors from *A. biennis* are targeted towards proteases belonging to the chymotrypsin clan. The trypsin inhibitor purified by Odani et al. [15] from fruit bodies of *L. edodes* is probably the only other known fungal protein of this kind. Similarly to *A. biennis* inhibitors, it is more specific towards trypsin than to chymotrypsin, and it does not inhibit subtilisin.

Many protease inhibitors are thermostable proteins. This feature also concerns the majority of known fungal protein inhibitors [14, 16-18, 22, 25] and has been used in the procedure of purification described in this paper. Protease inhibitors from *A. biennis* were stable from 20 to 100°C when treated for 30 min. Only at 100°C, a 15% decrease in inhibitory activity was observed. Heat stability is a feature seldom precisely described, but these results are comparable with those obtained for $GLPAI_1$, the aspartic proteinase A inhibitor produced by submerged cultures of *G. lucidum* [17].

Effects of *A. biennis* protease inhibitors on different serine proteinases as measured with hemoglobin and fluorogenic substrates. Values shown are means ± standard deviation

Enzyme	Inhibitory activity (mU), hemoglobin	Inhibitory activity (%), fluorogenic substrates*
Trypsin	39 ± 1	98 ± 1
Chymotrypsin α	14 ± 6	24 ± 5
Carlsberg subtilisin	0.4 ± 0.4	n.d.
Proteinase K	3 ± 2	n.d.

Note: n.d., not detected.

* Because the definition of the inhibitory unit (U) shown in "Materials and Methods" is not suitable for the method used for fluorogenic substrates, results for this section are presented as percentage of inhibition.

The physiological function of the described proteins is unknown. The inhibitors may participate in defense against insects or parasitic microorganisms. Their regulatory role in intracellular proteolysis of the fungus may also be considered, as inhibitors from *P. ostreatus* and *Saccharomyces cerevisiae* are directed against their endogenous enzymes [14, 26]. Further experiments are necessary to establish the precise physiological function and to complete the characterization of these proteins. According to accessible data, this is the first report about protease inhibitors from *A. biennis*. These acidic, thermostable proteins are active against trypsin and, to a smaller degree, chymotrypsin. Discovery of these inhibitors has extended our still limited knowledge about this group of fungal proteins and thus broadened the spectrum of known protease inhibitors available for potential use in medicine or crop protection.

REFERENCES

1. Rawlings, N. D., Tolle, D. P., and Barrett, A. J. (2004) *Biochem. J.*, **378**, 705-716.
2. Bode, W., and Huber, R. (2000) *Biochim. Biophys. Acta*, **1477**, 241-252.
3. Bode, W., and Huber, R. (1992) *Eur. J. Biochem.*, **204**, 433-451.
4. Krowarsch, D., Cierpicki, T., Jelen, F., and Otlewski, J. (2003) *Cell. Mol. Life Sci.*, **60**, 2427-2444.

5. Gettins, P. G. W. (2002) *Chem. Rev.*, **102**, 4751-4803.
6. Schwarze, F. W. M. R., Engels, J., and Mattheck, C. (2000) *Fungal Strategies of Wood Decay in Trees*, Springer-Verlag, Berlin-Heidelberg.
7. Shah, V., and Nerud, F. (2002) *Can. J. Microbiol.*, **48**, 857-870.
8. Lindeberg, G., and Holm, G. (1952) *Physiol. Plant.*, **5**, 100-114.
9. Lee, T.-M., and Lin, Y.-H. (1995) *Plant Sci.*, **106**, 43-54.
10. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
11. Uriel, J., and Berges, J. (1968) *Nature*, **218**, 578-580.
12. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.*, **68**, 850-858.
13. Schacterle, G. R., and Pollack, R. L. (1973) *Anal. Biochem.*, **51**, 654-655.
14. Dohmae, N., Takio, K., Tsumuraya, Y., and Hashimoto, Y. (1995) *Arch. Biochem. Biophys.*, **316**, 498-506.
15. Odani, S., Tominaga, S., Kondou, S., Hori, H., Koide, T., Hara, S., Isemura, M., and Tsunasawa, S. (1999) *Eur. J. Biochem.*, **262**, 915-923.
16. Zuchowski, J., and Grzywnowicz, K. (2006) *Curr. Microbiol.*, **53**, 259-264.
17. Tian, Y.-P., and Zhang, K.-C. (2005) *Enzyme Microb. Technol.*, **36**, 357-361.
18. Brzin, J., Rogelj, B., Popovic, T., Strukelj, B., and Ritonja, A. (2000) *J. Biol. Chem.*, **275**, 20104-20109.
19. Gzogyan, L. A., Proskuryakov, M. T., Ievleva, E. V., and Valueva, T. A. (2005) *Appl. Biochem. Microbiol.*, **41**, 538-541.
20. Doljak, B., Stegnar, M., Urleb, U., Kreft, S., Umek, A., Ciglaric, M., Strukelj, B., and Popovic, T. (2001) *Blood Coagul. Fibrinolysis*, **12**, 123-128.
21. Mlinaric, A., Kreft, S., Umek, A., Strukelj, B., and Popovic, T. (2000) *Acta Pharmacol.*, **50**, 39-48.
22. Escudero, B., Parra, F., and Suarez-Rendueles, P. (1993) *Biochimie*, **75**, 855-859.
23. Feldman, M. L., Oliva, C. R., Casalongue, C. A., and Daleo, G. R. (2000) *Physiol. Plant.*, **109**, 14-20.
24. Laskowski, M., Jr., and Kato, I. (1980) *Annu. Rev. Biochem.*, **49**, 593-625.
25. Schu, P., and Wolf, D. H. (1991) *FEBS Lett.*, **283**, 78-84.
26. Schu, P., Suarez-Rendueles, P., and Wolf, D. H. (1991) *Eur. J. Biochem.*, **197**, 1-7.