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Extracellular Proteinases of *Aspergillus oryzae*

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Although the production of proteolytic enzymes by different strains of *Aspergillus oryzae* has been investigated in submerged culture (5, 15), in static liquid culture (3, 11), and on solid substratum (8–10), there appear to be few reports pertaining to the comparative evaluation of these different methods on the elaboration of proteinases by specific strains of *A. oryzae*. K. Mogi (13) compared the proteinase activity of a few strains grown in submerged culture and on solid substratum and found the submerged culture superior. The properties of proteinases produced by *A. oryzae* under various conditions differ in many respects (3, 12, 14, 16). We wish, therefore, to report the influence of different methods upon the production of extracellular proteinase by *A. oryzae* E.I. 212 (our collection) and to present some of the properties of the enzyme.

A proteinase-rich fraction was prepared as follows. Moldy bran (100 g) obtained by growing *A. oryzae* on moist wheat bran (40% moisture) for 3 days at 30 C was extracted with water (1:4, w/v), and the aqueous extract was centrifuged for 10 min at 3,000 rev/min. The supernatant fluid, on treatment with alcohol, gave a proteinase-rich fraction, which was then suspended in water and fractionated with ammonium sulfate. The proteinase-rich fraction thus obtained was resuspended in water, dialyzed against several changes of distilled water, and further fractionated with alcohol to obtain solid, needle-shaped crystals having higher proteinase activity. Repeated attempts to crystallize this enzyme by the method of Crewther and Lennox (3) failed.

Proteinase activities were determined by viscosity reduction (7), gelatin digestion (17), hemoglobin digestion (1), and caseinolysis (2).

TABLE 1. *Influence of fermentation methods upon the formation of proteinase by Aspergillus oryzae*

Method	Proteinase activity				Collagenolytic activity[c]	
	Gelatin digestion[a]		Caseinolysis[b]			
	*p*H 6.8	*p*H 10.5	*p*H 6.5	*p*H 10.5	*p*H 6.8	*p*H 10.5
Submerged culture[d]	nil	nil	nil	nil	nil	nil
Static liquid culture[d]	nil	nil	nil	nil	nil	nil
Solid substratum (wheat bran)	3.17	0.51	0.319	0.377	nil	nil

[a] Gelatin (5.0%, E. Merck) was used as substrate; digestion was continued for 1 hr at 40 C in the presence of suitable buffers with 25.0 mg of enzyme. The results (average of triplicate trials) are expressed as milligrams of amino acid N per milligram of protein.

[b] Activity is expressed as optical density change at 650 nm; 2% casein (E. Merck) was used as substrate; 1 mg of enzyme acted for 10 min at 40 C in the presence of suitable buffers (average of triplicate trials).

[c] Collagen (2%) was digested with 25.0 mg of enzyme in the presence of suitable buffers at 40 C.

[d] Various media reported in the literature were tried; incubation period varied from 5–10 days at 25 C–28 C.

Collagenolytic activity was assayed by the hydroxyproline determination method (4) with pure collagen isolated from fish swimming bladder (6) as the substrate. Since strain E.I. 212 produces extracellular proteinase when cultivated on wheat bran as solid substratum but fails to do so in submerged and static liquid culture with different media (Table 1), it differs from the strains studied by Mogi (13) and Crewther and Lennox (3). A comparative study of this proteinase with other common proteinases (Table 2) reveals that the proteinase from our strain differs in many respects from that reported by

Crewther and Lennox (3). It does not possess any collagenolytic activity (Table 2) and thus differs from the proteinase isolated from *A. oryzae* by Nordwig and Jahn (14). The optimal *p*H values for casein digestion by this enzyme are 6.5 and 10.5; for gelatin digestion, *p*H 6.8 is optimal (Fig. 1). The optimal temperature for casein digestion at *p*H 6.5 is 60 C; at *p*H 10.5, it is 37 to 42 C. The enzyme at *p*H 6.5 loses all activity at 75 C; at *p*H 10.5, it loses activity at 55 C (Fig. 2). The dependency of proteinase activity on Ca^{++} and Mg^{++} in the presence of

TABLE 2. *Comparison of proteolytic activity of Aspergillus oryzae proteinase(s) with some common proteolytic enzymes*

Enzyme	Relative proteolytic activity[a]/mg of protein		
	Viscosity reduction	Gelatin digestion	Hemoglobin digestion
Pepsin (Rosekilde)...	30	3	638
Trypsin (E. Merck)..	800	55	74
Papain (Eagle Laboratory).....	145	16	61
Proteinase(s).....	100	100	100

[a] Activity was determined at the optimum *p*H of the respective enzyme at 40 C; for proteinase, however, *p*H 6.8 was used (average of triplicate trials).

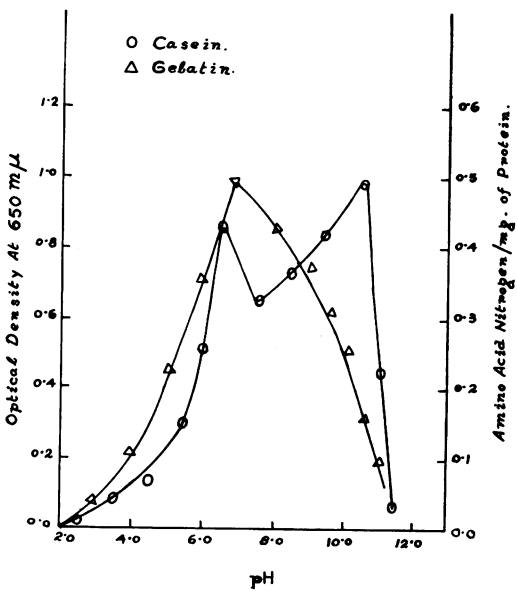

FIG. 1. *Optimal pH values for casein and gelatin digestion by extracellular proteinase.*

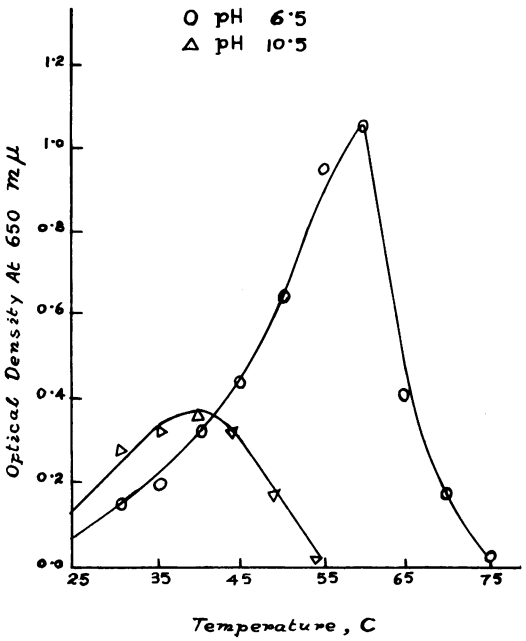

FIG. 2. *Effect of temperature on extracellular proteinase activity at pH 6.5 and 10.5.*

TABLE 3. *Effect of metal ions on proteinase activity in the presence of EDTA*

Treatment[a]	Relative proteinase activity	
	*p*H 6.5	*p*H 10.5
Control.....	100	100
Mg^{++} + EDTA.....	59	99
Ca^{++} + EDTA.....	88	119

[a] Added as 0.5 ml of a 10^{-2} M solution of salt per 7 ml of reaction mixture.

ethylenediaminetetraacetic acid (EDTA) is also different at the two optimal *p*H levels (Table 3). This behavior suggests that this enzyme system is either a mixture of two enzymes or that it possesses two different kinds of functional groups that act differently at two optimal *p*H levels. It appears that strain E.I. 212 not only differs from other strains in its mode of elaboration of proteolytic enzyme but also in the type of extracellular proteinase(s) produced.

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LITERATURE CITED

1. Anson, M. L. 1938. Estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22:79.

2. Cowman, R. A., and M. L. Speck. 1967. Proteinase enzyme system of lactic streptococci. I. Isolation and partial characterization. Appl. Microbiol. **15**:851–856.
3. Crewther, M. G., and F. G. Lennox. 1950. Preparation of crystals containing protease from *Aspergillus oryzae*. Nature **165**:680.
4. Gurzon, G., and J. Giltorw. 1954. Aromatic aldehydes as specific chromatographic color reagents for amino acids. Nature **173**:314.
5. Dworschack, R. G., et al. 1952. Proteolytic enzymes of micro-organisms. Evaluation of proteinase produced by molds of the *Aspergillus flavus-oryzae* group in submerged culture. Arch. Biochem. Biophys. **41**:48.
6. Gallop, P. M. 1955. Particle size and shape in a citrate extract of ichthyocol. Arch. Biochem. Biophys. **54**:486.
7. Lennox, F. G., and W. J. Ellis. 1945. Euphorbain, a protease occurring in the latex of the weed *Euphorbia lathyris*. Biochem. J. **39**:465.
8. Matsushima, K. 1953. The proteolytic enzymes of molds. I. Comparison of the methods for determination of the proteolytic activity. J. Ferment. Technol. **31**:367.
9. Matsuyama, M. 1950. Studies on *Aspergillus*. I. Enzyme production in the solid culture. J. Ferment. Technol. **28**:149.
10. Maxwell, M. E. 1950. Production of protease by cultivation of *Aspergillus oryzae* on wheat bran. Australian J. Appl. Sci. **1**:348.
11. Maxwell, M. E. 1952. Enzymes of *Aspergillus oryzae*. I. The development of a culture medium yielding high protease activity. Australian J. Sci. Res. (Ser. B) **5**:42.
12. Miura, Y., and W. Montonaga. 1954. Examination of protease in taka-diastase by paper chromatography. Ann. Rept. Takamine Lab. **6**:14.
13. Mogi, K. 1958. Studies on the Soy-sauce production by submerged culture. J. Ferment. Technol. **36**:87.
14. Nordwig, A., and W. F. Jahn. 1966. Specificity properties of a protease from *Aspergillus oryzae*. Z. Physiol. Chem. **345**:284.
15. Specht, H. 1957. Proteolytic activities of molds on raw material. Zentr. Bakteriol. Parasitenk. Abt. II **110**:540.
16. Specht, H. 1957. Proteinases of *Aspergillus oryzae*. Naturwissenschaften **44**:37.
17. Taylor, W. H. 1959. Studies on gastric proteolysis. I. The proteolytic activity of human gastric juice and pig and calf gastric mucosal extracts below *p*H 5. Biochem. J. **71**:73.