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Production of a New Type of Acid Carboxypeptidase of Molds of the *Aspergillus niger* Group

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The ability of 88 fungi, which had been obtained as high-potency strains for acid proteinase production, to produce a new type of acid carboxypeptidase (having an optimal pH of about 3 for hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine) in surface koji culture was determined. Among the aspergilli, substantial amounts of this new acid carboxypeptidase were produced by *Aspergillus saitoi*, *A. usamii*, *A. awamori*, *A. inuii*, and *A. niger*. Maximum yields of acid carboxypeptidase per gram of substrate were obtained by submerged culture in a medium containing 0.9% defatted soybean and 0.6% wheat bran. However, the maximum enzyme concentration per milliliter was obtained with a medium containing 3% defatted soybean and 2% wheat bran. The terminal pH could be controlled by varying the concentrations of soybean oil meal and wheat bran. The maximum enzyme production was reached after 4 days or more at 30 C.

Acid carboxypeptidase was observed in culture filtrates of *Aspergillus saitoi* by Ichishima in 1969 (2). The enzyme has been isolated, purified, and partially characterized (4-9); it liberates basic, neutral, and acidic amino acids, including proline, from the carboxyterminal of the substrates at acidic pH values. Previous studies (5) have demonstrated that the enzyme exhibits a preference for aromatic and dicarboxylic amino acids in the position "X" when the X-Y bond of small synthetic substrates of the type R-X-Y are hydrolyzed (R = peptide residue, benzyloxycarbonyl-, benzoyl-, or acetyl-).

Ethylenediaminetetraacetate and *o*-phenanthroline had no effect on the acid carboxypeptidase activity at pH 5.2, suggesting that there is no requirement for metal ions (5). Diisopropylfluorophosphate and tosylphenylalanine chloromethylketone inhibited the enzyme (8); hydrocinnamic acid, indole-3-acetic acid, and 4-phenylbutyric acid were competitive inhibitors (7).

In this communication we describe the effects of conditions on the production of a new type of acid carboxypeptidase from molds of the *A. niger* group (11).

MATERIALS AND METHODS

Organisms. All strains tested were subcultured from culture collections. Table 1 is a list of genera and numbers of strains tested. These strains had been obtained as high-potency strains for acid proteinase production.

A. saitoi (10, 12) R-3813 (now designated as ATCC-14332) and *A. usamii* IAM-2186, IAM-2185 (ATCC-14331), *A. awamori* IAM-2351, IAM-2347, IAM-2390 (ATCC-14335), *A. awamori* var. *acidus* IAM-2279, *A. inuii* IAM-2255, IAM-2258, and *A. niger* NRRL-330 were used throughout this investigation. *A. oryzae* IAM-2600, IAM-2608, IAM-2609, IAM-2616, IAM-2640, IAM-2649, IAM-2730, IAM-2734, IAM-2736, *A. oryzae* var. *globosus* M-9, *A. oryzae* var. *magnasporus* IAM-2620, IFO-4221, IFO-4230, IFO-4250, IFO-4277, IFO-4278, *Penicillium daleae* IFO-6087, *P. frequentance* AHU-8328, *P. funiculosum* IAM-7013, *P. janthinellum* IFO-8070, *P. lividum* IAM-7200, and *P. oxalicum* AHU-8326 were used for preliminary comparative study. Stock cultures were maintained on normal solid nutrient medium containing rice koji extract (11).

Koji culture. Eighty-eight strains of fungi were examined for enzyme production by the koji method (1). Wheat bran (3 g) was throughly mixed with 2.1 ml of tap water in a 100-ml Erlemmeyer flask and autoclaved at 15 lb/in^2 for 30 min. The sterilized bran was inoculated with spores of the above molds and then incubated at 30 C for at least 60 h. A large number of molds grow satisfactorily on this medium. The flasks were shaken twice a day to redistribute the medium and produce better temperature control.

The major part of the acid carboxypeptidase produced in koji culture was extracted with 10 volumes of distilled water or 0.05 M acetate buffer at pH 3.0, by a standard technique (3). The mixture was adjusted to

TABLE 1. *List of microorganisms tested for acid carboxypeptidase production*

Microorganisms	No. tested
Aspergillus niger group	
A. aureus	3
A. awamori	7
A. inuii	4
A. nakazawai	1
A. niger	3
A. saitoi	1
A. usamii	2
Aspergillus oryzae group	
A. oryzae	9
A. oryzae var. *magnasporus*	8
A. oryzae var. *globosus*	1
Penicillium, 30 species	38
Rhizopus, 6 species	6
Mucor, 2 species	2
Paecillomyces	1
Fusarium	1
Alternaria	1

a pH of 4 with 1 N HCl and allowed to stand in the cold for 2 h.

Submerged culture. A liquid medium prepared from wheat bran and defatted soybean was used. Wheat bran (0.3 g) and crushed defatted soybean (0.45 g) were mixed with 0.75 ml of tap water containing 0.0075 ml of concentrated HCl in a 500-ml Sakaguchi flask and sterilized first at 15 lb/in^2 for 1 h. To the sterilized mixture in the flask was added 49.25 ml of tap water. The flask was then sterilized twice at 15 lb/in^2 for 30 min. The pH was adjusted to about 5.5 for spore germination of the *A. niger* group. After inoculation with spores, the culture was incubated at 30 C and shaken continuously at 120 (10-cm) strokes per min for 100 h or more.

Preparation of crude enzyme from culture filtrates. After extraction from cultures, the culture filtrates were pooled and the pH was adjusted to 4 to 5. The enzyme was precipitated with 530 g of solid $(NH_4)_2SO_4$ per liter of culture filtrate at 5 C. The precipitate was removed by filtration and stored in the cold.

After the pooled culture filtrate was precipitated with sufficient cold acetone to obtain a final acetone concentration of 60 to 67%, the precipitate was separated by centrifugation and dried in vacuo. The dried material was ground up in a mortar and stored in the cold.

For large-scale production, the acid carboxypeptidase contained in the culture medium was separated from acid proteinase by passage through an Amberlite CG-50 column. The resin was equilibrated to pH 3.0 with 0.01 M acetate buffer. After the column was packed at 4 C, the culture filtrate (pH 3.0) was passed through the bed. The acid carboxypeptidase was almost quantitatively eluted, whereas the acid proteinase was almost quantitatively adsorbed. The specific activity of the column eluate was about two- or threefold higher than that of the enzyme in the culture medium.

TABLE 2. *List of potent strains of molds of genus Aspergillus and Penicillium for acid carboxypeptidase production by the koji method*

Strain	Activity (U × 10^3/ml of filtrate)
Aspergillus niger group	
A. aureus IAM-2337	230
A. awamori IAM-2347	321
A. awamori IAM-2351	342
A. awamori IAM-2390	367
A. awamori var *acidus* IAM-2279	331
A. inuii IAM-2255	296
A. inuii IAM-2258	321
A. nakazawai IAM-2293	112
A. niger NRRL-330	270
A. saitoi R-3813	510
A. usamii IAM-2185	455
A. usamii IAM-2186	495
Aspergillus oryzae group[a]	
A. oryzae IAM-2600	510
A. oryzae IAM-2608	445
A. oryzae IAM-2609	407
A. oryzae IAM-2616	557
A. oryzae IAM-2640	723
A. oryzae IAM-2649	480
A. oryzae IAM-2730	398
A. oryzae IAM-2734	608
A. oryzae IAM-2736	528
A. oryzae var. *magnasporus* IAM-2620	770
A. oryzae var. *magnasporus* IFO-4221	605
A. oryzae var. *magnasporus* IFO-4230	454
A. oryzae var. *magnasporus* IFO-4250	411
A. oryzae var. *magnasporus* IFO-4251	413
A. oryzae var. *magnasporus* IFO-4277	357
A. oryzae var. *magnasporus* IFO-4278	439
A. oryzae var. *globosus* M-9	428
Penicillium group[a,b]	
P. daleae IFO-6087	836
P. frequentance AHU-8328	602
P. funiculosum IAM-7013	780
P. janthinellum IFO-8070	1070
P. janthinellum IAM-7026	306
P. lividum IAM-7200	454
P. oxalicum AHU-8336	831

[a] Activity was measured at pH 3.7.
[b] Inoculated at 25 C for 180 h.

The enzyme preparations were stable when stored in the cold.

Chromatography. Chromatographic elution was accomplished at 5 C.

Partial purification of the acid carboxypeptidases from strains of the *A. niger* group was performed with gel filtration according to the preceding paper (5).

Molecular weight determination. Molecular weight determination was performed by the gel filtration method with the partially purified enzymes obtained from Sephadex G-100. The following standard preparations were used at a concentration of 3 to 5 mg/ml: from Nutritional Biochemicals Corp., Cleve-

land, Ohio, human γ-globulin, fraction II (mol wt 156,000) and bovine hemoglobin (mol wt 67,000); from the Sankyo Co., Tokyo, Taka-amylase A (mol wt 51,200).

Assay. Acid carboxypeptidase activity was determined by the increase in ninhydrin color after hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine (pH 3.1) at 30 C, according to our previous paper (5). One unit of acid carboxypeptidase activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min at pH 3.1 and 30 C.

Substrates. Benzyloxycarbonyl-glutamyl-tyrosine and benzyloxycarbonyl-glycyl-prolyl-leucyl-glycine were purchased from the Protein Research Foundation, Osaka.

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

RESULTS

Production of acid carboxypeptidase on koji culture. A survey of fungi for their ability to produce acid carboxypeptidase demonstrated that the following strains were good producers of the enzyme: *A. saitoi* R-3813 (ATCC-14332), *A. usamii* IAM-2186, IAM-2185 (ATCC-14331), *A. awamori* IAM-2351, IAM-2347, IAM-2390 (ATCC-14335), *A. awamori* var. *acidus* IAM-2279, *A. inuii* IAM-2255, IAM-2258 and *A. niger* NRRL-330 (Table 2). *A. saitoi* R-3813, *A. usamii* IAM-2186, and *A. usamii* IAM-2185 produced the highest enzyme activities of any organism belonging to the *A. niger* group tested by the koji method.

The production of acid carboxypeptidase by

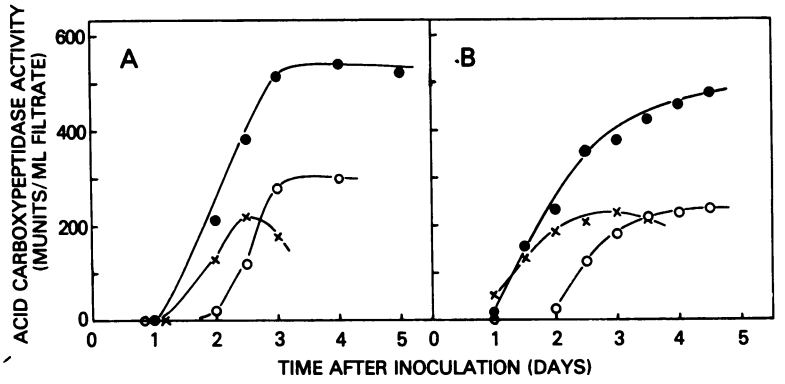

FIG. 1. *Effect of temperature on acid carboxypeptidase production in koji culture by A. saitoi R-3813 and A. usamii IAM-2186 at 30 C. A, A. saitoi R-3813; B, A. usamii IAM-2186. Symbols: O, incubated at 25 C; ●, incubated at 30 C; ×, incubated at 35 C.*

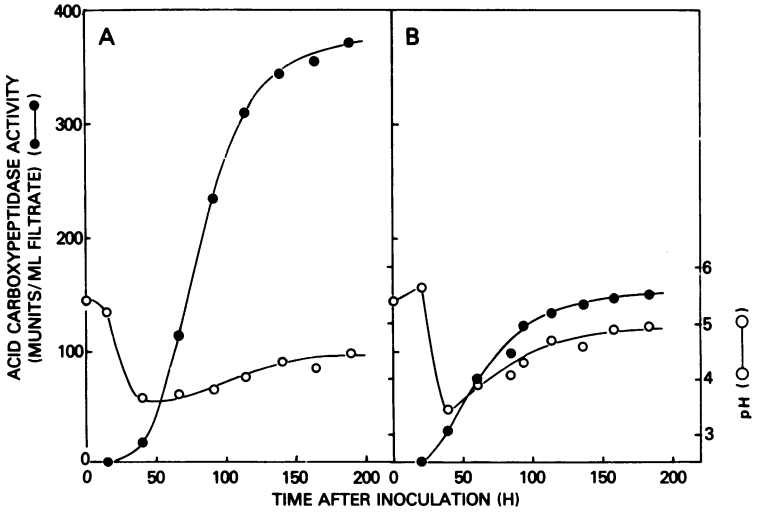

FIG. 2. *Acid carboxypeptidase production by A. saitoi R-3813 in submerged culture at 30 C. A, 3% defatted soybean and 2% wheat bran medium; B, 0.9% defatted soybean and 0.6% wheat bran medium. Symbols: ●, acid carboxypeptidase activity; O, pH.*

A. saitoi and *A. usamii* was determined at various temperatures. The maximum production was at 30 C. Maximum enzyme titers were reached at about 4 days (Fig. 1).

Production of acid carboxypeptidase in submerged culture. Production of acid carboxypeptidase in submerged culture is shown in Fig. 2 and Table 3. The effect of different medium compositions on acid carboxypeptidase production by *A. saitoi* was determined at 24-h intervals up to 190 h. A medium composed of 0.9% defatted soybean and 0.6% wheat bran supported substantial quantities of growth and acid carboxypeptidase production (150 mU/ml of filtrate). However, maximum enzyme production (360 mU/ml of filtrate) was obtained with a medium containing 3% soybean and 2% wheat bran.

When the initial pH was adjusted to between 5.0 and 6.0, maximum enzyme levels were obtained. As the concentration of soybean was increased, the terminal pH usually increased, but as the level of wheat bran was increased, the terminal pH decreased. When the pH of the culture was adjusted at 30 h or more to 2.5 or below or to 6.0 or above, no enzyme was produced. By controlling the concentration of soybean and wheat bran, we were able to control the terminal pH. A terminal pH of 4.0 to 5.5 in submerged culture was favorable to acid carboxypeptidase production and stability of the enzyme. Maximum enzyme production was usually reached after 4 days or more at 30 C. The enzymatic activity was completely stable in the medium at pH 5.5 and 30 C for 350 h.

TABLE 3. *Effect of concentrations of defatted soybean and wheat bran on acid carboxypeptidase production by A. saitoi in submerged culture*

Expt	Concn of medium (%)		Final pH	Activity (U × 10^3/ml of filtrate)
	Soybean	Wheat bran		
1[a]	0.8	0.2	4.5	110
	0.6	0.4	4.5	124
	0.4	0.6	4.5	108
	0.2	0.8	4.2	91
		1.0	3.2	51
2[b]	0.6	0.4	5.1	76
	0.9	0.6	4.3	119
	1.2	0.8	4.4	154
	1.8	1.2	4.5	176
	2.4	1.6	4.0	191
	3.0	2.0	4.1	215
	3.6	2.4	4.5	167

[a] Incubation for 92 h at 30 C.
[b] Incubation for 91 h at 30 C.

Enzymatic properties of acid carboxypeptidase. Preliminary comparative studies of the acid carboxypeptidases from *A. saitoi* R-3813, *A. usamii* IAM-2186, *A. awamori* var. *acidus* IAM-2279, *A. inuii* IAM-2258, *A. nakazawai* IAM-2293, and *A. niger* NRRL-330 showed no significant differences in stability, enzymatic activity, or molecular weight (Table 4). The acid carboxypeptidases from these strains showed no difference in residual activity after storage for 14 h at pH 2.3 to 5.0 at 30 C. These enzymes also showed no significant differences in thermal stability. Figure 3 shows the thermal stability of the acid carboxypeptidase from *A. usamii*.

DISCUSSION

Over the past 20 years, extensive work has been published on the applied aspects of enzymology. We applied the koji method to screen fungi for acid carboxypeptidase production. We found that a number of *Aspergillus* species produced a new type of enzyme, having an optimal pH of 3.1 for hydrolysis of benzyloxycarbonyl-glutamyl-tyrosine.

Although strains of the *A. oryzae* group were found to be rich sources of acid carboxypeptidase (Table 2). *A. oryzae* and *A. oryzae* var. *magnasporus* produced acid carboxypeptidases having relatively high optimal pH values (3.7 for benzyloxycarbonyl-glutamyl-tyrosine and 4.8 for benzyloxycarbonyl-glycyl-prolyl-leucyl-glycine hydrolysis) and low-molecular-weight

TABLE 4. *Enzymatic properties of partially purified acid carboxypeptidases from strains of the A. niger group*

Acid carboxypeptidase isolated from	Mol wt	Optimal pH for hydrolysis	
		Benzyloxy-carbonyl-Glu-Tyr	Benzyloxy-carbonyl-Gly-Pro-Leu-Gly
A. usamii IAM-2186	150,000	3.2[a]	3.9[b]
A. inuii IAM-2258	150,000	3.3[a]	3.8[b]
A. awamori var. *acidus* IAM-2279	150,000	3.3[a]	4.0[b]
A. nakazawai IAM-2293	150,000	3.3[a]	3.3[a]
A. niger NRRL-330	150,000	3.3[a]	3.5[b]
A. saitoi R-3813[c]	155,000 51,000[d]	3.1[a]	3.2[b]

[a] Acetate buffer.
[b] Sörensen's citrate buffer.
[c] Data from reference 5.
[d] Dissociation was observed partly in the purified preparation.

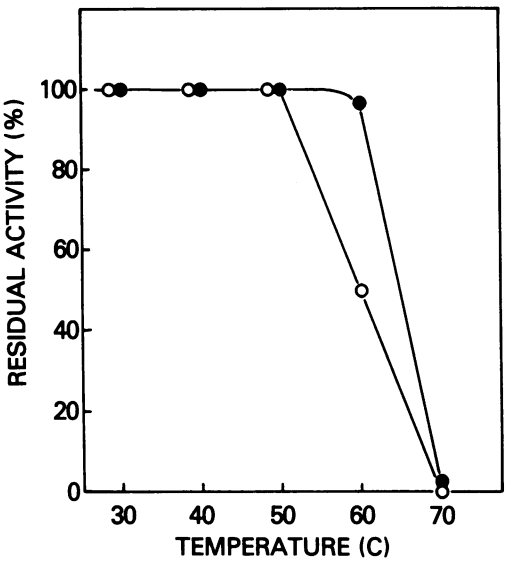

FIG. 3. *Thermal stability of acid carboxypeptidase from A. usamii IAM-2186 at pH 3.1* (○) *and 4.5* (●) *for 10 min.*

values (80,000). The enzymes from both strains were apparently less stable than the enzyme from *A. saitoi* (8).

After 38 strains of *Penicillium* were investigated, six wild-type strains (*P. daleae* IFO-6087, *P. frequentance* AHU-8328, *P. funiculosum* IAM-7013, *P. janthinellum* IFO-8070, *P. lividum* IAM-7200, and *P. oxalicum* AHU-8326) were found to be excellent for acid carboxypeptidase production. The preliminary enzymatic examination of these strains indicated that the enzymes were similar to that from *A. oryzae*. A detailed account of the properties of the enzymes from penicillia will appear elsewhere.

The strains of *Rhizopus*, *Mucor*, *Paecilomyces*, and *Fusarium* tested produced no detectable amounts of acid carboxypeptidase.

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LITERATURE CITED

1. Arima, K. 1964. Microbial enzyme production, p. 277-294. *In* M. P. Starr (ed.), Global impacts of applied microbiology. Almqvist & Wikell, Stockholm, Göteborg, Uppsala.
2. Ichishima, E. 1969. A new type of acid carboxypeptidase from *Aspergillus*. Proc. Agr. Chem. Soc. Jap. **44**:55.
3. Ichishima, E. 1970. Purification and mode of assay for acid proteinase of *Aspergillus saitoi*, p. 397-406. *In* G. E. Perlmann and L. Lorand (ed.), Methods in enzymology, vol. 19. Academic Press Inc., New York.
4. Ichishima, E. 1972. Acid proteinase and acid carboxypeptidase of molds of the genus *Aspergillus*, p. 259-270. *In* G. Terui (ed.), Proceedings of the International Fermentation Symposium: Fermentation technology today. Society of Fermentation Technology, Suita, Osaka.
5. Ichishima, E. 1972. Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*. Biochim. Biophys. Acta **258**:274-288.
6. Ichishima, E., and T. Arai. 1972. C-terminal sequence determination by a new type of acid carboxypeptidase from *Aspergillus saitoi*, p. 326-331. *In* K. Sekita (ed.), Proceedings of the Symposium on Biochemistry. Japanese Biochemical Society, Tokyo.
7. Ichishima, E., and T. Arai. 1973. Specificity and mode of action of acid carboxypeptidase from *Aspergillus saitoi*. Biochim. Biophys. Acta **293**:444-450.
8. Ichishima, E., S. Sonoki, K. Hirai, Y. Torii, and S. Yokoyama. 1972. Comparative studies on enzymatic activities of acid carboxypeptidase of molds of the genus *Aspergillus*. J. Biochem. Tokyo **72**:1045-1048.
9. Ichishima, E., and K. Yomogida. 1973. Esterolytic activity of acid carboxypeptidase from *Aspergillus saitoi*. Agr. Biol. Chem. **37**:693-694.
10. Iizuka, H. 1955. The electron microscope investigation on classification of conidia of the genus *Aspergilli*. J. Gen. Appl. Microbiol. **1**:10-17.
11. Raper, K. B., and D. I. Fennell. 1965. The genus *Aspergillus*. The Williams & Wilkins Co., Baltimore.
12. Sakaguchi, K., H. Iizuka, and S. Yamasaki. 1951. A study on black *Aspergilli*. J. Agr. Chem. Soc. Jap. **24**:138-142.