Large Scale Production and Crystallization of Acid Carboxypeptidase from Submerged Culture of *Penicillium janthinellum*, and Stability of the Crystalline Enzyme

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Acid carboxypeptidase of *Penicillium janthinellum* IFO-8070 was produced effectively in submerged culture on a medium of 4~5% rice bran. The enzyme production was enlarged to volume cultivation of 150-liters in a 200-liters jar fermentor, and the yield of acid carboxypeptidase per milliliter filtrate reached to the maximum 3 days after inoculation.

Acid carboxypeptidase of low molecular weight (M.W.=51,000) produced in the liquid culture broth was purified and crystallized in a large scale. Purification steps include Amberlite CG-50 treatment, ammonium sulfate precipitation, dialysis using "Diaflow," activated charcoal treatment, and condensation using collodion-bag, or condensation and dialysis using "Diaflow."

The crystals of the acid carboxypeptidase suspended in 50 mm acetate buffer (pH 3.7) were completely stable for six months at 5°C. On the other hand, at low enzyme concentration (0.01 U/ml) in 50 mm acetate buffer (pH 3.7), crystallized enzyme was somewhat labile, whereas, this inactivation was completely depressed by covering enzyme solution with toluene.

Carboxypeptidases have been isolated and purified from many origins such as *Penicillium* spp.,^{1~6)} *Aspergillus* spp.,^{7~14)} bovine pancreas,^{15,16)} citrus fruit and leaves^{17,18)} barley,^{19,20)} French beans,^{21,22)} yeast,²³⁾ and cotton seeds.²⁴⁾

Aspergillus acid carboxypeptidase^{7~13)} has been purified and characterized by Ichishima and co-workers, which had an acidic pH optimum of 3.1 for the hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine (Z-Glu-Tyr). *A. oryzae* and *A. oryzae* var. *magnasporus* produced acid carboxypeptidases which had the same hydrolytic activities toward synthetic substrates but the different pH optima, molecular weights, and stabilities towards the inhibitors such as *p*-chloromercuribenzoate (PCMB), L-1-tosylamide-2-phenylethyl chloromethylketone (TPCK), monoiodoacetic acid and diisopropylfluorophosphate (DFP).⁹⁾

We previously reported the production and some properties of acid carboxypeptidase of

Penicillium molds in the koji culture.^{1,2)} A low molecular weight (M.W.=51,000) acid carboxypeptidase of *P. janthinellum* IFO-8070 produced in wheat bran koji culture was purified 330-fold from koji extract, and crystallized as a disc-electrophoretically homogeneous protein with the recovery of 20%.³⁾ Furthermore, we investigated the submerged culture conditions on a small scale (100 ml) and medium composed of wheat bran, defatted soybean, and KH_2PO_4 .³⁾

In this report, we describe the large scale production of acid carboxypeptidase by *P. janthinellum* IFO-8070 on the liquid medium of 4% rice bran. Furthermore, we report a large scale purification and crystallization method of the low molecular weight enzyme (M.W.=51,000) produced in this submerged culture, and stabilities of the crystalline enzyme.

MATERIALS AND METHODS

Materials. Benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z-Glu-Tyr) was purchased from the Protein Research Foundation, Osaka. Amberlite CG-50 (Type-1) was purchased from Rohm & Haas (Philadelphia, USA). Other chemicals were obtained commercially.

Organism. Strain used in this experiment is *Penicillium janthinellum* IFO-8070.¹⁻³⁾

Submerged culture on a small scale. A liquid medium of 100 ml prepared from rice bran and wheat bran was used according to the previous paper.³⁾

A large scale cultivation. A large scale cultivation was carried out using a 30-liters jar fermentor equipment (Marubishi Co., Tokyo). 800 g of rice bran was added to 19.2 liters of tap water and the mixture was adjusted to pH 4.0 with conc. HCl. The medium was sterilized at 121°C for 20 min. After inoculation with two slants of spores, a culture was incubated at 25°C.

Further large scale cultivation was performed using a 200-liters jar fermentor unit (Marubishi Co., Tokyo). 6 kg of rice bran was added to 144-liters of well water, and the mixture was adjusted to pH 4.0 with conc. HCl. The medium was sterilized at 121°C for 20 min, and inoculated with 1.5 liters of seed culture. Cultivation was performed at 25°C aerating 75 liters/min, and agitating as follows: 0~24 hr; 250 rpm, 24 hr~; 400 rpm. Seed culture was cultivated in 2-liters volume of shaking Erlenmeyer flask containing 500 ml of 4% rice bran (initial pH=4.0) inoculated with one platinum loop of spores. Cultivation was done for 3 days on a rotary shaker (126 rpm) at 27°C.

Large scale crystallization of acid carboxypeptidase. An outline of the purification step is shown in Table II. Jar fermentation using a 200-liters volume jar fermentor unit was employed twice. The culture broth was filtered through a filter press equipment (Noritake Tekkosho Co., Osaka) at the pressure of about 0.5~1.0 kg/cm². The culture filtrate, 251 liters (1.40 U/ml), was adjusted to pH 3.5 with conc. HCl, and then was applied to the Amberlite CG-50 (Type-1) column (8.2×56 cm) equilibrated with 70 liters of 10 mM acetate buffer (pH 3.5). Top of the column was covered by toluene (1 cm depth layer) for preventing from contamination. The column was washed with 70 liters of the starting buffer at a flow rate of 3 liters per hr. The acid carboxypeptidase was then eluted with 0.5 M acetate buffer (pH 5.2). Eluate was fractionated in 20 ml using a fraction collector. Active fractions (Fraction No. 126~500) were combined (6,910 ml), and subjected to salting out by the addition of 4.2 kg of solid ammonium sulfate at 5°C.

The precipitate was separated by centrifugation (5000×g, 20 min) and dissolved in 700 ml of 50 mM acetate buffer (pH 3.7). Enzyme solution was then centrifuged (5000×g, 20 min) and insoluble substances were discarded. Supernatant was then dialyzed against 50 mM acetate buffer (pH 3.7) using a "Diaflow" equipment with a two-liters volume of reservoir to remove ammonium sulfate and low molecular weight substances. Furthermore, 14 g of activated charcoal was added to the dialyzate and the mixture was adjusted to pH 3.7 with 1 N HCl, and kept to stand for 10 min at room temperature. The enzyme solution was then filtered through Toyo Roshi No. 5C filter paper to remove charcoal.

50 ml of the enzyme solution from step 5 in the Table II was concentrated in a collodion-bag in the cold (5°C). Enzyme crystals (Fig. 3a) appeared within a week. Crystallization could be greatly hastened, however, by seeding the solution with enzyme crystals obtained earlier. Crystals were washed with 50 mM acetate buffer (pH 3.7) or 5 mM acetate buffer (pH 4.0) as the enzyme crystals were almost insoluble at pH values between 3.5 and 4.5 in these salt concentration.

In another case, 580 ml of the enzyme solution from step 5 was concentrated to about 40 ml and dialyzed in a "Diaflow" equipment against 5 mM acetate buffer (pH 4.0) using a 2-liters volume of reservoir. Crystallization (Fig. 3b) was allowed to take place as the dialysis was continued for 5 hr or more. Enzyme crystals were washed with 5 mM acetate buffer (pH 4.0) and stored at 5°C.

Stabilities of crystalline acid carboxypeptidase. Stabilities of the enzyme at several purification steps were investigated. Enzyme solution was diluted with 50 mM acetate buffer (pH 3.7) to 0.01±0.001 U/ml, and 20 ml each was stored in an Erlenmeyer flask (100 ml volume) or test tube (16.5×165 mm) at 25°C, 5°C, or 0°C. 0.5 ml aliquot was pipetted at indicated time, and used directly to the reaction with 0.5 ml portion of Z-Glu-Tyr (pH 3.7).

Acid carboxypeptidase assay. The enzyme was assayed routinely with Z-Glu-Tyr in 50 mM sodium acetate buffer (pH 3.7).²⁾ One unit of Z-Glu-Tyr hydrolase activity was defined as the amount of enzyme required to liberate 1 μmole of tyrosine per min at pH 3.7 and 30°C.

Protein determination. Protein concentration was estimated from the absorbance at 280 nm ($A_{1\text{cm}}^{1\%} = 25.4$) using a Hitachi 101 spectrophotometer.

Ultrafiltration. For the concentration or dialysis of enzyme solution, collodion-bags (Sartorius, Germany) or ultrafiltration unit were used. A "Diaflow" ultrafiltration assembly equipped with a HFA-180 membrane (Abcor, USA) was used for ultrafiltration.

Disc electrophoresis. Acrylamide gel electrophoresis was performed at 25°C with the standard pore formulation of Davis²⁵) at pH 9.4.

RESULTS

Submerged production of acid carboxypeptidase

Variation in acid carboxypeptidase production by *P. janthinellum* IFO-8070 in submerged culture is shown in Table I. A medium composed of 3% rice bran supported substantial growth and acid carboxypeptidase production (Table I, Expt. 1, 2, and 3). Maximum yield of acid carboxypeptidase per milliliter filtrate (1.31 U/ml) was obtained with a medium containing 5% rice bran, and this value was rather higher than that (1.235 U/ml) obtained in the previous experiment³) on a wheat bran: defatted soybean medium (Table I).

In a 30-liters jar fermentor with 4% of rice bran, substantial amount of the enzyme (1.00 U/ml) was obtained 3 days after inoculation, and no inactivation of the enzyme was observed when the agitation was changed from 250 rpm to 400 rpm after 48 hr of inoculation (Fig. 1).

Enzyme production with 4% rice bran could be scaled up to a 200-liters jar fermentor (Fig. 2). The maximum enzyme production (1.30 U/ml of filtrate) was reached after 72 hr of inoculation at 25°C.

In the production of acid carboxypeptidase using the rice bran medium, presterilization could be omitted in the large scale cultivation using 30-, and 200-liters jar fermentors by controlling aeration and agitation (Fig. 1, Fig. 2).

TABLE I. EFFECT OF CONCENTRATION AND COMPOSITION OF RICE BRAN AND WHEAT BRAN ON ACID CARBOXYPEPTIDASE PRODUCTION BY *P. janthinellum* IFO-8070

Expt. ^{a)}	Cultivation times	Concentration of nutrient (%)		Final pH	Activity U/ml
		Rice bran	Wheat bran		
1	72 hr	3.0	0	4.26	0.773
		2.5	0.5	4.31	0.895
		2.0	1.0	4.44	0.732
		1.5	1.5	4.59	0.893
		1.0	2.0	4.58	0.564
		0.5	2.5	5.21	0.845
		0	3.0	5.03	0.466
2	96 hr	3.0	0	4.73	0.974
		2.5	0.5	4.73	1.000
		2.0	1.0	4.80	0.964
		1.5	1.5	4.89	0.889
		1.0	2.0	5.00	0.807
		0.5	2.5	5.62	0.572
		0	3.0	5.55	0.663
3	120 hr	3.0	0	5.33	1.067
		2.5	0.5	5.50	1.140
		2.0	1.0	5.51	1.023
		1.5	1.5	5.42	1.098
		1.0	2.0	5.42	0.923
		0.5	2.5	6.21	0.675
		0	3.0	5.72	0.599
4	96 hr	1.0		4.67	0.109
		2.0		4.61	0.605
		3.0		4.53	0.974
		4.0		4.59	1.284
		5.0		4.50	1.310
		7.0		4.12	1.215
		10.0		4.13	0.809
Reference 3)		Wheat bran, 2.0%; defatted soybean, 1.0%; KH ₂ PO ₄ , 0.2%; initial pH=5.5		6.20	1.235

^{a)} The initial pH was adjusted to 4.0.

FIG. 1. Enzyme Production by *P. janthinellum* IFO-8070 in a 30-liters Jar Fermentor on the 4% Rice Bran Medium at 25°C.

	Expt.	Cultivation hr		
Aeration (liters/min)	{ 1	0~48	48~71	71~
	{ 2	5	5	5
		20	20	28
Agitation (rpm)	{ 1	250	400	400
	{ 2	250	400	450

When the initial pH was adjusted to 4.0, the cultural pH was almost constant throughout the cultivation, and it was very preferable

FIG. 2. Enzyme Production by *P. janthinellum* IFO-8070 in a 200-liters Jar Fermentor on the 4% Rice Bran Medium at 25°C.

TABLE II. PURIFICATION OF ACID CARBOXYPEPTIDASE FROM *P. janthinellum* IFO-8070

Step	Volume or weight	Specific activity (U/A ₂₈₀)	Recovery (%)
1. Culture filtrate	251 liters	0.08	100
2. Amberlite CG-50 (Type 1) treatment	6910 ml	4.40	90.6
3. Precipitation by ammonium sulfate	700 ml	5.86	72.5
4. Dialysis with "Diaflow"	700 ml	9.16	64.3
5. Activated charcoal treatment	630 ml	11.9	58.0
6A. Condensation with collodion-bag	160 mg ^{a)}	16.5	1.76
6B. Condensation and dialysis with "Diaflow"	1000 mg ^{a)}	16.5	10.8

^{a)} Calculated from specific extinction ($A_{1\text{cm}}^{1\%} = 25.4$).

(a)

(b)

FIG. 3. Crystals of Acid Carboxypeptidase with Molecular Weight of 51000 from *P. janthinellum* IFO-8070 ($\times 150$). Details are described in the text. a, collodion-bag method; b, membrane method.

at the point of stability of the enzyme produced in the medium, and of preventing the liquid culture from contamination. There are almost no foaming throughout the cultivation, and antifoaming reagent was unnecessary. Since this fungus forms filamentous mycelia when grown in this medium under these aeration and agitation conditions described in the text, filtration of culture broth can be efficiently accomplished, in a laboratory scale, by vacuum filtration through Toyo Roshi No. 2 filter paper. Use of a filter press equipment is desirable when hundreds of liters are to be treated.

Large scale preparation of crystalline enzyme

Large scale purification and crystallization include Amberlite CG-50 treatment, ammonium sulfate precipitation, dialysis using "Diaflow," activated charcoal treatment, and condensation using a collodion-bag or condensation and dialysis using "Diaflow" (Table II). Crystallization with a collodion-bag or "Diaflow" produced the same type of needle-shaped crystals (Fig. 3a and 3b), and both crystalline preparations had the same specific activity of 16.5 U (Table II). These crystalline acid carboxypeptidases were disc-electrophoretically homogeneous at pH 9.4 same as the crystals³⁾ obtained from koji culture.

Stabilities of the acid carboxypeptidase

The crystals of acid carboxypeptidase suspended in 50 mM acetate buffer (pH 3.7) were completely stable in six months at 5°C (Table III). Furthermore, no loss of enzyme activity was observed when the crystallized enzyme solution of 0.5 U/ml was stored at 25°C for 96 hr (Table III). When the enzyme concentration was 0.1 U/ml, the crystallized enzyme was considerably stable on the storage at 25°C (Table III). The enzyme was, however, somewhat labile when diluted with 50 mM acetate buffer (pH 3.7) to such a low concentration as 0.01 ± 0.001 U/ml, and in this case, more than 80% of the enzyme activity was lost after incubation at 25°C for 48 hr (Table III). On the storage at low temperature (5°C or 0°C), the crystallized enzyme was a little more stable than that at 25°C (Table III).

Inactivation of enzyme solution in low concentration (0.01 ± 0.001 U/ml) could be completely retarded by covering surface of the solution with toluene, and in this case, the enzyme solution showed no loss of the activity within 8 days at 25°C (Table IV). Dilution of the enzyme with boiled and vacuum-packed buffer solution was rather preferable to prevent enzyme inactivation (Table IV).

Addition of 10 mM L-ascorbic acid as an

TABLE III. STABILITIES OF CRUDE AND CRYSTALLINE PREPARATIONS OF ACID CARBOXYPEPTIDASE AT pH 3.7

Enzyme preparation from	Stored at (°C)	Residual activity (%)							
		0 hr	5 hr	10 hr	24 hr	48 hr	72 hr	96 hr	6 month
Culture filtrate (Step 1) ^{a)}	25	100	98	105	91	94			
Amberlite CG-50 (Step 2) ^{a)}	25	100	99	94	90	77			
Dialyzate (Step 4) ^{a)}	25	100	90	87	93	77			
Crystals (Step 6B) ^{a)}	25	100	63	47~67	15~38	0~18			
Crystals (Step 6B) ^{a)}	5	100	—	87	60	37			
Crystals (Step 6B) ^{a)}	0	100	—	76	75	50			
Crystals (Step 6B), 0.1U/ml ^{b)}	25	100	—	—	71	66	57	55	
Crystals (Step 6B), 0.5U/ml ^{b)}	25	100	—	—	104	111	106	103	
Crystal suspension (Step 6A) ^{c)}	5	100	—	—	—	—	—	—	100

^{a)} Enzyme solution was diluted with 50 mM acetate buffer (pH 3.7) to about 0.01 ± 0.001 U/ml, and stored at indicated temperature.

^{b)} Enzyme solution was diluted with 50 mM acetate buffer (pH 3.7) to 0.1 U/ml and 0.5 U/ml, and stored at 25°C.

^{c)} Enzyme crystals were suspended in 50 mM acetate buffer, and stored at 5°C.

TABLE IV. EFFECT OF TOLUENE AND L-ASCORBIC ACID ON THE STABILITY OF *P. janthinellum* IFO-8070 ACID CARBOXYPEPTIDASE

Expt. ^{a)}	Deaeration ^{b)}	Stored in	Condition		Residual activity (%)				
			Covered with toluene	10 mM L-ascorbate	0	24	(hr) 48	72	(days) 8
1	no	Erlenmeyer flask (100 ml volume)	no	no	100	49	40	23	0
		Test tube (16.5 × 165 m/m)	yes	no	100	91	98	93	98
2	no	Erlenmeyer flask (100 ml volume)	no	yes	100	37	28	38	
		Test tube (16.5 × 165 m/m)	yes	yes	100	92	94	89	
3	yes	Erlenmeyer flask (100 ml volume)	no	no	100	55	50	51	
		Test tube (16.5 × 165 m/m)	yes	no	100	94	85	90	

^{a)} Enzyme volume is 20 ml.^{b)} 50 mM acetate buffer (pH 3.7) to be used to dilute enzyme solution was boiled for 30 min and aspirated for 1 hr before use.

antioxidant had no appreciable protecting effect on the enzyme activity (Table IV). 10 mM SH-reagents such as 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) had no retarding effect on the inactivation. 0.4 M KCl and 1 M sucrose also showed no preventing effect on the enzyme inactivation at low enzyme concentration (0.01 ± 0.001 U/ml).

DISCUSSION

We now observed high acid carboxypeptidase production on the rice bran medium, and the maximum enzyme production (1.31 U/ml) was observed 96 hr after inoculation. Production of acid carboxypeptidase could be enlarged to 30-liters and 200-liters volume jar fermentor unit.

The rice bran medium has many advantages in the industrial scale, such as low cost of material, readily for sterilization, unnecessary of antifoaming reagent and pH control during the enzyme production, and readily for filtration of mycelia. Acid carboxypeptidase produced by *P. janthinellum* IFO-8070 was almost completely adsorbed onto the Amerlite CG-50 column, and easily eluted with 0.5 M acetate buffer (pH 5.2). Through this procedure, the culture filtrate was condensed to one 40th and was purified 55-times in specific activity (Table II).

We previously obtained 15 mg of crystalline acid carboxypeptidase of low molecular weight (M.W. = 51,000) from 1.5 liters koji extract.³⁾ Purification method included ammonium sulfate precipitation, Amberlite CG-50 chromatography, dialysis, acetone fractionation, Amberlite CG-50 rechromatography, and condensation in a collodion-bag.³⁾ However, column chromatography was laborous and was not proper for large scale enzyme preparation. We investigated a simple purification method from the submerged culture filtrate.

Purification procedure summarized in Table II is very suitable for the large scale preparation of crystalline enzyme. Successful crystalline preparation (Fig. 3a and 3b) was made. Condensation by collodion-bag forms the same type of crystals (Fig. 3a) as that obtained from koji culture previously,³⁾ on the other hand, condensation and dialysis by "Diaflow" forms smaller crystals (Fig. 3b). Crystalline enzyme from a liquid culture was eluted from the Sephadex G-75 column at the same elution volume as that of crystalline enzyme purified from a solid koji culture, indicating that both enzyme preparation have the same molecular weight (51,000). These crystalline enzyme purified from solid and liquid cultures showed the same specific activity of 16.5 U,

and a single protein band on disc-electrophoresis at pH 9.4 gel.

Since partially purified acid carboxypeptidases of *Penicillium* molds have been recognized to be stable at the pH between 3 and 7,²⁾ stabilities were investigated at pH 3.7. In a crude preparation, acid carboxypeptidase was stable at 25°C even in the low concentration of 0.01 ± 0.001 U/ml, whereas, the crystalline enzyme was somewhat labile under the same condition. At low temperature (5°C or 0°C), the inactivation was partially retarded. Furthermore, covering surface of the enzyme solution with toluene completely protected the enzyme activity from inactivation. These phenomena seem to indicate the oxidative denaturation of the diluted enzyme solution.

The previous inhibition studies²⁾ suggested that a thiol group is in or very near the active site of the acid carboxypeptidase. Whereas, antioxidant of L-ascorbic acid and SH-protecting reagents of 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) showed no protecting effect in the present experiments.

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