ISOLATION AND CHARACTERIZATION OF AN ACID PROTEINASE FROM *Aspergillus oryzae*

V. I. Shitova, M. A. Samartsev,
S. V. Kulikov, and N. V. Belyakov

UDC 577.1.156

The heat stability, substrate specificity, and the pH optima of activation and inhibition of an acid proteinase isolated from the industrial preparation amilorizin Pkh have been studied. The enzyme has been found active in the hydrolysis of chromophoric peptide substrates of the type of Dnp-Gly-Gly-X-Arg-OH, where X = Phe, Met, Trp. Inhibition of the enzymatic activity by pepstatin and covalent inhibitors of carboxylic proteinases show that this enzyme belongs to the proteinases of the pepsin type.

It is known that various strains of *Aspergillus oryzae* produce considerable amounts of a proteinase which is active in the acid pH region [1]. Tsujita and Endo [2] have isolated from a surface culture of *Asp. oryzae* two extracellular proteinases, A_1 and A_2 with molecular weights of 63,000 and 32,000 differing by the presence in the former of 49% of carbohydrate component. Very close in amino composition to these proteinases is an enzyme with a molecular weight of 39,000, the assignment of which to the carboxylic proteinases of the pepsin type has been suggested without direct proofs [3]. At the same time, a homology with pepsin of the acid proteinases of several other species of the genus *Aspergillus* has been shown strictly [4].

We have isolated an acid proteinase from the technical preparation "amilorizin Pkh" and have compared a number of its properties with those of enzymes isolated by other authors. The enzymatic properties of the proteinase and its inactivation by specific inhibitors of carboxylic proteinases were studied.

The subsequent extraction of the comminuted biomass of *Asp. oryzae* with sodium acetate buffer, pH 4.7, showed that three extractions ensured isolation of 78% of the extracellular acid proteinase present in the sample (Table 1). An amylase and other water-soluble proteins were extracted simultaneously. The extract was concentrated by ultrafiltration using Biopor T-50 membranes from an aromatic polyamidoimide [5], and the proteinase was separated from the amylase by gel chromatography on Ultragel AcA 54 (Fig. 1).

The results of a study of the physiochemical properties of the proteinase isolated permitted it to be characterized as a glycoprotein corresponding to proteinase A_1 isolated by Tsujita and Endo [2]. This conclusion was made on the basis of a correspondence of the values of the molecular weights, the practical coincidence of the heat stability curves (Fig. 2), the glycoprotein nature of the enzyme, confirmed by qualitative reactions for carbohydrates (coloration with phenol and sulfuric acid and with anthrone) and the capacity of the enzyme for being specifically sorbed on concanavalin-A-Sepharose, and the closeness of the values of the isoelectric points (Table 2).

The proteinase that we obtained behaves as an individual substance on disc electrophoresis in polyacrylamide gel, but on isoelectric focusing in a thin layer of polyacrylamide gel it is separated into two components obviously corresponding to components A_{1a} and A_{1b} separated by Tsujita and Endo with the aid of preparative isoelectric focusing in a column [6]. They showed that both isoenzymes contain about 50% of polysaccharide and differ only in the composition of their carbohydrate moieties: component A_{1a} with pI 3.15 contains as monosaccharide units galactose, glucose, and mannose, while in component A_{1a} with pI 3.5 glucose predominates.

All-Union Scientific-Research Institute of Ultrapure Biopreparations, Leningrad. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 366-373, May-June, 1981. Original article submitted November 12, 1980.

 0009-3130/81/1703-0288$07.50

TABLE 1. Extraction of Amylase and an Acid Proteinase from the Biomass of *Aspergillus oryzae* (4 g)

Serial number of the extraction	Volume of solution, ml		Protein			Proteolytic activity, pH 2.6		Amylolytic activity	
	taken for extraction	extract	total amount	yield, % on the total	concentration, mg/ml	specific, units/mg	yield, % of total	specific, units/mg	yield, % of total
1	20	9	12.4	28	1.38	9	31	155	29
2	20	18	11.0	25	0.60	11.3	34.5	89	34
3	15	17	6.5	14.5	0.38	6.9	12.5	66	23
Total of 1—3	55	44	29.9	67.5	0.68	9.4	78	94	86
4—15	180	189	14.2	32.5	0.08	5.6	22		14
Total of 1—15			44.1	100			100		100

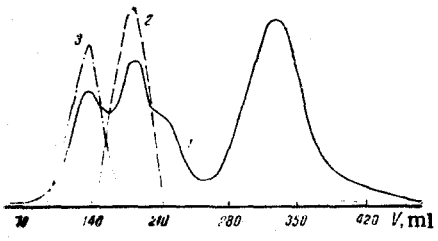

Fig. 1

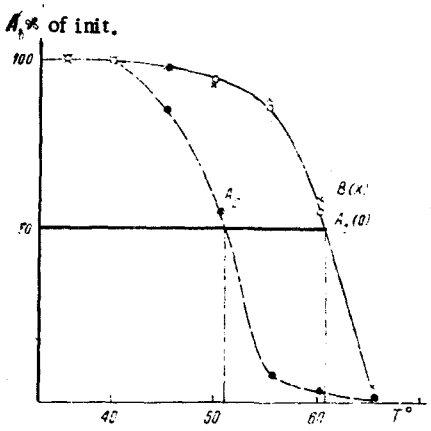

Fig. 2

Fig. 1. Gel chromatography of the water-soluble proteins of amilorizin Pkh on Ultragel AcA 54: 1) protein (D_{280}); 2) amylolytic activity; 3) proteolytic activity.

Fig. 2. Inactivation of the proteinases of *Aspergillus oryzae* after incubation for 10 minutes at various temperatures: A_1 and A_2) proteinases A_1 and A_2 according to the literature; B) results of the present work.

TABLE 2. Comparison of the Properties of the Acid Proteinases Isolated from Surface Cultures of *Aspergillus oryzae* by Various Authors

Index	Davidson et al. [6]	Tsujita and Endo [2]		Results of the present work
		A_2	A_1	
Molecular weight	39400	32000	63000	65000
Temperature at which 50% inactivation takes place in 10 min	Not det.	51°	61°	62°
Carbohydrate content, %	Not det.	0	49	+
Isoelectric points	3.9; 4.1	3.90	3.15; 3.50	3.1; 3.2
Optimum pH from the hydrolysis of denatured hemoglobin	Not det.	4.3	4.3	4.8

It is known that glycoproteins including glucose and mannose residues bind specifically with concanavalin A. This has been shown, for example, for the carboxylic proteinase from *Mucor miehei* MMP, containing only 4.7% of carbohydrate [7]. It is characteristic that after the destruction of the carbohydrate moiety by periodate oxidation, this enzyme completely lost its capacity for binding with concanavalin A. In our case, the specific nature of the binding of the proteinase with concanavalin immobilized on Sepharose is confirmed by the stability of the complex in solutions with high ionic strength (1 M NaCl); biospecific elution was achieved by the use of solutions of methyl α-D-mannoside.

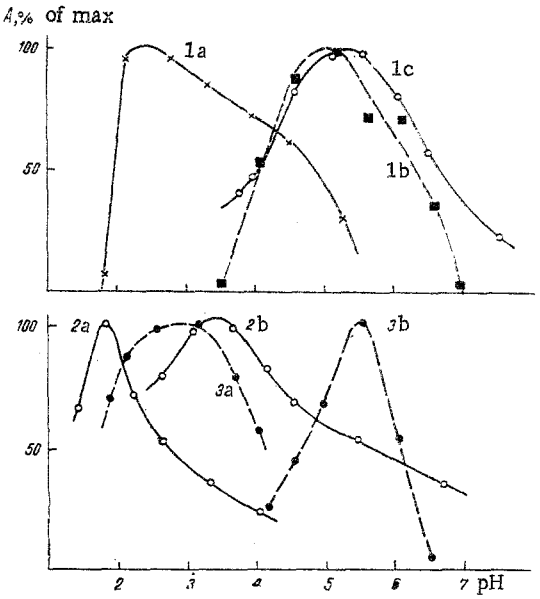

Fig. 3. Dependence of the activities of acid proteinases on the pH: enzymes: 1) proteinase of *Aspergillus oryzae*; 2) porcine pepsin; 3) proteinase from *Aspergillus awamori*. Substrates: a) hemoglobin; b) hemoglobin in the presence of urea; c) the tetrapeptide Dnp-Gly-Gly-Phe-Arg-OH (II).

TABLE 3. Peptides Tested as Substrates of the Acid Proteinase

Peptide*	Yield, %	mp, °C	Activity, units/mg
I. Dnp-Gly-Gly-Val-Arg-OH	89	172—174	0
II. Dnp-Gly-Gly-Phe-Arg-OH	97	188—196	25.8
III. Dnp-Gly-Gly-Met-Arg-OH	71	166—174	26.2
IV. Dnp-Gly-Gly-Trp-Arg-OH	84	218—222	10.7
V. Dnp-Gly-Gly-D-Phe-Arg-OH	72	178—188	0
VI. Dnp-Gly-Gly-Thr-Arg-OH	53	166—186	0

*The amino acids, apart from the D-phenylalanine in peptide (V) and the glycine had the L-configxrations.

With respect to the optimum pH for the hydrolysis of hemoglobin, the acid proteinase from *Asp. oryzae* is similar to the enzyme from *Asp. awamori* and differs substantially from pepsin (Fig. 3). A capacity of the enzyme for hydrolyzing, in addition to pepsin, low-molecular-weight peptides at a Phe—Phe bond as, for example, in Z-His-Phe-Phe-OEt (pH 3.4), has been reported previously [3]. We have also found that the enzyme from *Asp. oryzae* cleaves the chromophoric peptide substrates (II-IV) given in Table 3. It was shown with the aid of the TLC of the cleavage products that the hydrolysis of the peptides took place only at Gly-X bonds, which excludes the possibility of the presence of an acid carboxy peptidase in the preparation [8]. An analog of substrate (II) with the L-phenylalanine replaced by its D-isomer (V) acted neither as a substrate nor as an inhibitor to the enzyme. On the other hand, peptide (VI) inhibited the hydrolysis of substrate (II) by the mechanism of complete competitive inhibition (Table 4).

Dinitrophenylpeptides of arginine have previously been suggested as substrates of natural proteinases [9, 10]. We have synthesized peptides by a method differing from that described by Lyublinskaya et al. [9] by using arginine with a protected carboxy group in the form of an internal salt [11]. The peptides obtained are not hydrolyzed at an appreciable rate by pepsin, rennin, or the carboxylic proteinases from *Aspergillus awamori* and *Aspergillus foetidus*. In spite of the difference in substrate specificity, the proteinase from *Asp. oryzae* is, like pepsin and related enzymes of microorganisms, inhibited by pepstatin and irreversible inhibitors of carboxylic proteinases when either hemoglobin or a synthetic peptide

is used as substrate (see Table 4). At the same time, the enzyme under investigation is insensitive to the action of inhibitors of serine proteinases (phenylmethanesulfonyl fluoride), thiol proteinases (p-hydroxymercuribenzoate), and metalloproteinases (EDTA). These results unambiguously permit the enzyme to be assigned to the carboxylic proteinases of the pepsin type.

EXPERIMENTAL

Industrial preparations of amilorizin Pkh, glucoawamorin Pkh, and pectofoetidin P10kh, hemoglobin from the Olaine chemical reagents factory, and pepsin, rennin, carboxypeptidase, pepstatin, phenylmethanesulfonyl fluoride, p-hydroxymercuribenzoate, methyl α-D-mannoside, and the dyes Brilliant Blue (Coomassie) R-250 and G-250 (USA) were used.

Isolation of the Enzyme from the Biomass. A preparation of amilorizin Pkh, which consists of the dried biomass of a surface culture of *Asp. oryzae*, was comminuted in a Homogenizer 302 disintegrator (Poland). With stirring for an hour at 20°C, 10 g of the preparation was extracted with 0.1 M sodium acetate buffer, pH 4.7 (100 ml and 2 × 50 ml). The cell material was separated off by centrifugation, the supernatant (160 ml) was treated with 15 ml of 1 M $CaCl_2$, and the precipitate that deposited was removed by centrifugation. The solution was concentrated by ultrafiltration through a Biopor T-50 membrane under a pressure of 2 atm to a volume of 15 ml. The concentrate was chromatographed, on a 2.5 × 70 cm column of Ultragel AcA 54 (LKB, Sweden). In the fractions protein was determined by the reaction with the dye Coomassie C-250 [12] and the proteolytic and amylolytic activities were measured. The enzymatic peaks were dialyzed against water and lyophilized.

Chromatography on Con A-Sepharose. A 1 × 10 cm column of con A-Sepharose (Sweden) was equilibrated with 0.1 M sodium acetate buffer having pH 4.7 and containing 10^{-3} M $CaCl_2$, $MgCl_2$, and $MnCl_2$, and 3 ml of a solution of the enzyme, in the same buffer (0.7 mg/ml of protein) was passed through it. Then stepwise elution was carried out with buffer solutions to which 0.2, 0.4, and 1 M NaCl had been added. A protein fraction possessing proteolytic and amylolytic activities was eluted with a buffer solution containing 0.1 M NaCl and 0.1 M methyl α-D-mannoside. A buffer solution containing 1 M NaCl and 25% isopropanol was used to regenerate the column.

Determination of Amylolytic Activity [13]. To 1 ml of 0.5% starch solution in 0.1 M sodium acetate buffer having pH 4.7 was added 2 ml of enzyme solution, and the mixture was kept at 30° for 5 min. Then 1 ml of a solution of iodide reagent (0.44% iodine and 0.16% of KI in 10% acetic acid) was added and the percentage transmission of the solution at 660 nm (T_{660}) was measured. The concentration of enzyme was selected in such a way that the transmission was between 30 and 70%. In a control, in place of the iodide reagent 1 ml of 10% acetic acid was added to the sample. As the nominal unit of activity we took the amount of enzyme causing an increase in the transmission under the selected conditions of analysis by 10%.

Determination of Proteolytic Activity with Respect to Hemoglobin. To 2 ml of a 2% solution of hemoglobin having pH 2.5 was added 0.5 ml of enzyme solution, and the mixture was kept at 35°C for 10 min. The reaction was stopped by the addition of 2.5 ml of 5% trichloroacetic acid (TA), the precipitate was separated off by filtration, and the optical density of the filtrate at 280 nm (D_{280}) was determined. The concentration of enzyme was selected in such a way that the D_{280} value did not exceed 0.8. In a control sample, TCA was added to the solution first, and then the solution of the enzyme. As the nominal unit of activity we took the amount of enzyme, corresponding to a rise in D_{280} by 1 o.u. under the selected conditions of analysis.

Solutions of hemoglobin for determining the pH optimum were prepared from a solution in 0.01 N HCl by adding solutions of HCl or NaOH to the required pH. Solutions of hemoglobin denatured by urea were prepared by dissolving 2 g of hemoglobin in 100 ml of 40% urea solution and were brought to the required pH.

Determination of Activity with respect to Tetrapeptides [9]. A 0.2 μM solution of the peptide in 0.1 M sodium acetate buffer with pH 5.35 was prepared. To 4 ml of the substrate solution was added 1 ml of enzyme solution (0.3 mg/ml), and the mixture was kept at 35°C for 10 min, after which 0.5 ml of 1 N HCl was added and the mixture was extracted with 5 ml of 10% solution to ethanol in ethyl acetate. From the organic layer the Dnp-Gly-Gly-OH was extracted into 4 ml of 1% $NaHCO_3$, and the optical density of the aqueous extract was determined at 360 nm (D_{360}). As the nominal unit of activity we took the amount of enzyme hydrolyzing

TABLE 4. Inhibition of the Acid Proteinase

Inhibitor	Concentration of inhibitor, mmole/ml	Substrate	Percentage inhibition	
			of the Asp. oryzae proteinase	of pepsin
1,2-Epoxy-3-p-nitrophenoxypropane	$4.27 \cdot 10^{-2}$	Hemoglobin	66	73
		The tetrapeptide II	54	
N-Diazoacetyl-N'-dinitrophenylethylenediamine	$7.1 \cdot 10^{-4}$	Hemoglobin	73	93
	$2.9 \cdot 10^{-3}$	Hemoglobin	86	72
		The tetrapeptide II	53	
The tetrapeptide Dnp-Gly-Gly-Thr-Arg-OH (VI)	3.9	The tetrapeptide II	73	

1 μmole of peptide per minute. The specific activity was calculated from the formula $A_{sp} = 2.67 \cdot D_{360}/C$, where C is the amount of enzyme in the sample, and 2.67 is a calculation factor taking into account the molar extinction of the reaction product and the dilution of the sample during the experiment and the time of incubation. In a control sample, the HCl was added before the enzyme solution. Solutions of the tetrapeptide (II) with different pH values were prepared by adding 0.4 ml of a 2 μM solution of the peptide in water to 3.6 ml of 0.1 M sodium acetate buffer with predetermined pH value.

In order to determine the directions of cleavage of the peptides by the enzyme, an ethyl acetate extract of the hydrolysis products was chromatographed on plates of SI 60 silica gel (GFR) in the butan-1-ol-acetic acid–water (4:1:1) system. The peptides DNP-Gly-Gly-OH and Dnp-Gly-Gly-Phe-OH were used as markers; the latter tripeptide was isolated in the hydrolysis of the tetrapeptide (II) with carboxypeptidase B.

Example of the Synthesis of a Peptide: Dnp-Gly-Gly-Phe-Arg-OH (II). To a suspension of 0.321 g of H-Phe-Arg-OH [14] in 10 ml of dimethylformamide was added 0.5 g of Dnp-Gly-Gly-ONp (obtained by the carbodiimide method in dimethylformamide, mp 215-218°C), and the mixture was stirred at 25°C for 3-4 days, the completeness of the reaction being monitored with the aid of TLC. Then the reaction mixture was poured into a mixture of 100 ml of ethyl acetate and 50 ml of ether, and the resulting precipitate was washed with ethyl acetate and ether. To eliminate the Dnp-Gly-Gly-OH completely, the product was dissolved in 300 ml of water and extracted with ethyl acetate and ether. The aqueous solution was subjected to lyophilization. Yield 0.55 g (97%).

Inhibition. For inhibition by N-diazoacetyl-N'-dinitrophenylethylenediamine, to 10 ml of a solution of the enzyme in 0.1 M acetate buffer with pH 4.8 (1 mg/ml of protein) was added a solution of 2 mg of the freshly prepared inhibitor [15] in 0.5 ml of acetone, 0.1 ml of 0.1 M copper acetate, and samples were taken at 5 minute intervals over thirty minutes in order to determine residual activity. When 1,2-epoxy-3-p-nitrophenoxypropane, synthesized by Marle's method [16] was used, 100 ml of the inhibitor in the form of a fine powder was added to 10 ml of enzyme solution (0.1 mg/ml), and the reaction was performed with stirring at 20°C for 1.5 h with the taking of samples every 15 min [17]. In the experiments on inhibition by pepstatin, to each of a number of 5-ml samples of enzyme solution (0.1 mg/ml) was added 0.1 ml of an aqueous suspension of pepstatin (0.1 mg/ml) and the mixtures were stirred at 20°C for from 5 to 20 min [18]. On working with peptides (V) and (VI), to 1 ml of enzyme solution (1 mg/ml) was added 2 ml of 0.1 M acetate buffer pH 5.35 and 1.6 ml of a $2 \cdot 10^{-6}$ M solution of the peptide. The mixture was kept at 20°C for 10 min, and then 0.4 ml of a $2 \cdot 10^{-6}$ M solution of peptide substrate (II) was isolated and the activity was determined at 35°C.

Isoelectric focusing in a thin layer of polyacrylamide gel was carried out on a Multiphore instrument (Sweden) under the recommended conditions using Servalyte AG 2-11 ampholytes (GFR). Development was performed with the dye Coomassie R-250 or with a solution of cytochrome C [19].

The synthetic inhibitors and tetrapeptides were obtained by N. Yu. Sokolova and assistance in performing some of the experiments was given by E. B. Leonov.

SUMMARY

1. It has been shown that the acid proteinase isolated from an industrial preparation of a surface culture of *Aspergillus oryzae* (amilorizin) corresponds to an enzyme of glycoprotein nature with a molecular weight of 63,000.

2. The fact that the enzyme belongs to the group of carboxylic proteinases of the pepsin type is shown by its inactivation by specific inhibitors.

3. A capacity of the enzyme for hydrolyzing certain synthetic dinitrophenyltetrapeptides has been found.

LITERATURE CITED

1. T. Nakadai and S. Nasuno, J. Ferment. Technol., 54, 872 (1976).
2. Y. Tsujita and A. Endo, Biochim. Biophys. Acta, 445, 194 (1976).
3. R. Davidson, A. Gertler, T. Hofmann, Biochem. J., 147, 45 (1975).
4. L. S. Lobareva and V. M. Stepanova, Usp. Biol. Khim., 19, 83 (1978).
5. N. P. Denisova, V. P. Zhemkov, and E. N. Morozova, Mikol. Fitopatol., 13, 127 (1979).
6. Y. Tsujita and A. Endo, J. Biochem., 81, 1063 (1977).
7. W. S. Rickert and P. A. McBride-Warren, Can. J. Biochem., 54, 120 (1976).
8. N. M. Azarenkova, T. I. Vaganova, A. Ya. Strongin, and V. M. Stepanov, Biokhimiya, 41, 20 (1976).
9. L. A. Lyublinskaya, L. V. Lastovetskaya, T. V. Shekhvatova, T. I. Vaganova, and V. M. Stepanov, Khim. Prir. Soedin., 75 (1976).
10. L. A. Lyublinskaya, T. I. Vaganova, L. D. Yakusheva, E. S. Oksenoit, and V. M. Stepanov, IVth All-Union Symposium on the Chemistry of Proteins and Peptides [in Russian], Minsk (1977), p. 197.
11. S. I. Virovets, V. F. Martynov, and M. I. Titov, Zh. Obshch. Khim., 38, 2337 (1968).
12. M. M. Bradford, Anal. Biochem., 72, 248 (1976).
13. R. M. Attia and S. A. Ali, Z. Bacteriol., II, 132, 193 (1977).
14. M. P. Filatova, R. M. Krit, G. S. Suchkova, G. A. Ravde', and V. T. Ivanov, Bioorgan. Khim., 1, 437 (1975).
15. V. M. Stepanov, L. S. Lobareva, and N. Y. Maltsev, Biochim. Biophys. Acta, 151, 719 (1968).
16. E. R. Marle, J. Chem. Soc., 101, 305 (1912).
17. J.-C. Cripon, Biochemie, 58, 747 (1976).
18. W.-J. Chang, S. Horiuchi, K. Takahashi, M. Yamasaki, and Y. Yamada, J. Biochem., 80, 975 (1976).
19. C. W. Ward, Anal. Biochem., 74, 242 (1976).