Submerged Production, Purification, and Crystallization of Acid Carboxypeptidase from *Penicillium janthinellum* IFO-8070

SADAJI YOKOYAMA, AKIRA OOBAYASHI, OSAMU TANABE, AND EIJI ICHISHIMA

Central Research Laboratories, Takara Shuzo Co., Ltd., Setahashimoto, Otsu, Shiga-Prefecture 520-21, Japan; and Laboratory of Enzymology and Microbiology, Tokyo Nôkô University, Fuchu, Tokyo 183, Japan

Received for publication 12 August 1974

*Penicillium janthinellum* IFO-8070 produced an acid carboxypeptidase of molecular weight 51,000 in a liquid medium at 25 C. Maximum enzyme concentration was obtained within 3 to 6 days in a medium containing 2% wheat bran, 1% defatted soybean, and 1% KH₂PO₄; the initial pH was 2 to 4. When submerged aerobic conditions were used, a 51,000-molecular-weight acid carboxypeptidase was produced and no detectable amounts of 160,000-molecular-weight acid carboxypeptidase were produced. Acid carboxypeptidase with a molecular weight of 51,000 was purified 330-fold from koji culture to yield a crystalline protein which was demonstrated by disc electrophoresis to be homogeneous. The purification method included ammonium sulfate fractionation, Amberlite CG-50 chromatography, acetone fractionation, Amberlite CG-50 rechromatography, and concentration in a collodion bag. The specific activity of the enzyme was about three times more than that of the acid carboxypeptidase from *Aspergillus* *saitoi*.

We reported that several species of the genus *Penicillium* produce large quantities of acid carboxypeptidases in the surface koji culture (15). Some differences were observed among the acid carboxypeptidases obtained from these strains in the pH optima for hydrolysis of several peptides, effects of some inhibitors on enzyme activity, and molecular weight (15). In this communication, we describe the conditions for the production of the new type of acid carboxypeptidase from *Penicillium janthinellum* IFO-8070 in submerged aerobic culture. Furthermore, this report includes a method of purification and crystallization of the 51,000-molecular-weight acid carboxypeptidase.

**MATERIALS AND METHODS**

**Materials.** Benzylxycarbonyl-glutamyl-tyrosine was supplied by the Protein Research Foundation, Osaka. Hammarsten milk casein was obtained from E. Merck. Sephadex G-75 and Amberlite CG-50 were purchased from Pharmacia, Sweden, and Rohm and Haas, respectively. Collodion bags were obtained from Sartorius, Germany.

**Submerged culture.** The microorganism was *P. janthinellum* IFO-8070. This strain had the highest carboxypeptidase levels of the *Penicillia* tested in previous screening experiments (15).

A liquid medium prepared from 2 g of wheat bran and 1 g of defatted soybeans was used. The ingredients were mixed with 3 ml of tap water containing 0.03 ml of concentrated HCl in a 500-ml Sakaguchi flask and sterilized at 121 C for 1 h. After the addition of 97 ml of tap water with or without 0.2 g of KH₂PO₄ to the Sakaguchi flask, and adjusting the pH value to between 2 and 4 with 1 N HCl, the medium was resterilized at 121 C for 30 min. After inoculation with one platinum loop of spores which was maintained on a koji-agar slant, a culture was incubated at 25 C and shaken continuously at 120 (10 cm) strokes per min for 72 h or more.

**Enzyme preparation and purification.** For the preparation of acid carboxypeptidase, *P. janthinellum* IFO-8070 was cultured as described previously (15). Cultures were extracted, culture filtrates were pooled, and the pH was adjusted to 4 with 1 N HCl. The enzyme was precipitated with 608 g of solid (NH₄)₂SO₄ per liter of culture filtrate at 5 C. The precipitate was removed by filtration through Toyo no. 2 filter paper with the aid of a 0.5-cm deep Hyflo super-cell and stored in the cold (5 C). Curde enzyme preparation was dissolved in 0.05 M acetate buffer (pH 3.5) and dialyzed overnight at 5 C against 0.02 M acetate buffer (pH 3.5). Concentrated and dialyzed enzyme solution was applied to an Amberlite CG-50 column (see Fig. 3). The fractions of peak 1 (Fig. 3) were pooled and dialyzed overnight against 0.005 M acetate buffer (pH 4.0) at 5 C.

The enzyme preparation was brought to 65% saturation with chilled acetone at 6 C or below. The
precipitate was collected by centrifugation and was then dissolved in 0.05 M acetate buffer (pH 3.5). Insoluble materials were removed by centrifugation and discarded. The supernatant was rechromatographed on the Amberlite CG-50 column (see Fig. 4).

Active fractions were combined and then were concentrated by pervaporation in a collodion bag. Crystallization was allowed to occur as the concentration of enzyme increased in the collodion bag.

**Assays.** Acrylamide gel electrophoresis was performed at 20 C with the standard pore formulation of Davis (3) at pH 9.4.

Acid carboxypeptidase was determined by the increase in ninhydrin color after hydrolysis of benzoxycarbonyl-glutamyl-tyrosine at pH 3.7 and 30 C, according to the previous paper (15). One unit of acid carboxypeptidase activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min at pH 3.7 and 30 C.

Endopeptidase activity was determined according to Ichishima (6). One unit of acid proteinase activity was defined as the amount of enzyme which yields the color equivalent to 1 μmol of tyrosine per min at pH 3.0 and 30 C.

Protein concentrations were estimated by measuring the absorbance at 280 nm using a Hitachi model 101 spectrophotometer.

**RESULTS**

**Production of acid carboxypeptidase.** Variations in acid carboxypeptidase production by *P. janthinellum* IFO-8070 in submerged culture at 25 C are shown in Fig. 1 and 2 and Table 1. A medium composed of 2% wheat bran and 1% defatted soybean supported substantial quantities of growth and acid carboxypeptidase production (1.235 U/ml of filtrate, Table 1). As the concentration of defatted soybean was increased, the terminal pH usually increased; as the level of wheat bran was increased, the terminal pH decreased.

Increases in the terminal pH of broth cultures could be retarded by the addition of 0.2% KH₂PO₄ (Table 1). Addition of 1% KH₂PO₄ was more effective than other phosphate levels on enzyme yield (Fig. 1). The maximal enzyme concentration per milliliter was obtained with a medium containing 2% wheat bran, 1% defatted soybean, and 1% KH₂PO₄ (Fig. 1).

![Fig. 1. Effect of the concentration of KH₂PO₄ on the production of acid carboxypeptidase at 25 C. The basal medium was composed of 2% wheat bran and 1% defatted soybean; the initial pH was pH 5.5. Symbols: •, 1.0% KH₂PO₄; ○, 0.5%; ●, 0.2%; ○, none.](image1)

![Fig. 2. Effect of the initial pH on the production of acid carboxypeptidase. The medium was composed of 2% wheat bran and 1% defatted soybean. Symbols: •, initial pH 2.0; ×, 3.0; □, 4.0; ○, 5.0; Δ, 6.0.](image2)
When the initial pH was adjusted to 2.0 and 3.0, maximal enzyme levels were obtained at about 5 or 6 days (Fig. 2). When the initial pH was adjusted to 4.0, the maximal enzyme level was obtained at about 4 days; this maximum was lower than the maxima observed when initial pH values of 2.0 or 3.0 were used. The terminal pH could also be controlled by adjusting the initial pH to 2.0 or 3.0.

**Purification and crystallization of acid carboxypeptidase.** Gel filtration on Sephadex G-75, using 0.01 M acetate buffer of pH 4.0, did not resolve carboxypeptidase of high (160,000) and low (51,000) molecular weights (15). Therefore, we resorted to other means of enzyme purification. The result of the purification and crystallization procedure that was finally adopted is summarized in Table 2. Amberlite CG-50 chromatography was used for the initial step in the purification of the acid carboxypeptidase. The concentrated solution from (NH₄)₂SO₄ precipitate was dialyzed against 0.02 M acetate buffer (pH 3.5) and then subjected to ion-exchange chromatography on Amberlite CG-50. As shown in Fig. 3, the acid carboxypeptidase activity was eluted as two components. The specific activity for the fraction of peak 1 (major component of acid carboxypeptidase) was 2.00 U, and the second fraction (peak 2) was 0.04 U.

The active fraction (peak 1) eluted from Amberlite CG-50 was precipitated with chilled acetone at 6 C or below after dialysis against 0.005 M acetate buffer, pH 4.0. As shown in Table 2, the specific activity of the precipitate was 7.00 U.

Further purification of the acetone precipitate was attempted using Amberlite CG-50. The final step of the rechromatography is shown in Fig. 4. The specific activity of the active fraction was 11.43 U (Table 2).

The highly-purified fractions eluted from

---

**Table 1. Effect of concentration and composition of defatted soybean, wheat bran, and KH₂PO₄ in a submerged medium on acid carboxypeptidase production by P. janthinellum IFO-8070**

| Expt | Conc of medium (%) | Final pH | Activity (U/ml of filtrate) |
|------|--------------------|----------|----------------------------|
|      | Wheat bran | Defatted soybean | KH₂PO₄ (%) |                      |
| 1    | 0         | 3         | 0   | 7.99 | 0.014 |
|      | 1         | 2         | 0   | 7.85 | 0.031 |
|      | 2         | 1         | 0   | 6.96 | 0.670 |
|      | 3         | 0         | 0   | 5.60 | 0.436 |
| 2    | 0         | 3         | 0.2 | 7.05 | 0.259 |
|      | 1         | 2         | 0.2 | 6.96 | 0.775 |
|      | 2         | 1         | 0.2 | 6.20 | 1.255 |
|      | 3         | 0         | 0.2 | 5.72 | 0.715 |
| 3    | 0.67      | 0.33      | 0.2 | 6.09 | 0.548 |
|      | 1.33      | 0.67      | 0.2 | 6.36 | 0.925 |
|      | 2.00      | 1.00      | 0.2 | 6.36 | 1.025 |
|      | 2.67      | 1.33      | 0.2 | 6.48 | 0.805 |
|      | 3.33      | 1.67      | 0.2 | 6.57 | 0.756 |
|      | 4.67      | 2.33      | 0.2 | 6.08 | 0.446 |
|      | 6.70      | 3.30      | 0.2 | 5.46 | 0.266 |

*The pH was adjusted to 5.5. Incubation times at 25 C were: 110 h (experiment 1), 72 h (experiment 2), and 79 h (experiment 3).
Amberlite CG-50 were concentrated in a colloid bag. Crystallization was allowed to take place as the concentration of the enzyme increased in the colloid bag for about 1 week. It could be greatly hastened, however, by seeding the solution with enzyme crystals obtained earlier. The enzyme crystals (Fig. 5) were insoluble in 0.05 M acetate buffer (pH 3.7), so they were washed five times with 50 ml of 0.05 M acetate buffer (pH 3.7). The crystalline enzyme suspension in the 0.05 M acetate buffer (pH 3.7) was stable at least 1 year at 5°C. The specific activity of the crystal of acid carboxypeptidase from \textit{P. janthinellum} IFO-8070 was 16.50 U (Table 2).

**Purity of crystalline acid carboxypeptidase.** A single amido black stained band was observed when the crystalline acid carboxypeptidase was run on analytical gel electrophoresis at pH 9.4 (Fig. 6). This observation shows that the crystalline acid carboxypeptidase was electrophoretically homogeneous.

**DISCUSSION**

Previously, we reported the effects of culture conditions on the production of acid carboxypeptidases from molds of the \textit{Aspergillus niger} group (10). In the submerged culture of \textit{A. saitoi}, maximal yields of acid carboxypeptidase per gram of raw material were obtained in a medium containing 0.6% wheat bran and 0.9% defatted soybean, and the maximal enzyme concentration was obtained in a medium containing 2% wheat bran and 3% defatted soybean (10). However, the maximal yields of acid carboxypeptidase per gram of raw materials using \textit{P. janthinellum} were obtained in a medium containing 0.67% wheat bran plus 0.33% defatted soybean. The maximal enzyme concentration was obtained with a medium containing 2% wheat bran, 1% defatted soybean, and 1% KH$_2$PO$_4$. When the initial pH of the medium was low (pH 2 to 3), the pH of the culture medium did not rise sufficiently high to cause enzyme inactivation (Fig. 2). When submerged cultivation was used, the main component was the low-molecular-weight acid carboxypeptidase and no detectable amount of high-molecular-weight acid carboxypeptidase was produced.

The maximal yields of acid carboxypeptidase in submerged culture were obtained as 54.8 U/g
of raw materials, whereas the maximal yields of the enzyme in koji culture (15) were 10.7 U/g of raw materials. It has been recognized that the submerged culture is preferable to koji culture (15) at the point of controlling culture conditions and scale up of enzyme production. For the basal experiment of large-scale acid carboxypeptidase production, some of the culture conditions of mold acid carboxypeptidase were studied.

We previously (15) reported that, in koji culture, *P. janthinellum* IFO-8070 produced a major component of acid carboxypeptidase with a molecular weight of 51,000 and a minor component of acid carboxypeptidase with a molecular weight of 160,000 (15). The major active component of acid carboxypeptidase with a molecular weight of 51,000 was absorbed on an ion-exchange resin and eluted from the column when the pH and sodium acetate concentration were increased. The minor active component of acid carboxypeptidase, with a molecular weight of 160,000, was not absorbed on the resin. The purification gave a 330-fold purification of acid carboxypeptidase from culture filtrate to a final product with a direct yield of 20%. The specific activity for the crystalline acid carboxypeptidase from *P. janthinellum* IFO-8070 was 16.50 U for absorbance at 280 nm. The specific activity is about three times more than that of the enzyme from *Aspergillus* *saitoi* R-3813 (7).

Carboxypeptidases have been obtained from pancreas (1, 4), French bean (2, 14), barley (13), citrus fruit (16), yeast (5), *Aspergillus* spp. (E. Ichishima, Abstr. Annu. Meet. Agr. Chem. Soc. Japan, p. 55, 1969; T. Arai and E. Ichishima, J. Biochem., in press; 7, 8, 9, 10), and *Penicillium* spp. (11, 12, 15). Although almost all of the above carboxypeptidases have been isolated, only carboxypeptidase A (EC 3.4.2.1.) from bovine pancreas has been obtained in the crystalline form (1). Acid carboxypeptidase from *P. janthinellum* IFO-8070 was purified and crystallized with a direct yield of 20%. Crystalline acid carboxypeptidase from *P. janthinellum* IFO-8070 was insoluble in 0.05 M acetate buffer between pH 3.0 and 4.5. However, essentially all of the crystals were dissolved in 0.05 M acetate buffer at pH 3.7 when 0.6 M NaCl was present. A detailed account of properties of the crystalline acid carboxypeptidase will appear elsewhere.

**LITERATURE CITED**

1. Anson, M. L. 1935. Crystalline carboxypeptidase. Science 81:467.
2. Carey, W. F., and J. R. E. Wells. 1972. Phaseolisin, a plant carboxypeptidase of unique specificity. J. Biol. Chem. 247:5573-5579.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121:404-427.
4. Folk, J. E., K. A. Piez, W. R. Carroll, and J. A. Glandner. 1960. Carboxypeptidase B. IV. Purification and characterization of the porcine enzyme. J. Biol. Chem. 235:2272-2277.
5. Hayashi, R., S. Moor, and W. H. Stein. 1973. Carboxypeptidase from yeast, large scale preparation and the application to COOH-terminal analysis of peptides and protein. J. Biol. Chem. 248:2296-2302.
6. Ichishima, E. 1970. Purification and mode of assay for acid proteinase of *Aspergillus* *saitoi*, p. 387-406. In G. E. Perlmann and L. Lorant (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
7. Ichishima, E. 1972. Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*. Biochim. Biophys. Acta 255:274-288.
8. Ichishima, E., and T. Arai. 1973. Specificity and mode of action of acid carboxypeptidase from *Aspergillus* *saitoi*. Biochim. Biophys. Acta 293:444-450.
9. Ichishima, E., S. Sonoki, Y. Torii, and S. Yokoyama. 1972. Comparative studies on enzymatic activities of acid carboxypeptidase of molds of the genus *Aspergillus*. J. Biochem. 72:1045-1048.
10. Ichishima, E., A. Yamane, T. Nitta, M. Kinoshita, S. Ninkuni, T. Oka, and S. Yokoyama. 1973. Production of a new type of acid carboxypeptidase of molds of the *Aspergillus niger* group. Appl. Microbiol. 26:327-331.
11. Jones, S. R., and T. Hofmann. 1972. Penicilliocarbox-
ypeptidase-S, a nonspecific SH-dependent exopeptidase. Can. J. Biochem. 50:1297-1310.
12. Shaw, R. 1964. Proteolytic enzyme of Penicillium janthinellum. II. Properties of peptidase B. Biochem. Biophys. Acta 92:558-567.
13. Visuri, K., J. Mikora, and T.-M. Enari. 1969. Isolation and partial characterization of a carboxypeptidase from barley. Eur. J. Biochem. 7:193-199.
14. Wells, J. R. E. 1965. Purification and properties of a proteolytic enzyme from French Beans. Biochem. J. 97:229-235.
15. Yokoyama, S., A. Oobayashi, O. Tanabe, S. Sugawara, E. Araki, and E. Ichishima. 1974. Production and some properties of a new type of acid carboxypeptidase of Penicillium molds. Appl. Microbiol. 27:953-960.
16. Zuber, H. 1964. Purification and properties of a new carboxypeptidase from citrus fruit. Nature (London) 201:613.