Fungal Proteolytic Enzymes

Part I. Isolation of two Kinds of Acid-proteases Excreted by *Aspergillus niger* var. *macrosporus**

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Two kinds of proteolytic enzyme, tentatively named acid protease A and B which showed a single peak on electrophoresis individually, were isolated from the crude enzyme powder obtained from the broth filtrate cultured with *Aspergillus niger* var. *macrosporus*. Acid protease B is similar too the fungal acid protease previously reported, because the enzyme exhibits optimum activity on milk casein at about pH 2.6 and 55°C when the incubation was done at pH 2.6. Acid protease A is a new proteolytic enzyme, because the enzyme exhibits optimum activity on milk casein at about 2.0 and 70°C or 60°C when the incubation was done at pH 2.6 or 1.5 respectively.

INTRODUCTION

Microbial acid-proteases have been isolated from *Aspergillus oryzae*¹⁾, *Asp. saitoi*²⁾, *Paecilomyces varioti*³⁾, *Trametes sanguinea*⁴⁾ and so-called Butyl bacteria⁵⁾. These enzymes digest milk casein well to produce a large amount of amino acid, and their optimal pH and optimal temperature for milk casein digestion lie in the region of pH 2.5~3.0 and 55~60°C.

In the course of studies on microbial enzymes in our laboratory, it has been observed that the broth filtrate cultured with the strain DBD-0406, which was newly isolated and identified with *Aspergillus niger* var.

macrosporus by the authors⁶⁾, showed a strong proteolytic activity on milk casein in the pH range of 1.5~6.0. The crude enzyme powder, prepared from the broth filtrate cultured with this strain, was purified by means of column chromatography using Sulfo Ethyl Cellulose after fractionation with ammonium sulfate. Two kinds of proteolytic enzymes, tentatively named acid protease A and B, were isolated and each of these two enzymes showed single peak of them own on electrophoresis. Acid protease B is similar to other fungal acid protease previously reported, because its optimal pH and optimal temperature incubated at pH 2.6 for milk casein digestion lie about pH 2.6 and 55°C, and under the optimal condition this enzyme digests milk casein well to produce a large amount of amino acid.

On the other hand, acid protease A is a new proteolytic enzyme, because its optimal

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1) K. Nakanishi, *J. Biochem. Japan*, **46**, 1263 (1959).

2) F. Yoshida and M. Nagasawa, *This Journal*, **20**, 257 (1956).

3) K. Tomoda and H. Shimazono, *Proceeding of the 14th Symposium on Enzyme Chem. Japan*, 1962, p. 1.

4) T. Misaki et al., *J. Agr. Chem. Soc. Japan*, **35**, 1264 (1961).

5) F. Uchino, K. Ito and S. Doi, *ibid.*, **35**, 719 (1961).

6) Y. Koaze et al., *in press*.

pH and optimal temperature incubated at pH 2.6 or 1.5 for milk casein digestion lie at about pH 2.0 and 70°C or 60°C, and the activity of this enzyme on amino acid formation from milk casein is less than that of acid protease B. Present paper deals with the isolation procedure of these two kinds of fungal acid proteases and effects of pH and temperature on their proteolytic activities on milk casein digestion.

MATERIALS AND METHODS

The Crude Enzyme Powder.

The crude enzyme powder used in this experiment was prepared from the broth filtrate cultured with *Asp. niger* var. *macrosporus* DBD-0406 as reported elsewhere⁹⁾, and effects of pH and of the temperatures kept at pH 2.6 or 1.5 on the activity on milk casein digestion were shown in Figs. 1 & 2.

Preparation of Sephadex.

The two types of Sephadex, G-25 and G-75, which were products of the Pharmacia Co., were used. For purification of the crude enzyme powder, the Sephadex G-75 was equilibrated with 0.02 M acetate buffer of pH 3.5 containing 10^{-3} M Ca acetate, and, for desalting of the partially purified enzyme, the Sephadex G-25 was equilibrated with McIlvaine buffer ($\mu=0.03$) of pH 2.6 containing 10^{-3} M Ca acetate.

FIG. 1. The Effect of pH on Milk Casein Digestion by the Crude Enzyme Powder.

Five ml of 0.6% milk casein solution were digested with 1 ml of the enzyme solution containing 0.167 mg of the crude enzyme powder at 30°C for ten minutes at various pH from 1.5 to 6.0. The proteolytic activity was assayed by the modified Anson's method.

FIG. 2. The Effect of Temperature on Milk Casein Digestion by the Crude Enzyme Powder.

Five ml of 0.6% milk casein solution were digested with 1 ml of the enzyme solution containing 0.07 mg of the crude enzyme powder at pH 2.6 for ten minutes at various temperatures from 30°C to 70°C, and 0.15 mg of the crude enzyme powder at pH 1.5.

Preparation of Sulfo Ethyl Cellulose.

The Sulfo Ethyl Cellulose was a product of the Serva Co. And, for the isolation of acid protease A and B, this was equilibrated with the same buffer-salt solution as used for Sephadex G-25 as mentioned above.

Protease Assay.

Protease activity was assayed by a modified Anson's method⁷⁾ Usually reaction was carried out on milk casein (Casein according to Hammarsten, Merck) at 30°C for 10 minutes in pH 2.6 or 1.5. And when the effect of pH on proteolytic activity was investigated, pH was varied in the region of 1.0~5.6. At

7) S. Akabori, "Method of Enzyme Research", Asakura Book Co., Ltd., (2), 1956, p. 240.

pH 2.3 or below, Walpole or Clark buffer was used and, at pH 2.6 or above, McIlvaine buffer was used. When the effect of temperature on proteolytic activity was investigated, temperature was varied in the region of 30~85°C. The amount of non-proteinous product digested from milk casein during the incubation period was estimated either spectrophotometrically at 275 $m\mu$ or by use of the Folin reagent at 660 $m\mu$.

Quantitative Determination of Protein.

Amount of protein in eluates obtained from the column chromatogram was determined by optical density at 280 $m\mu$ using Photo-Electro Spectrophotometer EPU 2A, Hitachi Ltd.

FIG. 3. Thermal Inactivation of the Proteolytic Activity of the Crude Enzyme Powder Kept at pH 2.6.

1.25 g of the crude enzyme powder were dissolved in Walpole buffer of pH 2.6 and filled up 10 ml after adjusting to pH 2.6. The enzyme solution was poured into 15 ml of the same buffer as mentioned above pre-heated at 55°C and kept at the same temperature successively. One ml aliquot of the enzyme solution kept as mentioned above was taken out at each time mentioned in the figure and their proteolytic activities for milk casein digestion were assayed at pH 2.6 and 1.5.

Electrophoresis.

Specimens were dissolved in 0.02 M phosphate buffer of pH 7.0 containing 0.1 M NaCl ($\mu=0.18$) and, using Tiselius type instrument HTB-2, Hitachi Ltd., the electrophoresis was done under condition of 6 mA and 3°C.

RESULTS AND DISCUSSION

(1) Thermal Inactivation of the Crude Enzyme Powder.

When the crude enzyme powder was kept at 55°C, pH 2.6, proteolytic activities assayed at pH 2.6 and 1.5 gradually decreased with the lapse of time. After forty minutes, both proteolytic activities assayed at pH 2.6 and 1.5 became almost the same (Fig. 3). On the other hand, when the crude enzyme powder was kept at 55°C, pH 1.5, proteolytic activities assayed at pH 2.6 and 1.5 rapidly de-

FIG. 4. Thermal Inactivation of the Proteolytic Activity of the Crude Enzyme Powder Kept at pH 1.5.

2.5 g of the crude enzyme powder were dissolved in Walpole buffer of pH 1.5. The enzyme solution was poured into 15 ml of the same buffer as mentioned above pre-heated at 55°C and kept at the same temperature successively. One ml aliquot of the enzyme solution kept as mentioned above was taken out at each time mentioned in the figure and their proteolytic activities for milk casein digestion were assayed at pH 2.6 and 1.5.

creased. After five minutes, the proteolytic activity assayed at pH 1.5 became higher than that assayed at pH 2.6 (Fig. 4).

(2) Isolation of Acid Protease A and B.

All treatments were performed at 4°C. As shown in Fig. 5, the crude enzyme powder was purified and subsequently the acid protease A and B were isolated as follows; (a) The crude enzyme powder was partially purified by means of column chromatography using Sephadex G-75. Then fractions having proteolytic activity, eluted from the Sephadex column with 0.02 M acetate buffer of pH 3.5 containing 10^{-3} M Ca acetate, were collected. (b) These fractions were added with ammonium sulfate to 0.5 saturation, then the precipitate was collected by centrifugation and freeze-dried (Enzyme Powder I). (c) The supernatant was further added with ammonium sulfate to 0.75 saturation, then the precipitate was collected by centrifugation and freeze-dried (Enzyme Powder II). (d) The Enzyme Powder I and II were individually desalted and buffer-changed by means of col-

umn chromatography using Sephadex G-25, and fractions having proteolytic activity were applied to Sulfo Ethyl Cellulose column and eluted with McIlvaine buffer ($\mu=0.03$) containing 10^{-3} M Ca acetate with gradual change of pH from 2.6 to 7.2. As shown in Fig. 6, the distribution pattern of protein having five peaks was obtained from the column chromatography of Enzyme Powder I. Among them, protein fractions of the second and the fifth peak showed remarkable proteolytic activity. And protein fractions of the first and the third peak also showed weak proteolytic activity. On the other hand, as shown in Fig. 7, the distribution pattern of protein having the only one obvious peak which exhibits proteolytic activity was obtained from the column chromatography of Enzyme Powder II. These fractions exhibiting proteolytic activity were freeze-dried individually. Each proteolytic activity of protein fraction of the fifth peak obtained from the Enzyme Powder I (EP-Ie) and of the peak obtained from the Enzyme Powder II (EP-IIa) assayed at pH 2.6 was higher than that assayed at pH 1.5. However, on the contrary, the proteolytic activity of the protein fraction of the second peak obtained from the Enzyme Powder I (EP-Ib) assayed at pH 2.6 was lower than that assayed at pH 1.5. Each proteolytic activity of protein fraction of the first and the third peak obtained from the Enzyme Powder I (EP-Ia & -Ic) assayed at pH 2.6 was also lower than that assayed at pH 1.5.

FIG. 5. The Isolation Procedure of Acid Protease A and B from the Crude Enzyme Powder

(3) Effects of pH and Temperature on the Activity of Acid Protease A and B.

Effects of pH on proteolytic activities of the EP-Ie and of the EP-IIa were shown in Fig. 8. The optimal pH for milk casein digestion by the two preparations lies equally at about pH 2.6, and their proteolytic activities assayed at pH 1.5 were about 30% of that assayed at pH 2.6, and proteolytic activities

FIG. 6. Distribution of the Proteolytic Activity of the Enzyme Powder I on Sulfo Ethyl Cellulose Column Chromatogram.

Five grams of the Enzyme Powder I were desalted by means of column chromatography using 100 g of Sephadex G-25. About 50 ml of active fractions having proteolytic activity were applied to 30 g of Sulfo Ethyl Cellulose column equilibrated with McIlvaine buffer ($\mu=0.03$) of pH 2.6 containing 10^{-8} M Ca acetate. And then this was eluted with the same buffer-salt solution with gradual change of pH from 2.6 to 7.2 (these two pH solutions were both 1,000 ml), and each 10 ml of eluate were collected in test tube. On each fraction, the quantity of protein was measured at 280 m μ (solid line) and the proteolytic activity was assayed at pH 2.6 (broken line).

FIG. 7. Distribution of the Proteolytic Activity of the Enzyme Powder II on Sulfo Ethyl Cellulose Column Chromatogram.

Five grams of the Enzyme Powder II were desalted by means of column chromatography using 100 g of Sephadex G-25. About 50 ml of active fractions having proteolytic activity were applied to 40 g of Sulfo Ethyl Cellulose column equilibrated with the same buffer-salt solution as described in Fig. 6. Other conditions were the same as described in Fig. 6.

FIG. 8. Effects of pH on Milk Casein Digestion by Purified Preparations Tentatively Named Acid Protease B.

Five ml of 0.6% milk casein solution were digested with 1 ml of the enzyme solution containing 0.0285 mg of EP-Ic (left) and 0.0448 mg of EP-IIa (right) at 30°C for ten minutes at various pH values from 1.0 to 5.6.

FIG. 9. Effects of Temperature on Milk Casein Digestion by Purified Preparations Tentatively Named Acid Protease B.

Five ml of 0.6% milk casein solution were digested at pH 2.6 (—○—) with 1 ml of the enzyme solution containing 0.0288 mg of EP-Ic (left) or 0.0283 mg of EP-IIa (right) for ten minutes at various temperatures from 30°C to 70°C. And also the digestion of milk casein was carried out at pH 1.5 (---□---) with 0.288 mg of EP-Ic (left) or 0.283 mg of EP-IIa (right).

assayed at pH 1.0 were almost undetectable. Furthermore, effects of temperature on pro-

FIG. 10. The Effect of pH on Milk Casein Digestion by the Purified Preparation Tentatively Named Acid Protease A.

Five ml of 0.6% milk casein solution were digested with 1 ml of the enzyme solution containing 0.51 mg of EP-Ib at 30°C for ten minutes at various pH values from 1.0 to 5.6.

teolytic activities of the two preparations were shown in Fig. 9. The optimal temperature incubated at pH 2.6 for milk casein digestion by both preparations lies equally at about 55°C and that incubated at pH 1.5 decreased almost linearly with the rise of temperature above 30°C. Therefore these two preparations were obviously identical with each other and the enzyme was tentatively named acid protease B and this type of enzyme is considered to be similar to well-known fungal acid protease previously reported by many investigators¹⁻⁴). On the other hand, the effect of pH on the proteolytic activity of the EP-Ib was shown in Fig. 10. The optimal pH for milk casein digestion by this preparation lies at about pH 2.0, and its proteolytic activities assayed at pH 1.5 and 1.0 were about 125% and 115% of that assayed at pH 2.6 respectively. Furthermore the effect of tem-

FIG. 11. The Effect of Temperature on Milk Casein Digestion by Purified Preparation Tentatively Named Acid Protease A.

Five ml of 0.6% milk casein solution were digested at pH 2.6 (—○—) with 1 ml of the enzyme solution containing 0.51 mg (left) or 0.284 mg (right) of EP-Ib for ten minutes at various temperatures from 30°C to 85°C. And also the digestion of milk casein was carried out at pH 1.5 (—□—) with 0.51 mg of the same enzyme.

FIG. 12. Photographs of Electrophoresis of Acid Protease A and B Using the Tiselius type instrument.

The acid protease A (EP-Ib, left) or the acid protease B (EP-Ic, right) was individually dissolved in 0.02 M phosphate buffer pH 7.0 containing 0.1 M NaCl ($\mu=0.18$). Using the Tiselius type instrument HTB-2, Hitachi Ltd., the electrophoresis on these enzymes was individually carried out under conditions of 6 mA and 3°C.

perature on the proteolytic activity of this preparation was shown in Fig. 11. The optimal temperature incubated at pH 2.6 for milk casein digestion by this preparation lies at about 70°C and that incubated at pH 1.5 lies at about 60°C. Therefore this preparation is believed to be a new proteolytic enzyme not yet reported and tentatively named acid protease A.

As shown in Fig. 12, both acid protease A and B gave a single peak on the electro-

phoresis using the Tiselius type instrument.

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