APPLIED MICROBIOLOGY, Apr. 1973, p. 584-588
Vol. 25, No. 4


# Production and Purification of Acid Protease from the Thermophilic Fungus, *Penicillium duponti* K1014

HIKOTAKA HASHIMOTO, YUKIO KANEKO, TAKASHI IWAASA, AND TAMOTSU YOKOTSUKA

*Central Research Laboratories, Kikkoman Shoyu Co., Ltd., 399 Noda, Noda-shi, Chiba-ken, Japan*

Received for publication 14 November 1972

A thermophilic fungus, *Penicillium duponti* K1014, produced an acid protease in a submerged culture. Maximum enzyme production, in a 30-liter fermentor at a preselected optimum growth temperature of 50 C, occurred after 3 days. The productivity of the enzyme was associated with changes of viscosity and pH of culture broth during the growth. The acid protease was isolated from the culture filtrates and was purified ninefold by alcohol precipitation, column chromatography on *O*-(diethylaminoethyl)cellulose, batchwise treatment with *O*-(carboxymethyl)cellulose, and by gel filtration. The purified enzyme was homogeneous on disk electrophoresis and sedimentation analysis. The sedimentation constant $s_{20,w}$, calculated at a concentration of 0.64%, was 3.98*S*.

Thermophilic fungi have been isolated from self-heating materials and other sources by various investigators (4, 7, 8, 15). Cooney and Emerson (4) demonstrated that certain thermophilic fungi are associated with the process of microbial thermogenesis.

*Penicillium duponti* K1014, which was isolated from the compost in Japan, was found to produce a large amount of extracellular acid protease and other hydrolytic enzymes such as cellulases, amylases, and hemicellulases (10).

Proteolytic enzymes of thermophilic fungi have been purified and characterized by several investigators (3, 13, 16, 17, 21; P. S. Ong and G. M. Gaucher, Abstr. Int. Ferment. Symp. Japan, 4th, Kyoto, p. 47, 19 March 1972). Chapuis and Zuber (3) described a thermostable metallo-aminopeptidase (AP 1) from *Talaromyces duponti*; cobalt ions conferred the highest activity upon activation and also upon recombination from the apoenzyme. A thermophilic fungus, *Malbranche pulchella*, produced a thermostable alkaline protease (P. S. Ong and G. M. Gaucher, Abstr. Int. Ferment. Symp. Japan, 4th, Kyoto, p. 47, 19 March 1972). Arima et al. (2) reported the identification of a fungus capable of growing between 15 and 50 C, *Mucor pusillus* var. *Lindt*, which produced a potent milk-clotting acid protease. An acid protease from a strain of *Mucor miehei* has been isolated and partially characterized (16, 17).

We have already found (10) that a true thermophilic fungus, identified as *P. duponti* K1014, produces thermostable acid protease. This paper (presented at the Annual Meeting of the Agricultural Chemical Society of Japan, 1 April 1970, at Fukuoka) deals with the production in submerged cultivation, purification, and physical homogeneity of the acid protease from *P. duponti*.

## MATERIALS AND METHODS

**Organism.** *P. duponti* K1014 which was isolated from a compost in Tochigi-Prefecture, Japan, in July, 1968 was used throughout this work. The culture was deposited with the American Type Culture Collection and assigned the designated ATCC number: *P. duponti* K1014 (ATCC 20186). Stock cultures were maintained on wheat bran extract agar slants and transferred at intervals of 5 or 6 months.

**Cultivation.** Cultivation of *P. duponti* K1014 was conducted in a 30-liter, baffled, stainless-steel Waldhof-type fermentor (Marubishi Rika Co., Ltd., Tokyo, Japan) containing 20 liters of a medium composed of 3% rice bran, 2% $NH_4Cl$, and soybean oil as an antifoaming agent. Five grams of sporulation culture of wheat bran koji, after 3 days of growth at 45 C, was used for inoculation. The culture was maintained at 50 C, aerated at 20 liters/min, and stirred at 300 rpm.

**Purification of enzyme.** The purification of acid protease was performed by a combination of alcohol precipitation, column chromatography on *O*-(diethylaminoethyl)cellulose (DEAE-cellulose; Brown

Co.), batchwise treatment with *O*-(carboxymethyl)-cellulose (CM-cellulose; Brown Co.), and gel filtration on Sephadex G-200 (Pharmacia Co.). These procedures were carried out at 0 to 4 C, and the enzyme in the crude or purified state was stable for more than 1 year when stored at 4 C.

**Assay of protease activity.** Protease activity was assayed according to the modified Anson method (9). A reaction mixture containing 1 ml of enzyme solution and 5 ml of 0.6% Hammarsten milk casein in 0.05 M acetate buffer (pH 2.5) was incubated at 30 C for 10 min. The enzyme reaction was stopped by adding 5 ml of 0.11 M trichloroacetic acid containing 0.22 M acetic acid and 0.33 M sodium acetate. The reaction mixture was allowed to stand for 30 min at 30 C and then filtered. To 2 ml of the filtrate, 5 ml of 0.55 M sodium carbonate was added, followed by the addition of 1 ml of three-times-diluted phenol reagent. The blue color was measured at 660 nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbance equivalent to 1 $\mu$g of tyrosine per min, under the above conditions.

**Assay of ribonuclease activity.** Ribonuclease (RNase) activity was measured according to a modification of Ishikawa's method (12). The reaction mixture containing 1.0 ml of a 0.5% yeast ribonucleic acid solution, 2 ml of 0.2 M acetate buffer (pH 3.5), and 1 ml of enzyme solution was incubated at 30 C for 10 min. The enzyme solutions were properly diluted with 0.2 M acetate buffer (pH 3.5) before they were assayed. The reaction was stopped by adding 1 ml of 1 M perchloric acid containing 0.25% uranyl acetate. After standing for 30 min at 30 C, the mixture was filtered through filter paper (Toyo Roshi no. 5 C). The filtrate was diluted 20-fold with water, and the optical density was measured at 260 nm. One unit of activity was defined as the amount of enzyme producing a change of absorbance of 1.0 per 10 min, under the above conditions.

**Protein concentration.** Protein concentrations were determined by the method of Lowry et al. (14) by using bovine serum albumin as a standard.

**Viscosity.** Viscosity of culture broth was measured at 20 C by using a Viscotester VT-02 (Rion Co., Ltd., Tokyo, Japan).

**Carbohydrate concentration.** Soluble carbohydrates were determined by the phenol-sulfuric acid method (6) and calculated as glucose equivalents per milliliter of culture filtrate.

**Electrophoresis.** Disk gel electrophoresis was carried out with polyacrylamide gel (pH 9.4) by the method of Davis (5). About 100 $\mu$g of the protein was applied to the column, and electrophoresis was performed at 18 C for 90 min with a current of 3 mA per gel column. Protein bands were detected by standing the gel with a 1.0% solution of amido schwartz dissolved in 7% acetic acid.

**Sedimentation analysis.** Sedimentation analysis was performed on a Hitachi analytical ultracentrifuge model UCA-1A. The enzyme concentration used was 0.64% in 0.01 M acetate buffer (pH 4.0) containing sodium chloride to adjust the ionic strength to 0.1. The photographs were taken at a phase-plate angle of 70 after reaching the full speed (55,430 rpm) at 11.25 C.

## RESULTS

**Production of acid protease by P. duponti K1014 in submerged cultivation.** Figure 1 shows the effect of cultivation temperature on protease production. Maximal accumulation of protease occurred at 50 C which was the optimal temperature of growth (10). The lowest temperature, 40 C, showed the slowest rate of accumulation.

Figure 2 shows the pH, viscosity, and carbohydrate and acid protease activity changes of culture broth occurring during the growth of the fungus at 50 C. There is a fall in pH during incubation; the pH has to be maintained between 4.0 and 5.5 because of the instability of the enzyme above 5.5 and below 4.0. In this preparation, the pH after the appearance of the enzyme was maintained at 4.3 to 4.8 by adding ammonia water. Maximal levels of viscosity of culture broth were reached after 20 h and decreased during further incubation. Accumulation of protease occurred largely after the stationary phase of viscosity was reached. Microscopy observations revealed that the hyphae were markedly longer at maximal levels of viscosity than later in the fermentation. It was also found (data not shown) that good growth and high viscosity of broth are obtained with low aeration and agitation rates, but little acid protease was produced. The culture was harvested after 3 days of incubation.

**Purification of enzyme: extraction.** The harvested broth was adjusted to pH 4.5 and

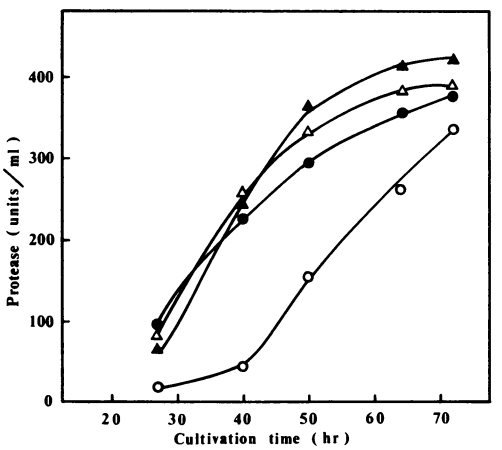


FIG. 1. *Effect of cultivation temperature on protease production by a thermophilic fungus, P. duponti K1014, grown in submerged cultivation. Symbols:* ○, *40 C;* ●, *45 C;* △, *47.5 C;* ▲, *50 C.*

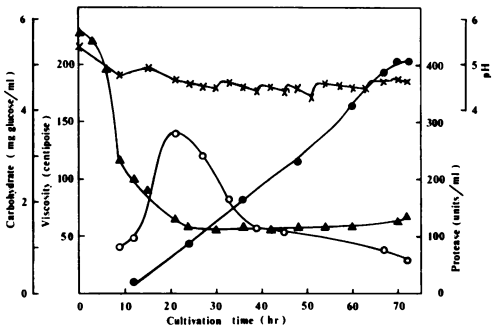


FIG. 2. *Acid protease activity, pH, viscosity, and carbohydrate changes during the culture of P. duponti K1014 in the 30-liter fermentor. Symbols: ●, acid protease activity; ×, pH; ○, viscosity; ▲, carbohydrate.*

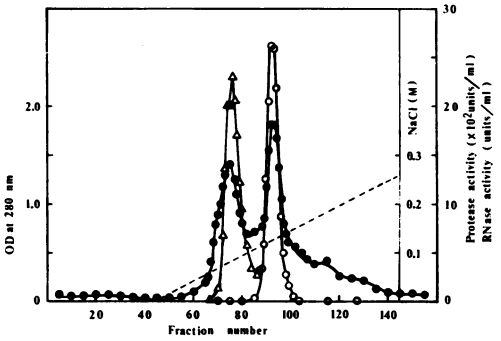


FIG. 3. *Chromatographic separation of acid protease on DEAE-cellulose. Symbols: ●, OD at 280 nm; ○, acid protease activity; △, RNase activity; dashed line, NaCl.*

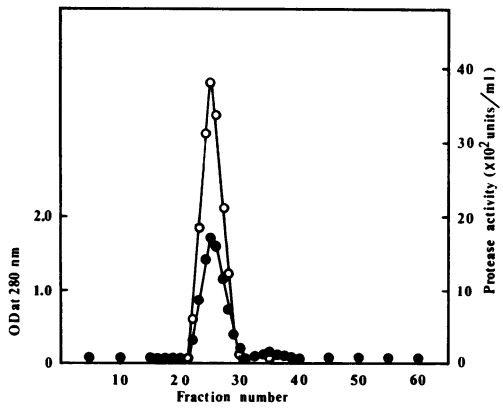


FIG. 4. *Gel filtration of acid protease on Sephadex G-200. Symbols: ●, OD at 280 nm; ○, acid protease activity.*

filtered with analytical grade of Celite. The filtrate was concentrated on a Centritherm Evaporator, type CT-1B (Alfa-Laval, Sweden), and then passed through sterilizing membrane filters (type HA, Millipore Corp.).

**Alcohol precipitation.** Three volumes of 95% ethanol (−20 C) were added dropwise with continuous stirring to the clarified broth. After standing overnight, the precipitate formed was collected by centrifugation at 8,000 × *g* for 10 min and dried in vacuo.

**Column chromatography on DEAE-cellulose.** Three grams of alcohol precipitation powder was dissolved in 100 ml of 0.01 M acetate buffer (pH 4.0), and undissolved materials were removed by centrifugation at 8,000 × *g* for 10 min. The clear solution was applied to a DEAE-cellulose column (3 by 57 cm) equilibrated with 0.01 M acetate buffer (pH 4.0). About 1.3% of the activity applied passed through the column. After washing the column with 2 liters of 0.01 M acetate buffer (pH 4.0), elution was performed by a linear gradient of sodium chloride concentrations from 0.0 up to 0.3 M in 0.01 M acetate buffer (pH 4.0). The flow rate was adjusted to 30 ml/h, and 10-ml fractions were collected. Figure 3 shows a typical elution pattern. The acid protease was detected in fractions 86 to 102; RNase activity was detected in fractions 65 to 83. Active fractions of protease free of RNase, numbers 90 to 96, were pooled and desalted by dialysis. The dialysate of pooled fractions was rechromatographed on a DEAE-cellulose column; a single peak was obtained.

**Batchwise treatment with CM-cellulose.** The rechromatographed enzyme preparation was dialyzed as described above, and then the dialysate (200 ml) was passed through a layer of CM-cellulose (3- by 5-cm-size layer) that had been equilibrated with 0.01 M acetate buffer (pH 4.0). The fraction that passed through the CM-cellulose was dialyzed against distilled water and lyophilized.

**Gel filtration on Sephadex G-200.** The lyophilized powder was dissolved in 3 ml of 0.01 M acetate buffer (pH 4.0), and applied to a Sephadex G-200 column (1.8 by 115 cm) equilibrated with the same buffer. Elution was performed with the same buffer, and 10-ml fractions were collected. Figure 4 shows a typical elution pattern. Fractions 22 to 28 were pooled, dialyzed, and lyophilized. The purified enzyme was free from RNase, cellulase, hemicellulase, and amylase activities. The results, showing the ninefold increase in specific activity achieved, of the overall purification procedures are summarized in Table 1.

**Disk electrophoresis.** The purified enzyme was homogeneous on disk electrophoresis (Fig. 5).

TABLE 1. *Purification of acid protease from P. duponti K1014*

| Procedure | Total activity (U) | Total protein (mg) | Specific activity (U per mg of protein) | Yield (%) |
|---|---|---|---|---|
| Crude extract | 1,096,400 | 6,821.5 | 160.7 | 100 |
| Alcohol precipitation | 996,725 | 2,783.5 | 358.1 | 90.9 |
| DEAE-cellulose (first) | 602,537 | 708.0 | 850.7 | 55.0 |
| DEAE-cellulose (second) | 437,253 | 447.5 | 976.7 | 39.9 |
| CM-cellulose | 314,822 | 262.8 | 1,197.8 | 31.9 |
| Sephadex G-200 | 279,842 | 193.0 | 1,450.2 | 25.5 |

**Sedimentation analysis.** The sedimentation patterns in Fig. 6 demonstrate the acid protease to be monodisperse. The sedimentation constant $s_{20,w}$, calculated at a concentration of 0.64%, was 3.98 *S*.

## DISCUSSION

It has been reported that certain fungi are capable of producing protease in submerged cultivation (1, 11, 18, 19, 20, 22). Sodek and Hofmann (19) reported that penicillopepsin, the acid proteinase of *Penicillium janthinellum*, could be produced at high levels in submerged culture in about 6 days at 25 C. Maximal production of the milk-clotting acid protease by a thermophilic fungus *M. pusillus* Lindt occurred after 5 days at 35 C. With an incubation temperature of 45 C, there was considerable enzyme production early in the incubation period followed by a drastic decline in activity (20).

Maximal production of acid protease by *P. duponti* K1014 in submerged cultivation, like solid cultivation (10), occurred after approximately 3 days at 50 C, which was the optimal temperature of growth. High yields of the enzyme were obtained when the pH of culture broth was maintained between 4.0 and 5.5. The fungus may be useful for industrial fermentations because of reduction in contamination that results from both the elevated temperature and acidic pH for growth.

Vigorous aeration and agitation were needed for high yields of acid protease. Under conditions of high aeration and agitation there were reductions in mycelial length and viscosity of the culture broth; these probably resulted in more effective transfer of oxygen to the mycelium. The procedure for production of acid protease by *P. duponti* K1014 that is presented here can be readily scaled up to commercial enzyme production.

Levels of acid protease and RNase attained in submerged culture were high, compared with levels attained on solid culture. However, levels of other hydrolases, such as cellulase, hemicellulase, and amylase, were poor in sub-

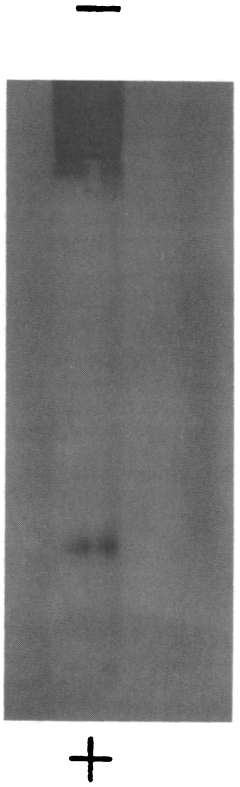

FIG. 5. *Disk electrophoresis of purified acid protease with polyacrylamide gel of pH 9.4. Top, negative; bottom, positive.*

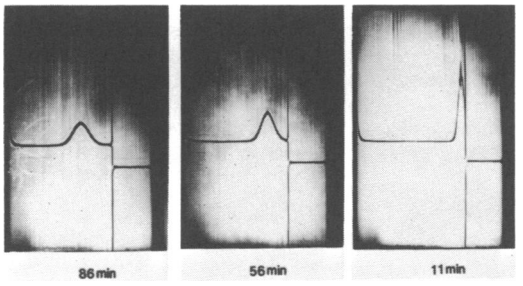


FIG. 6. *Ultracentrifuge patterns of purified acid protease.*

merged culture (H. Hashimoto et al., unpublished data). Purification of acid protease from *P. duponti* K1014 was performed with clarified broth of a submerged culture as the starting material. The results presented above with disk electrophoresis and sedimentation analysis indicate the physical homogeneity of the enzyme preparation.

## ACKNOWLEDGMENTS

We thank I. Koshiyama for the operation of the ultracentrifuge. Thanks are also due to T. Hayakawa for her technical assistance.

## LITERATURE CITED

1. Akiba, T., and T. Fukimbara. 1967. Studies on the production of acid protease by submerged culture of *Rhizopus*. I. Cultural conditions and some properties of the acid protease. J. Ferment. Technol. Jap. **45**:66–71.
2. Arima, K., S. Iwasaki, and G. Tamura. 1967. Milk clotting enzyme from microorganisms. I. Screening test and the identification of the potent fungus. Agr. Biol. Chem. (Tokyo) **31**:540–545.
3. Chapuis, R., H. Zuber. 1970. Thermophilic aminopeptidases: AP 1 from *Talaromyces duponti*, p. 552–555. *In* G. E. Perlman and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
4. Cooney, D. G., and R. Emerson. 1964. Thermophilic fungi. An account of their biology, activities, and classification. W. H. Freeman Publishing Co., San Francisco.
5. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**:404–427.
6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
7. Eggins, H. O. W., and D. G. Coursey. 1964. Thermophilic fungi associated with Nigerian oil palm produce. Nature (London) **203**:1083–1084.
8. Fergus, C. L. 1964. Thermophilic and thermotolerant molds and actinomycetes of mushroom compost during peak heating. Mycologia **56**:267–284.
9. Hagiwara, B. 1956. Proteolytic enzyme, p. 240. *In* S. Akabori (ed.), Kosokenkyuho, vol. 2. Asakura Shoten, Tokyo.
10. Hashimoto, H., T. Iwaasa, and T. Yokotsuka. 1972. Thermostable acid protease produced by *Penicillium duponti* K1014, a true thermophilic fungus newly isolated from compost. Appl. Microbiol. **24**:986–992.
11. Ichishima, E., and F. Yoshida. 1962. Studies on the proteolytic enzymes of Black *Aspergilli*. VIII. Effect of inorganic nitrogen compounds for the production of acid protease by *Kuro-Koji* mold group in submerged cultivation. Agr. Biol. Chem. (Tokyo) **26**:554–562.
12. Ishikawa, T., A. Tohe, I. Uno, and K. Hasunuma. 1969. Isolation and characterization of nuclease mutants in *Neurospora crassa*. Genetics **63**:75–92.
13. Iwasaki, S., T. Yasui, G. Tamura, and K. Arima. 1967. Milk clotting enzyme from microorganisms. III. The purification of the enzyme and its properties. Agr. Biol. Chem. (Tokyo) **31**:1421–1426.
14. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. Okafor, N. 1966. Thermophilic microorganisms from rotting maize. Nature (London) **210**:220–221.
16. Ottesen, M., and W. Rickert. 1970. The isolation and partial characterization of an acid protease produced by *Mucor miehei*. C. R. Trav. Lab. Carlsberg **37**:301–325.
17. Rickert, W. 1970. The degradation of the B-chain of oxidized insulin by *Mucor miehei* protease. C. R. Trav. Lab. Carlsberg **38**:1–17.
18. Shinmyo, A., M. Okazaki, and G. Terui. 1968. Kinetic studies on enzyme production by microbes. IV. Some physiological basis for kinetic studies on acid protease production by *Aspergillus niger*. J. Ferment. Technol. Jap. **46**:733–742.
19. Sodek, J., and T. Hofmann. 1970. Large-scale preparation and some properties of penicillopepsin, the acid proteinase of *Penicillium janthinellum*. Can. J. Biochem. **48**:425–431.
20. Somkuti, G. A., and F. J. Babel. 1967. Conditions influencing the synthesis of acid protease by *Mucor pusillus* Lindt. Appl. Microbiol. **15**:1309–1312.
21. Somkuti, G. A., and F. J. Babel. 1968. Purification and properties of *Mucor pusillus* acid protease. J. Bacteriol. **95**:1407–1414.
22. Tomoda, K., and H. Shimazono. 1964. Acid protease produced by *Trametes sanguinea*, a wood-destroying fungus. I. Purification and crystallization of the enzyme. Agr. Biol. Chem. (Tokyo) **28**:770–773.