ACTA CHEMICA SCANDINAVICA 17 (1963) 1541–1551

The Proteolytic Enzymes of *Aspergillus oryzae*

II. Properties of the Proteolytic Enzymes

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The properties of three proteolytic enzymes produced by *Aspergillus oryzae* are presented. The nature of the enzymes has been confirmed by electrophoresis, pH optimum, temperature optimum, substrate specificity, stability under different conditions, and effect of metal ions and other potential inhibitors.

Although the presence of proteolytic enzymes among the products produced by *Aspergillus oryzae* was reported already in 1932 [1], more comprehensive investigations of these enzymes were only recently initiated. The isolation of these products has long been attempted by many workers but the degree of purification has in most cases been very low. Attempts have been made to classify and characterize the proteolytic enzymes of different moulds including those derived from *Asp. oryzae*, by studying the apparent pH-activity curves with respect to protein digestion. The obvious limitations of this technique have already been pointed out and experimentally elucidated. In 1950, Crewther and Lennox [2] obtained crystals containing proteases from *Asp. oryzae* but the product was not characterized. Two years later, Astrup and Alkjaersig [3] showed by inhibitor studies that the product was not homogeneous but consisted of a mixture containing at least two proteolytic enzymes. Since then the proteolytic enzymes have been the subject of many investigations, but limited results have been presented and these have, in most cases, been obtained with crude, unfractionated, and partly inactivated preparations.

Following these less fruitful attempts to purify these enzymes by means of fractionation by adsorption, fractional precipitation, and other classical methods, we have in an earlier publication presented methods for the separation and estimation of all the proteolytic enzymes produced by a mould [4]. Methods have been presented for the quantitative estimation of the different components of the protease system without previous fractionation. The enzymes have been separated by a variety of methods without any appreciable loss in quantity or activity, both in the laboratory and on the industrial scale. Application to the protease mixture produced by *Asp. oryzae* resulted in the isolation

of three different proteolytic enzymes, referred to as protease I, II, and III. These enzymes have been purified, and this paper reports the results of a detailed study of their properties.

EXPERIMENTAL AND RESULTS

Isolation and purification of the proteolytic enzymes

The strain of *Asp. oryzae*, its cultivation as well as methods for the isolation and purification of the proteases have been described in an earlier publication [4]. Optimal production of proteolytic enzymes was reached by submerged cultivation of the strain on a protein and carbohydrate rich medium for 3 to 4 days. The culture filtrate was concentrated by tannin precipitation at pH 5.5. Small amounts of the different proteases could be separated by continuous electrophoresis, but the best results were obtained by the use of ion exchange cellulose. The proteases could be directly separated by column chromatography on DEAE-cellulose. The substances adsorbed on the column were eluted with phosphate buffers of pH 6.0 by stepwise changes in salt concentration. The proteases could also be separated in larger amounts and more rapidly after a preliminary fractionation with a multistage batch procedure using CM-cellulose. By this method the proteases I, II, and III were selectively adsorbed on CM-cellulose from a solution adjusted to pH 5.5, 4.5, and 3.0, respectively. The elution of the enzymes from the different batches of CM-cellulose could be achieved by use of a 0.05 M phosphate buffer of pH 7.0 for protease I and II and a buffer of pH 6.0 for protease III. In this way the proteases were separated and freed from the bulk of impurities. Further purification was obtained by chromatography on DEAE-cellulose according to the procedure mentioned above. The purified proteases were concentrated by precipitation with tannin or dialysis, followed by lyophilization. The products thus obtained were used for the following studies of the proteases and their properties.

Properties of the proteases

On re-chromatography each of the proteases was eluted as a single peak at the same salt concentration as in the first experiment. This behaviour upon re-chromatography indicated that a true fractionation had taken place.

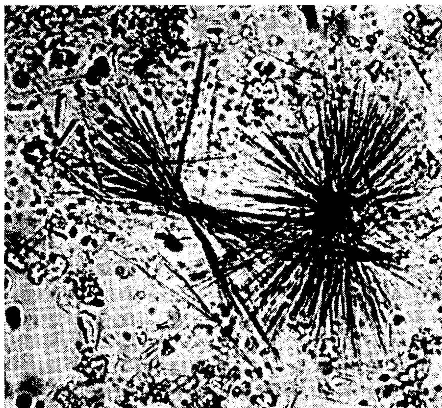

Fig. 1. Crystals of protease I.

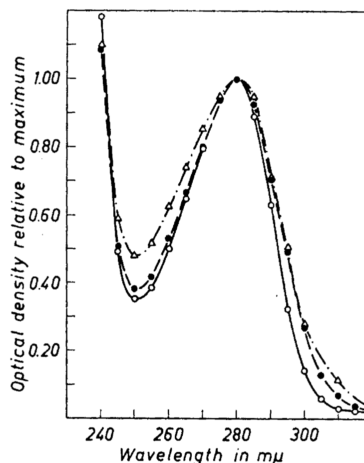

Fig. 2. Ultraviolet absorption spectra of the proteolytic enzymes in 0.05 M phosphate buffer of pH 7.0. ○, protease I; ●, protease II; △, protease III.

The proteases were subjected to detailed investigations of purity and they appeared homogeneous when examined by one-dimensional paper electrophoresis under a variety of conditions. They also appeared almost homogeneous in continuous electrophoresis at different pH conditions, a method by which one would normally expect to find greatest sensitivity in detecting foreign components. Acetate, phosphate, veronal, and phosphate-borate buffers were used, and the pH range covered was from 4.0 to 9.0.

Attempts were also made to crystallize protease I. Acetone was added dropwise with careful stirring to a concentrated solution of the enzyme until a faint cloudiness occurred. Upon standing for several days in the cold, the crystals grew into long fine needles as can be seen from Fig. 1. The crystals obtained could then be used to start further crystallization directly from concentrated water solutions of the enzyme.

The absorption spectra of the purified enzymes are shown in Fig. 2. The main absorption band occurs for all the proteases in the region characteristically associated with proteins and aromatic amino acids. The infrared spectra were also of the patterns to be expected for proteins of this type.

Action on protein substrates. The principal substrate used for measuring enzyme activity was casein, although other substrates were used in some determinations. Besides casein, urea-denatured hemoglobin, gelatin, fibrinogen and fibrin were broken down by these enzymes. The activity against fibrinogen and fibrin will be studied in greater detail in a subsequent paper.

pH optimum. The action of the purified proteases on casein, urea-denatured hemoglobin and gelatin was tested at 37°C over the pH range 2.0—12.0. The activity of the enzymes against these substrates was measured in the usual way as described earlier [4].

The method used for measuring the caseinolytic activity at different pH values was a modification of that described by Kunitz. Casein solutions adjusted to different pH values were incubated with the proteases. The degradation of casein was followed by the quantitative estimation of the digestion products

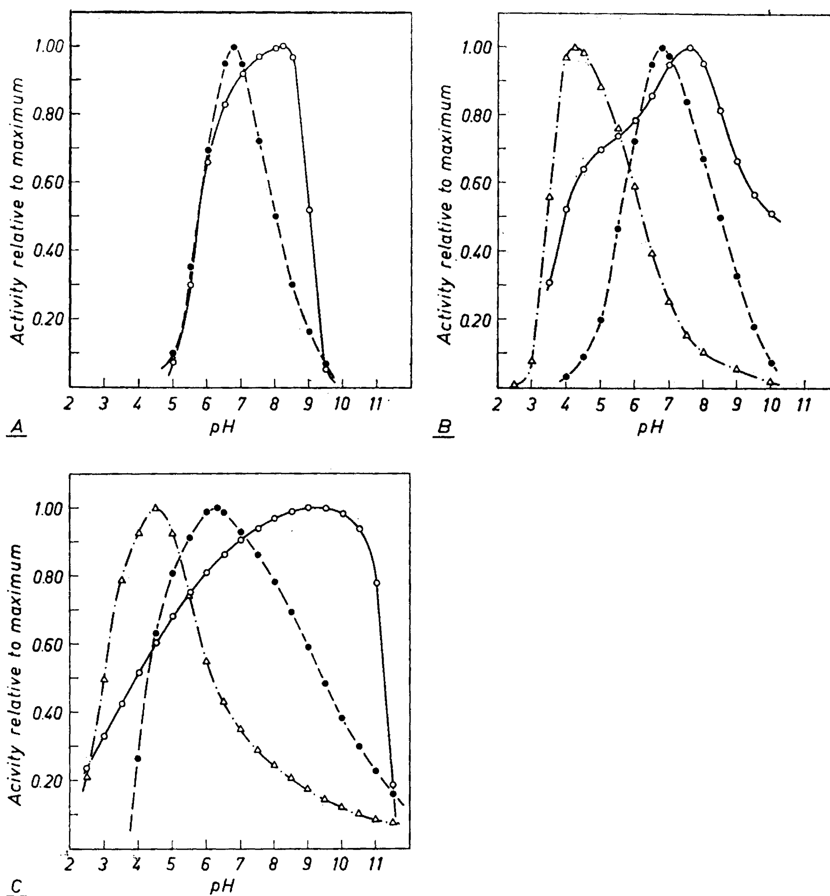

Fig. 3. Effect of pH on proteolytic activity. ○, protease I; ●, protease II; △, protease III.
A. Caseinase activity was determined by the rate of hydrolysis of 1.5 % casein in Johnson-Lindsay buffer after incubation for 30 min at 37°C. Increase in optical density of trichloroacetic acid filtrate was used as a measure of protease activity.
B. Hemoglobinase activity was determined by the rate of hydrolysis of about 2 % urea denatured hemoglobin after incubation for 10 min at 37°C. Increase in optical density of trichloroacetic acid filtrate was used as a measure of protease activity.
C. Gelatinase activity was estimated as the reduction in relative viscosity of a 5 % gelatin solution in Johnson-Lindsay buffer of the appropriate pH, after a digestion period of 5 min at 37°C.

by means of ultraviolet spectrophotometry, after precipitation of undigested proteins with trichloroacetic acid.

The effect of pH on the degradation of denatured hemoglobin was determined by the method of Anson as modified by Kunitz. Solutions of the purified proteases were incubated with solutions of urea-denatured hemoglobin adjusted

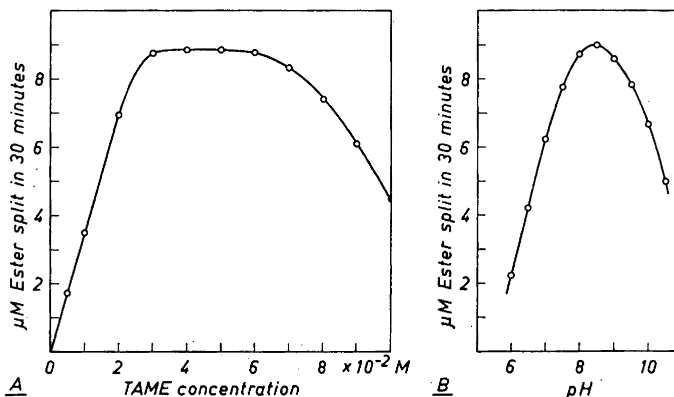

Fig. 4. Hydrolysis of TAME by protease I.
A. Substrate titration curve. TAME was incubated with protease I at pH 8.0 in 0.25 M TRIS buffer for 30 min at 37°C. Esterase activity was determined by the method mentioned in the text.
B. pH dependence of the hydrolysis of TAME by protease I. The activity was estimated as the hydrolysis rate of 0.04 M TAME in 0.25 M TRIS buffer of the appropriate pH, after incubation for 30 min at 37°C.

to various pH values. The concentration of decomposed products formed was estimated spectrophotometrically as described for the caseinase determination.

Gelatin hydrolysis was estimated by the viscosity reduction method. The enzymes under investigation were added to 5 % gelatin solutions buffered to various pH values, incubated for 5 min at 37°C and the viscosity determined. The enzymatic activity was then defined as the ratio of the reduction in viscosity after incubation to the initial viscosity.

Casein, which is soluble at pH 4 to 5, was not suitable for estimation of the pH optimum of protease III. This could, however, be estimated both with gelatin and also with denatured hemoglobin. The pH dependence of the enzymatic activity of the proteases is shown in Fig. 3. The pH-activity varied slightly with the buffers employed and with the substrate. The substrate had in some cases a stabilizing effect upon the enzyme, resulting in a higher pH optimum. Proteases I and II have pH optima at 7.6 to 9.5 and 6.3 to 6.8, respectively, while protease III is optimally active at pH 4.3 to 4.5.

Action on synthetic substrates. The activity of the proteases on synthetic lysine and arginine esters was also studied by the method originally described by Troll *et al.*[5] The synthetic substrates employed were unsubstituted lysine ethyl ester and tosyl arginine methyl ester, referred to as TAME. No significant enzymatic hydrolysis of the lysine ester could be detected even after incubation for several hours at 37°C with the different proteases. Protease I, however, was found to hydrolyse TAME, whereas proteases II and III had no action on this substrate. The substrate titration curve and the pH-optimum curve for the action of protease I on TAME are shown in Fig. 4. For enzyme assay, 0.04 M TAME was chosen as substrate concentration, the activity being

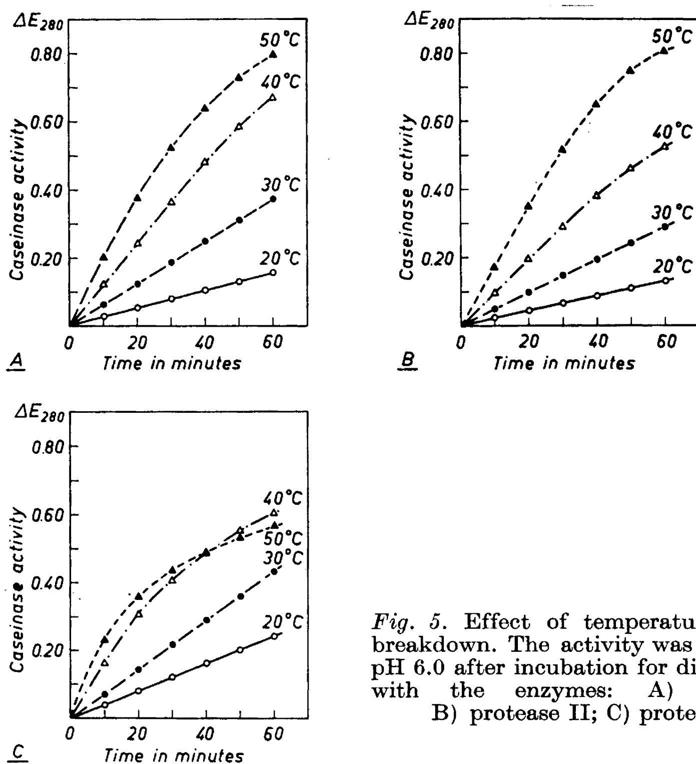

Fig. 5. Effect of temperature on casein breakdown. The activity was estimated at pH 6.0 after incubation for different times with the enzymes: A) protease I; B) protease II; C) protease III.

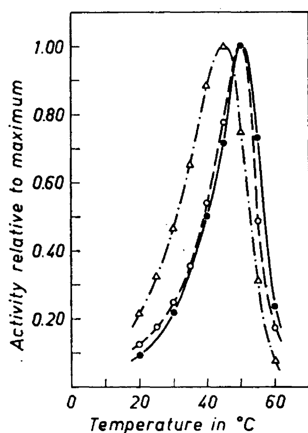

Fig. 6. Effect of incubation temperature on caseinase activity. The activity was estimated after incubation for 30 min at pH 6.0. ○, protease I; ●, protease II; △, protease III.

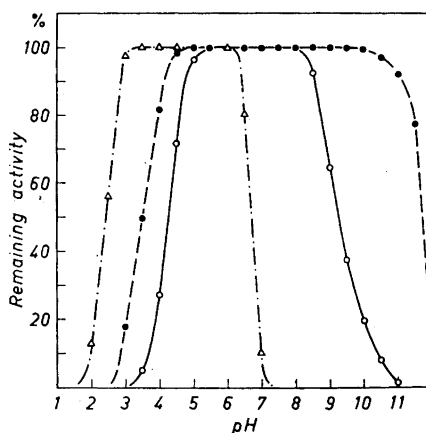

Fig. 7. Effect of pH on the stability of the proteases. The enzymes were incubated for 30 min at 37°C in Johnson-Lindsay buffer adjusted to the desired pH. The remaining caseinase activity was estimated at pH 7.4 for proteases I and II and at pH 6.0 for protease III. ○, protease I; ●, protease II; △, protease III.

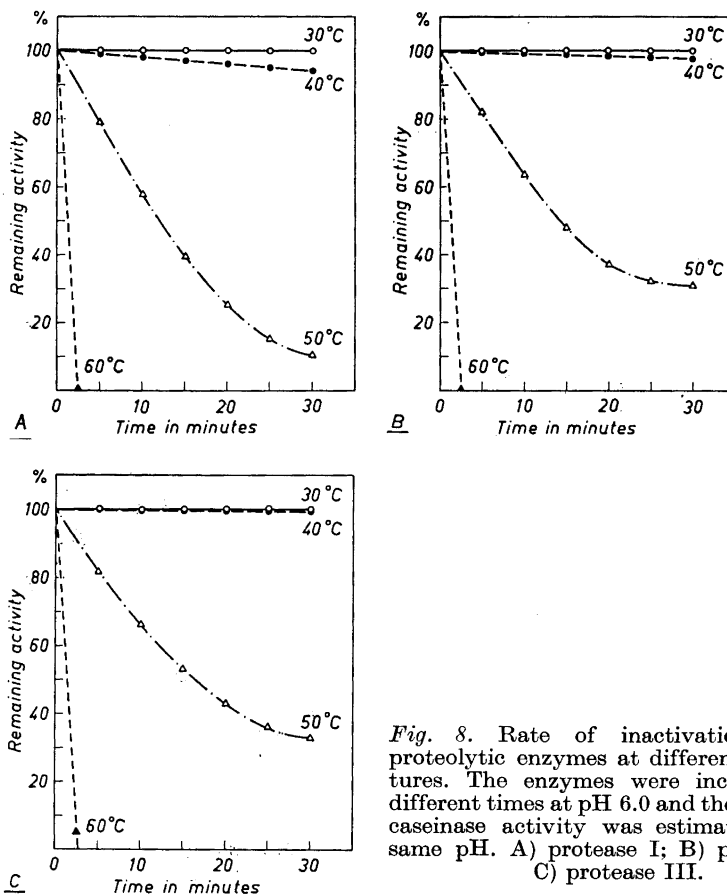

Fig. 8. Rate of inactivation of the proteolytic enzymes at different temperatures. The enzymes were incubated for different times at pH 6.0 and the remaining caseinase activity was estimated at the same pH. A) protease I; B) protease II; C) protease III.

expressed in μmoles per ml of acid liberated after incubation for 30 min at 37°C. At higher substrate concentrations, striking inhibition of the enzyme action became apparent. It can be seen that optimum activity is obtained at pH 8.5 or about the same as that for the hydrolysis of most proteins.

Temperature optimum. The effects of temperature on the velocity of enzymatic actions are extremely complex. They can, however, be readily analysed experimentally. The proteolysis of casein by the purified enzymes at 20 to 60°C were compared, as shown in Fig. 5. Although the initial velocity increases steadily as the temperature rises, the amount of substrate actually transformed during a given time interval first rises with increasing temperatures and then falls as the temperature passes beyond a certain optimum value. Two different temperature effects are operating simultaneously, namely, increase in initial velocity, and destruction of the enzyme at higher temperatures, resulting in a continuous fall in the concentration of active enzyme.

Table 1. Influence of metal ions on the proteolytic activity of the purified enzymes. The enzymes were incubated with the various metal ions for 15 min before assay. The caseinolytic activity at pH 6.0 was determined in the usual manner.

Metal ion	Final concn. of metal ion M	Proteolytic activity as % of control		
		Protease I	Protease II	Protease III
Ba^{2+}	10^{-2}	103	76	89
	10^{-3}	104	90	100
	10^{-4}	100	100	100
Ca^{2+}	10^{-2}	95	41	80
	10^{-3}	100	100	99
	10^{-4}	100	100	100
Mg^{2+}	10^{-2}	94	79	100
	10^{-3}	95	90	100
	10^{-4}	100	101	100
Zn^{2+}	10^{-2}	10	7	3
	10^{-3}	76	83	73
	10^{-4}	96	100	100
Mn^{2+}	10^{-2}	61	41	49
	10^{-3}	95	87	100
	10^{-4}	100	100	100
Cu^{2+}	10^{-2}	3	8	12
	10^{-3}	56	25	83
	10^{-4}	100	48	100
Ni^{2+}	10^{-2}	54	17	66
	10^{-3}	79	26	82
	10^{-4}	100	57	93
Co^{2+}	10^{-2}	84	51	72
	10^{-3}	95	82	100
	10^{-4}	100	100	100
Cd^{2+}	10^{-2}	23	6	10
	10^{-3}	60	16	35
	10^{-4}	100	35	80
Fe^{3+}	10^{-2}	48	8	8
	10^{-3}	74	19	42
	10^{-4}	92	82	84

The optimum temperature is not constant, but falls as the time interval increases. Fig. 6 shows the temperature effect for a 30 min incubation period. It can be seen that optimum activity is found at about 50°C for proteases I and II, and at 45°C for protease III.

Stability of the enzymes. Lyophilized or tannin precipitated preparations of the proteases are stable for years when kept at room temperature. In solution, however, the enzymes are rather sensitive towards heat and pH changes.

Table 2. Effect of some potential inhibitors on the activity of the purified proteases. The enzymes were pre-incubated with the inhibitor for 15 min before assay. The caseinolytic activity was estimated at pH 6.0.

Inhibitor	Final concn. of inhibitor	Proteolytic activity as % of control		
		Protease I	Protease II	Protease III
Soy bean inhibitor	0.02 %	100	100	100
ε-Aminocaproic acid	10^{-1} M	100	100	100
Potassium cyanide	10^{-2} M	100	95	93
	10^{-3} M	100	100	100
Iodine	10^{-3} M	50	0	0
	10^{-4} M	97	82	35
Iodoacetic acid	10^{-2} M	100	100	100
	10^{-3} M	100	100	100
Ascorbic acid	10^{-2} M	83	11	77
Na-laurylsulfonate	10^{-2} M	13	100	34
Laurylamine	10^{-1} M	27	26	0
L-Cysteine	10^{-2} M	100	36	100
	10^{-3} M	100	57	100
EDTA	10^{-2} M	100	10	100
	10^{-3} M	100	11	100
	10^{-4} M	100	15	100

(A) *Effect of pH.* The rate of inactivation of the proteases depends to a great extent on the pH of the solution, and this effect varies greatly between the proteases. The occurrence of irreversible destruction could be tested by exposing the enzymes to a range of pH values for 30 min at 37°C and then determining the remaining activity at the pH at which they are stable.

As shown in Fig. 7 the effect of pH varies greatly between the proteases. Protease III, which is optimally active at acid pH, shows also the greatest stability in acid solutions, while protease II shows stability over a much wider pH interval than the other proteases. The enzymes are of course more stable at lower temperature at any given pH value and the presence of a protein substrate has likewise a stabilizing effect.

Table 3. Recovery of inhibited protease II by various metal ions. EDTA was added to a solution of protease II to a final concentration of 10^{-3} M. After incubation for 15 min at room temperature various metal ions were added also to a final concentration of 10^{-3} M. As can be seen from Table 2 EDTA reduces the caseinase activity to 11 % of control. The recovery of activity after addition of metal ions was estimated at pH 6.0 using casein as substrate.

Metal ion	Ba^{2+}	Ca^{2+}	Mg^{2+}	Zn^{2+}	Mn^{2+}	Cu^{2+}	Ni^{2+}	Co^{2+}	Cd^{2+}	Fe^{3+}
Recovery %	0	0	0	98	84	32	34	87	26	92

Table 4. Some properties of the extracellular proteases produced by *Asp. oryzae*, estimated by the method mentioned in the text. +, strong inhibition; –, no inhibition.

Property		Protease		
		I	II	III
pH optimum with respect to	casein	8.2	6.8	–
	den. hemoglobin	7.6	6.8	4.3
	gelatin	9.0 – 9.5	6.3	4.5
	TAME	8.5	–	–
Temperature optimum, °C		50	50	45
pH stability		5.0 – 8.5	4.5 – 10.5	3.0 – 6.3
Inhibitors	Na-laurylsulfonate	+	–	+
	Laurylamine	+	+	+
	L-Cysteine	–	+	–
	EDTA	–	+	–
	ε-Aminocaproic acid	–	–	–
	Soy been inhibitor	–	–	–

(B) *Effect of temperature.* In order to determine the rate of thermal inactivation, the purified proteases were heated on a water bath for various times and the residual activity determined by the casein digestion method. The results are presented in Fig. 8, which shows that the inactivation of all proteases was complete after less than 2 min at 60°C. More than 50 % of the original activity was lost after 20 min at 50°C, but at 40°C the activity decreased at a very slow rate.

Inhibitors. The effect of various metal ions and other miscellaneous substances on the proteolytic activity of the proteases was studied by incubating the inhibitor with the enzymes for 15 min followed by the addition of the substrate (casein) and estimation of the activity in the usual manner. The results obtained with various metal ions at different concentrations are presented in Table 1. It can be seen that the proteases were unaffected or only slightly inhibited by barium, calcium, and magnesium ions even at the relatively high concentrations used. At the lowest concentration only ions of the heavy metals, *viz.* copper, nickel, cadmium and iron had any effect.

The results of studies with some other miscellaneous substances tested are presented in Table 2. The effect of crystalline soy bean trypsin inhibitor was investigated. The inhibitor, which is highly active against trypsin, did not show any effect on the protease. Another potent trypsin and plasmin inhibitor, ε-aminocaproic acid, was also without effect on all the three proteases. Potassium cyanide and iodoacetic acid exerted little or no effect on the enzymes. Proteases I and III were only slightly inhibited by ascorbic acid, which was, however, a powerful inhibitor of protease II. Conversely, proteases I and III were strongly inhibited by sodium laurylsulfonate and all three enzymes were inhibited by laurylamine. EDTA and L-cysteine had a powerful inhibi-

tory effect on protease II, but none on proteases I and III, demonstrating some striking differences among these proteases.

The re-activation of inhibited enzyme by metal ions was studied. To a solution of protease II containing EDTA was added metal ions, and the recovery of enzymatic activity estimated. As shown in Table 3, full recovery was observed in the case of Zn^{2+}, Mn^{2+}, Co^{2+} and Fe^{3+}. Partial recovery was shown by the addition of Cu^{2+}, Ni^{2+} and Cd^{2+}. The same results were obtained with an inhibited enzyme solution which had been dialysed against distilled water.

The sera of most animals are known to contain powerful inhibitors of proteolytic enzymes and these are also active against the proteolytic enzymes of *Asp. oryzae*, as will be described in a later publication.

Conclusion. The results presented above show clearly that three different proteolytic enzymes are produced by *Asp. oryzae*, the chief properties of which are summarized in Table 4.

From the evidences presented here and in the first paper of this series [4] it can be seen that all the proteolytic enzymes can be separated, isolated almost quantitatively and highly purified. This is the first complete separation and characterization of all the extracellular proteases produced by *Asp. oryzae*. The methods outlined in these two papers can be applied to the study of proteolytic enzymes derived from other materials after the necessary modifications of the experimental conditions.

REFERENCES

1. Grassmann, W. *Ergeb. Enzymforsch.* **1** (1932) 149.
2. Crewther, W. G. and Lennox, F. G. *Nature* **165** (1950) 680.
3. Astrup, T. and Alkjaersig, N. *Nature* **169** (1952) 314.
4. Bergkvist, R. *Acta Chem. Scand.* **17** (1963) 1521.
5. Troll, W., Sherry, S. and Wachman, J. *J. Biol. Chem.* **208** (1954) 85.

Received February 28, 1962.