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Purification and Characterization of Extracellular Proteinases of *Aspergillus oryzae*

A. K. KUNDU* AND S. MANNA

Department of Microbiology, East India Pharmaceutical Works Ltd., Calcutta-60, India

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The extracellular proteinases of *Aspergillus oryzae* EI 212 were separated into two active fractions by $(NH_4)_2SO_4$ and ethanol fractionation followed by diethylaminoethyl-Sephadex A-50 and hydroxyapatite chromatography. The molecular weight was estimated by gel filtration to be about 70,000 and 35,000 for proteinases I and II, respectively. Optimum pH for casein and hemoglobin hydrolysis was 6.5 at 60 C for proteinase I and 10.0 at 45 C for proteinase II, and for gelatin hydrolysis it was 6.5 at 45 C for both enzymes. The enzymes were stable over the pH range 6 to 8 at 30 C for 60 min. The enzyme activity for both the proteinases was accelerated by Cu^{2+} and inhibited by Fe^{2+}, Fe^{3+}, Hg^{2+}, and Ag^{+}. Halogenators (e.g., *N*-chlorosuccinimide) and diisopropyl fluorophosphate inhibited proteinase II. Sulfhydryl reagents such as *p*-chloromercuribenzoate and iodoacetate inhibited proteinase I. Sulfhydryl compounds accelerated the action of both enzymes.

In a previous communication (12), we reported that *Aspergillus oryzae* EI 212 differs from other *A. oryzae* strains in its production and type of extracellular proteinases. It was also suggested that the enzyme preparation obtained is either a mixture of two enzymes or it possesses two kinds of functional groups that act differently at two optimal pH levels. In the present paper, we report the isolation, purification, and characterization of the proteinase system in greater detail.

MATERIALS AND METHODS

Culture and growth media. The culture (designated *A. oryzae* EI 212) history and maintenance have been described (19).

Fermentation and enzyme isolation. Isolation of enzymes from moldy bran has been described by Kundu et al. (12).

Purification of the enzymes. Powdered $(NH_4)_2SO_4$ was added slowly with constant stirring at 5 C to the supernatant liquid from moldy bran to 60% saturation. The fine precipitates that formed after 2 h of stirring were collected by centrifugation at 6,000 × *g* for 20 min, suspended in distilled water, dialyzed against several changes of distilled water at 5 C for 48 h and reprecipitated with ethanol. The precipitated fraction thus obtained by centrifugation (6,000 × *g*) was dissolved in 50 ml of 0.02 M phosphate buffer, pH 7.0, and further purified by fractionating through a diethylaminoethyl-Sephadex A-50 (Pharmacia Fine Chemicals, Sweden) column (2 by 24 cm). Active proteinase fractions (6 to 20) were combined and reprecipitated with 3 volumes of ethanol. The precipitate obtained by centrifugation (6,000 × *g*) for 15 min was resuspended in 20 ml of 0.01 M phosphate buffer, pH 6.8, and further purified by column chromatography on hydroxyapatite gel (3 by 58 cm). Hydroxyapatite was prepared according to Tiselius et al. (26). Active fractions 61 to 73 (proteinase I) and fractions 103 to 113 (proteinase II) were pooled. These fractions were used for the characterization of the enzymes.

Proteinase assay. Proteinase activities were determined by Anson's (3) method using casein and hemoglobin as substrate and by the gelatin digestion method (6). For caseinolysis and hemoglobin digestion, 2% solutions of "casein alkalilöslich" and hemoglobin (E. Merck AG, Darmstadt, Germany) were employed, and for gelatin digestion 4% gelatin (E. Merck AG, Darmstadt, Germany) was used. Citrate-phosphate buffers (0.1 M) for pH 3.0 to 5.5, phosphate buffers for pH 6.0 to 8.0, boric acid buffers for pH 8.5 to 9.0, and carbonate-bicarbonate buffers for pH 9.5 to 10.5 were employed during the study. Eluted enzymes (66 μg of protein per ml for proteinase I and 46 μg of protein per ml for proteinase II) were diluted (1:5), and 1 ml of this diluted enzyme was added for every 5 ml of buffered substrate. The reaction mixture was incubated for 10 min in the case of casein and hemoglobin and for 3 h for gelatin at 40 C. For caseinolysis and hemoglobin digestion, 1 U of proteinase activity was defined as the amount of enzyme which produced 0.1 optical density unit change at 650 nm in 10 min under the conditions described. Specific activity was expressed as proteinase activity per milligram of protein. For gelatin digestion, the proteinase activity was expressed as milligrams of amino acid nitrogen liberated per milligram of protein.

Estimation of protein. Protein was estimated according to the method of Lowry et al. (17) using crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd.) as standard.

Molecular weight determination. Molecular weight was determined by the method of Andrews

(2) by comparing the elution volume of proteinases with that of known proteins, e.g., trypsin (23,800), pepsin (35,500), ovalbumin (45,000), bovine plasma albumin (65,000), and hemoglobin (68,000), through Sephadex G-75 (Pharmacia Fine Chemicals).

Electrophoresis. Homogeneity of the proteinase fractions was examined by paper electrophoresis (4). Electrophoresis of the proteinase fractions was carried out with a Systronix electrophoresis apparatus (type 601-1) on chromatographic paper (Whatman no. 1) strips with barbiturate buffer, $\mu m = 0.05$ and pH 8.6. A potential of 300 V (13.5 mA/strip) was applied for 6 h at 30 C. After migration, the strips were stained with bromophenol blue.

Activation energy. The study on activation energy was done as described by Han and Srinivasan (8).

RESULTS

Purification of the enzymes. Preliminary examinations of the concentrated enzyme preparation showed the presence of significant amounts of amylases, invertases, proteinases, cellulase, hemicellulase, and a new α-glucanase (13). The enzyme preparation was purified by fractionation on columns of diethylaminoethyl-Sephadex A-50 and hydroxyapatite. The elution pattern of proteins and distribution of proteolytic enzymes are shown in Fig. 1 and 2, respectively. The first protein peak (Fig. 1) from the diethylaminoethyl-Sephadex A-50 column contained most of the proteolytic activity, with a recovery of 40% of the initial proteinase activity. Finally, we obtained two proteinase fractions (peaks 4 and 5 in Fig. 2) from the hydroxyapatite column, purified approximately 75-fold in specific activity, with a total recovery of 20% of the initial proteinase activity. The results are given in Table 1.

Purity of the enzymes. Homogeneity of the proteinase fractions was checked by paper electrophoresis. A single protein band was observed for each enzyme fraction that migrated towards the cathode and travelled a distance of 2.5 and 3.5 cm from the line of application for proteinases I and II, respectively.

Effect of temperature on the activity and stability of enzymes. The optimum temperature for activity was found to be 58 to 60 C at pH 6.5 and 45 C at pH 10.0 for proteinases I and II, respectively (Fig. 3). The proteinases were

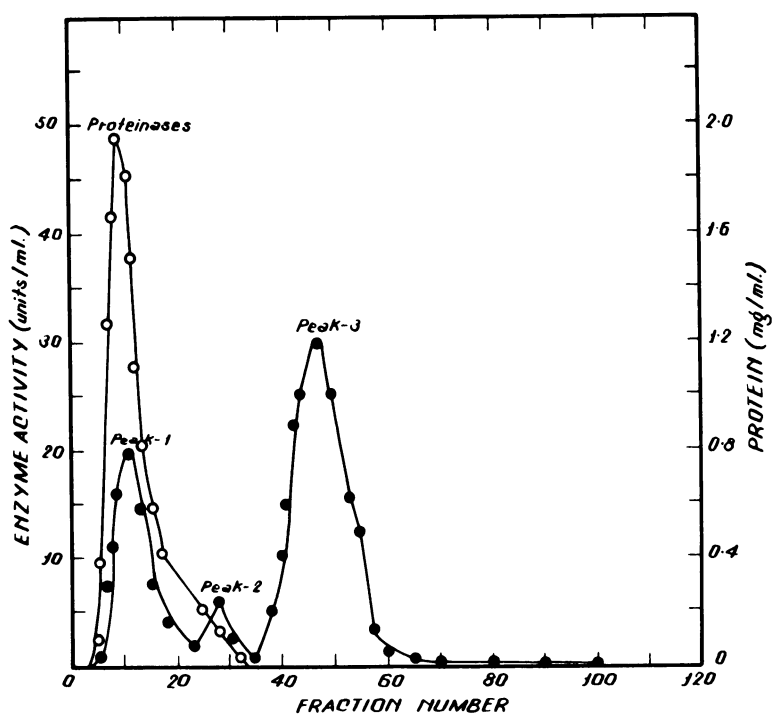

FIG. 1. *Diethylaminoethyl-Sephadex A-50 chromatography. Ethanol-precipitated protein preparation was dissolved in 0.02 M phosphate buffer, pH 7.0 (50 ml; protein, 6 mg/ml) and was applied to a diethylaminoethyl-Sephadex A-50 column (2 by 24 cm). The column was eluted with the same buffer with increasing concentrations of NaCl from 0.01 to 0.05 M. The flow rate was 50 ml/h, and 10-ml fractions were collected. Symbols: ●, protein concentration; ○, proteolytic activity measured with casein as substrate at pH 6.5 and 40 C.*

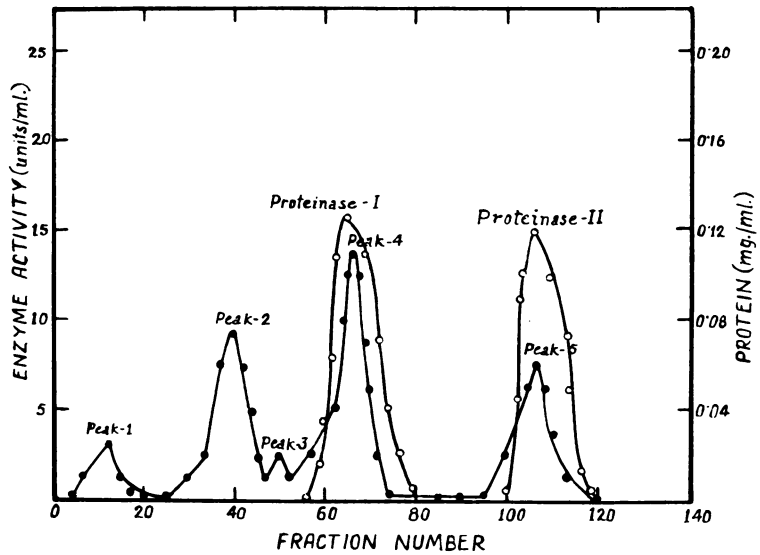

FIG. 2. *Calcium hydroxyapatite chromatography. Ethanol-precipitated protein fractions from the diethylaminoethyl-Sephadex A-50 column were dissolved in 0.02 M phosphate buffer, pH 6.8 (20 ml; protein, 2 mg/ml) and were applied to a calcium hydroxyapatite column (3 by 58 cm). The column was eluted with increasing concentrations of NaCl from 0.01 to 0.1 M in 0.02 M phosphate buffer, pH 6.8. The flow rate was 20 ml/h, and 10-ml fractions were collected. Symbols; ●, protein concentration; ○, proteolytic activity measured with casein as substrate at pH 6.5 and 40 C.*

TABLE 1. *Purification and overall recovery of A. oryzae proteinases*

Fraction	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg of protein)	Yield (%)
1. Crude aqueous extract	800	12,760	4,800	2.6	100
2. Ammonium sulfate precipitation (50 to 60%)	200	10,750	640	16.8	84.2
3. Alcohol precipitation (3 volumes)	50	8,400	300	28.0	65.8
4. Diethylaminoethyl-Sephadex A-50 chromatography	150	5,205	52.5	99.13	40.8
5. Alcohol precipitation (3 volumes)	20	5,090	40	127.25	39.5
6. Hydroxyapatite chromatography					
Fractions 61 to 73 (proteinase I)	120	1,460	8.0	182.5	11.5
Fractions 103 to 113 (proteinase II)	100	1,020	4.6	221.7	8.0

stable up to 40 C after which activity decreased sharply. Complete inactivation occurred at 70 C for proteinase I and at 60 C for proteinase II (Fig. 3).

Activation energy. The effect of temperature on the initial velocities of substrate hydrolysis was studied over the range of 25 to 60 C in 5° increments. Figure 4 shows a conventional Arrhenius plot of the data. In the case of proteinase I, a discontinuity is observed at 40 C with an activation energy of 28,853 J/mol above, and that of 56,966 J/mol below, this temperature. An activation energy of 51,345.5 J/mol was calculated for proteinase II, no discontinuity being observed in this case.

Effect of pH on the activity and stability of enzymes. The optimum pH values for casein and hemoglobin digestion by proteinases I and II are 6.5 at 60 C and 10.0 at 45 C, respectively (Fig. 5 and 6). With gelatin, however, the optimum pH for both the proteinases was found to be 6.5 at 45 C (Fig. 6). When both proteinases were kept at 30 C for 60 min at various pH values, proteinase I was found to be more stable at pH 6.0 to 7.5 and proteinase II at pH 6.0 to 8.0. Both proteinases lost activity gradually on both sides of their respective stability ranges (Fig. 5).

Molecular weight. The molecular weights of proteinases I and II, determined by the method

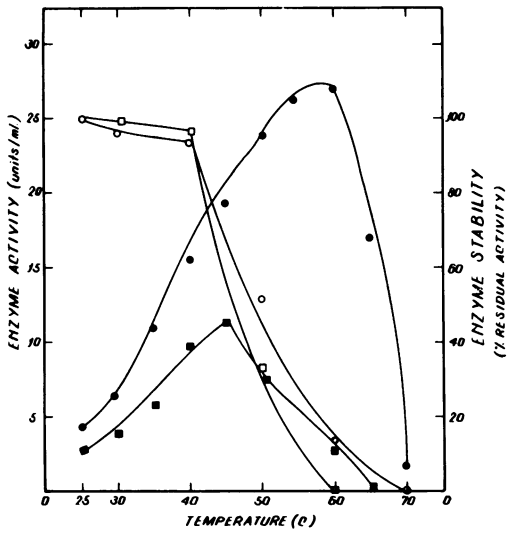

FIG. 3. *Effect of temperature on the activity and stability of A. oryzae proteinases. The stability of the enzyme was examined by preincubating the enzyme solution at various temperatures for 60 min at pH 7.0. The residual activity was assayed under standard conditions. The enzyme activity was measured at various temperatures in the reaction mixture. Casein was used as substrate. Symbols: ●, activity of proteinase I, measured at pH 6.5; ○, stability of proteinase I, residual activity measured at pH 6.5 and 60 C; ■, activity of proteinase II, measured at pH 10.0; □, stability of proteinase II, residual activity measured at pH 10.0 and 45 C.*

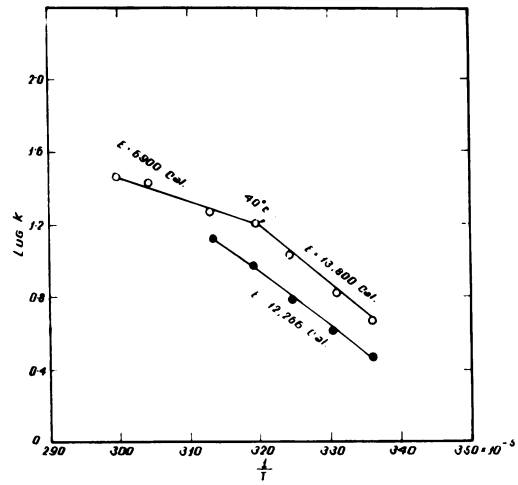

FIG. 4. *Arrhenius plots for activation energy. The effect of temperature on initial velocity of protein hydrolysis was studied over the range of 25 to 60 C in 5-C increments and was plotted in the conventional Arrhenius manner, log K versus 1/T, where K is the initial rate of hydrolysis and T is the absolute temperature. Activation energy (E_a) for the enzyme was calculated from the equation, 2.3 log $K_1/K_2 = E_a/R(1/T_2 - 1/T_1)$. Symbols: ○, proteinase I, measured at pH 6.5; ●, proteinase II, measured at pH 10.0.*

of Andrews (2), were found to be 70,000 and 35,000 (±5,000 each), respectively.

Kinetic parameters for casein hydrolysis. The effect of substrate concentration on the reaction velocity of casein hydrolysis is shown in Fig. 7. According to the Lineweaver-Burke plot (15) (Fig. 8), K_m and V_{max} for casein hydrolysis were calculated to be 1.39% and 9.4×10^{-4} mmol/min per ml for proteinase I and 0.68% and 8.5×10^{-4} mmol/min per ml for proteinase II.

Effect of urea on the stability of enzymes. The stability of the enzymes towards 8 M urea was studied. The enzymes are unstable in 8 M urea solution overnight at room temperature (30 C). It was observed that proteinase II is more stable than proteinase I. Stability is better at 4 C than at room temperature (Table 2).

Effect of metal ions and nonmetallic reagents on enzyme activity. No significant activation was observed with any of the metal ions except Cu^{2+} (Table 3). Both proteinases were inhibited by Fe^{2+}, Fe^{3+}, Ag^{+}, and Hg^{2+}. The behavior of these proteinases was also reported with different nonmetallic reagents (Table 3).

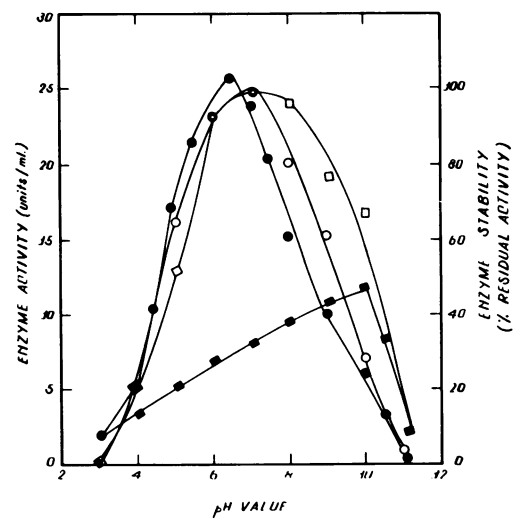

FIG. 5. *Effect of pH on the activity and stability of A. oryzae proteinases. The stability of the enzyme was examined by preincubating the enzyme solution, adjusted to various pH levels, for 60 min at 30 C. The residual activity in the sample was measured under standard conditions. The enzyme activity was measured at different pH levels in the reaction mixture. Casein was used as substrate. Symbols: ●, activity of proteinase I, measured at 60 C; ○, stability of proteinase I, measured at pH 6.5 and 60 C; ■, activity of proteinase II, measured at 45 C; □, stability of proteinase II, measured at pH 10.0 and 45 C.*

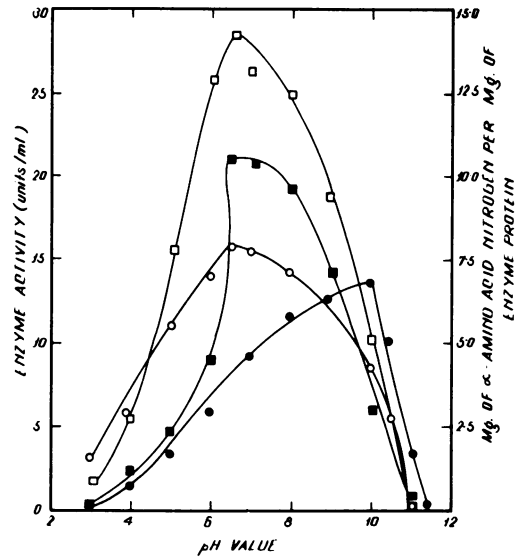

FIG. 6. *Effect of pH on the hydrolysis of hemoglobin and gelatin. The enzyme activity was measured at different pH levels in the reaction mixture. Symbols:* ○, *activity of proteinase I, measured at 60 C, and* ●, *activity of proteinase II, measured at 45 C (both were assayed by hemoglobin);* □, *activity of proteinase I, and* ■, *activity of proteinase II (both were measured at 45 C by using gelatin as substrate).*

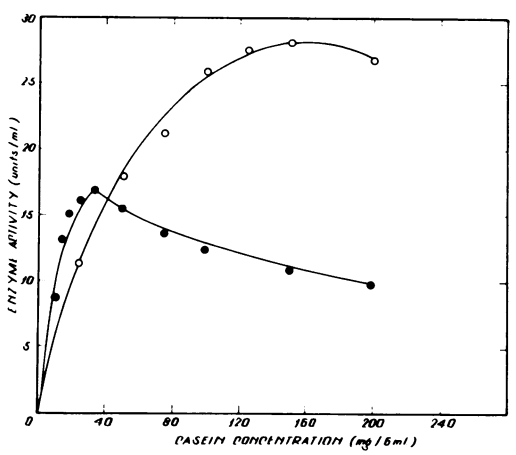

FIG. 7. *Effect of substrate concentration on the velocity of caseinolysis by A. oryzae proteinases. Symbols:* ○, *proteinase I, measured at pH 6.5 and 60 C;* ●, *proteinase II, measured at pH 10.0 and 45 C. The reaction mixture contained 1 ml of the diluted enzyme solution and 5 ml of the various concentrations of casein. The reactions proceeded for 10 min at their respective temperatures and were assayed as usual.*

DISCUSSION

Proteinase of *A. oryzae* EI 212 was separated into two fractions. The specific activity during purification increased by 75-fold. Fraction I was found to be a neutral proteinase and fraction II an alkaline proteinase. In several of its properties, proteinase I resembles the neutral proteases from *Aspergillus flavus* (16) and *A. oryzae* (21, 25), but it differs from them in many other properties. The activation by Cu^{2+} ions is unique in the case of neutral proteinase of this strain. It is known that Cu^{2+} inhibits the enzyme activity of many enzymes (19, 11, 13, 14, 22). Recently it has been reported that Cu^{2+} activates the renin-like enzyme from *Aspergil-*

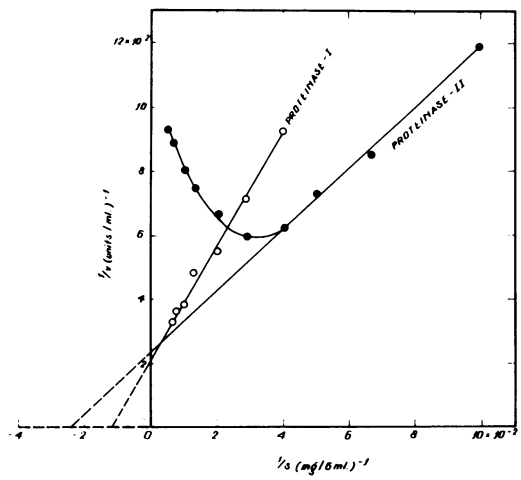

FIG. 8. *Effect of substrate concentration on the velocity of caseinolysis by A. oryzae proteinases. The data obtained from Fig. 7 are plotted according to the method of Lineweaver and Burke.*

TABLE 2. *Effect of urea on the stability of proteinases from A. oryzae*

Treatment[a]	Temp (C)	Relative proteolytic activity			
		Proteinase I		Proteinase II	
		0 h	24 h	0 h	24 h
Control (without urea)[b]	30	100	94	100	92
Control + urea	30	100	26	100	67
Control (without urea)	4	100	99	100	100
Control + urea	4	100	74	100	93

[a] The enzymes were incubated in the presence and absence of an 8 M urea solution (pH 6.5) overnight at room temperature (30 C) and at refrigerator temperature (4 C). The residual activity was assayed by the caseinolysis method as described in the text.

[b] Sodium azide (0.02%) was added to prevent the growth of molds and bacteria. Sodium azide had no effect on the activity and stability of the enzymes.

TABLE 3. *Effect of metal ions and nonmetalic reagents on the proteolytic activity of A. oryzae proteinases*

Treatment[a]	Relative activity	
	Proteinase I	Proteinase II
Metal ions		
Control (without any treatment	100	100
Ca^{2+}	80	108
Mg^{2+}	91	112
Fe^{2+}	12	18
Fe^{3+}	9	50
Cu^{2+}	240	188
Zn^{2+}	100	100
Co^{2+}	100	99
Mn^{2+}	85	67
Ba^{2+}	95	100
Ag^{+}	24	30
Hg^{2+}	40	24
Fe^{2+} + cupferron	27	41
Fe^{3+} + cupferron	38	73
Cu^{2+} + cupferron	147	95
Cu^{2+} + 8-hydroxy-quinoline	140	88
Ca^{2+} + EDTA	70	98
Mg^{2+} + EDTA	61	95
Fe^{2+} + EDTA	87	95
Fe^{3+} + EDTA	43	82
Cu^{2+} + EDTA	90	89
Zn^{2+} + EDTA	92	90
Co^{2+} + EDTA	98	95
Mn^{2+} + EDTA	100	96
Ba^{2+} + EDTA	92	91
Ag^{+} + EDTA	57	65
Hg^{2+} + EDTA	61	52
Ca^{2+} + histidine	95	100
Mg^{2+} + histidine	100	98
Fe^{2+} + histidine	102	70
Fe^{3+} + histidine	46	90
Cu^{2+} + histidine	177	98
Zn^{2+} + histidine	94	95
Ag^{+} + histidine	65	104
Hg^{2+} + histidine	88	80
Nonmetalic reagents		
Histidine	150	98
EDTA	100	88
Cupferron	100	105
8-Hydroxyquinoline	100	81
Nitroso R-salt	100	92
Sodium oxalate	100	91
Sodium molybdate	100	98
Iodine	6	2.5
$KMnO_4$	41	41
Ascorbic acid	21	131
N-chlorosuccinimide	100	23
p-CMB	37	100
Iodoacetate	58	100
Cysteine	120	111
Glutathione (reduced)	120	156
Sodium thioglycolate	161	109
DFP	97	29
2,4-Dinitro-fluorobenzene	100	100
β-Indolylacetic acid	100	94
Iodine + glutathione (reduced)	90	90
p-CMB + cysteine	100	98
p-CMB + sodium thioglycolate	95	100
p-CMB + glutathione (reduced)	92	100
Iodoacetate + cysteine	100	100
Iodoacetate + sodium thioglycolate	112	100
Iodoacetate + glutathione (reduced)	100	103

[a] A 0.5-ml amount of enzyme solution, 0.2 ml of 10^{-2} M reagent, and 0.3 ml of glass distilled water were mixed together and kept at 30 C for 60 min. The residual activity was assayed at pH 6.5 and 60 C and at pH 10.0 and 40 C for proteinases I and II, respectively. In the case of two reagents in combination, 0.1 ml of 2×10^{-2} M reagent was used. EDTA, Ethylenediaminetetraacetate; *p*-CMB, *p*-chloromercuribenzoate; DFP, diisopropyl fluorophosphate.

lus niger (1). There is no significant inhibitory effect of ethylenediaminetetraacetate on proteinase I, whereas the activity of neutral protease from *A. flavus* (16) is inhibited by 30 to 32% by ethylenediaminetetraacetate. The activation energy and molecular weight of our neutral proteinase could not be compared with other enzymes, since data are lacking in the literature with respect to known fungal neutral proteases. The activation energy curve of our neutral proteinase shows a discontinuity as observed with other enzymes by several workers (10, 20, 24).

In recent years, several workers have described alkaline proteases from different fungi. van Heyningen and Secher (27) reported on a new alkaline protease from *Acremonium kiliense*, having an optimum pH of 10.5. Alkaline proteases reported earlier (5, 22, 23) showed optimum pH values ranging from 7.5 to 9.0. *Acremonium kiliense* protease remains active over a wide range of pH values (down to below pH 5), but activity is rapidly lost at pH values above 12.5 because of irreversible denaturation. Like many proteolytic enzymes, it is stable at a low pH and can be stored at 4 C for many weeks at pH 3 to 7 without loss of activity. Its stability is not affected by an 8 M urea solution over-

night at room temperature. Yagi et al. (28, 29) isolated an alkaline protease from *Cephalosporium* sp. having an optimum pH of 11 at 30 C and an optimum temperature of 50 C. This enzyme is stable at pH 4 to 9 for 1 h at 40 C. Proteinase II from *A. oryzae* is an alkaline proteinase having a pH optimum of 10.0. Our proteinase II, however, differs from *Acremonium kiliense* protease in its stability towards urea. Stability of proteinase II is decreased in an 8 M urea solution either at room temperature or at 4 C. The molecular weight (35,000) of proteinase II is slightly higher than *Acremonium kiliense* protease (28,000) and much higher than alkaline protease from *Cephalosporium* sp. (22,500). Proteinase II is also activated by Cu^{2+} ions, a rare phenomenon. K_m and V_{max} values have not been reported in the case of protease from *Acremonium kiliense*. These values differ in comparing the *Cephalosporium* enzyme with proteinase II (K_m value, 0.125% for *Cephalosporium* sp. and 0.68% for proteinase II; V_{max}, 10.2×10^{-2} for *Cephalosporium* sp. and 8.5×10^{-4} mmol/min for proteinase II). Inhibition by *p*-chloromercuribenzoate and iodoacetate and its reversal by cysteine, glutathione (reduced), and sodium thioglycolate indicate the presence of thiol groups in proteinase I, but proteinase II does not contain such groups (7, 8, 18). Like *Cephalosporium* alkaline proteinase, the activity of proteinase II was also markedly inhibited by diisopropyl fluorophosphate. Moreover, it was also inhibited by *N*-chlorosuccinimide. These facts suggest that proteinase II might be classified as a serine enzyme (28). From this discussion, we conclude that the proteinase system of *A. oryzae* EI 212 contains two distinctly new proteinases.

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