Production of Aminopeptidase and Carboxypeptidase by *Streptomyces peptidofaciens*[†]

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Streptomyces peptidofaciens KY 2389, a new species isolated from a soil sample, exhibited the highest potencies in production of both aminopeptidase and carboxypeptidase among about 1,300 strains tested.

Optimum pH values of both aminopeptidase and carboxypeptidase for Leu- β -naphthylamide and Cbz-Gly-Leu were 8.0. Aminopeptidase was thermostable and the activity was not lost by treatment at 70°C for 1 hr, in the presence of Ca²⁺. Carboxypeptidase was heat-labile and over 50% of the activity was lost by treatment at 60°C for 1 hr. Of the synthetic peptides tested, Leu-Gly-Gly and Cbz-Gly-Leu were the most suitable substrates for aminopeptidase and carboxypeptidase, respectively.

Production of peptidases by microorganisms has been reported by many investigators. Especially, peptidases from *Escherichia coli*,¹⁾ *Bacillus subtilis*,²⁾ *Aeromonas proteolytica*,³⁾ *Phymatotrichum omnivorum*⁴⁾ and *Aspergillus saitoi*⁵⁾ were purified and their properties have been studied in detail.

As to actinomycetes, Narahashi *et al.*⁶⁾ and Morihara *et al.*⁷⁾ reported *Streptomyces griseus* and *Streptomyces fradiae*, respectively, capable of producing multiple proteolytic enzymes that include aminopeptidase and carboxypeptidase besides proteinases. Mizusawa *et al.*,⁸⁾ also reported that *Streptomyces rectus* var. *proteolyticus* produced carboxypeptidase as well as alkaline proteinase. However, these peptidases obtained from *Streptomyces* have not been homogeneous in the purities.

In the course of survey for microorganisms producing peptidases, we found that the culture filtrate of a newly isolated soil actinomycete, *Streptomyces peptidofaciens*, showed the highest hydrolytic activities toward Leu- β -naphthylamide and Cbz-Gly-Leu, but almost no caseinolytic activity.

In the previous communication,⁹⁾ it has been

briefly reported that extracellular aminopeptidase of *Streptomyces peptidofaciens* was purified and crystallized by a procedure involving acetone precipitation, DEAE-cellulose and hydroxyapatite column chromatographies and Sephadex G-75 gel filtration. The present paper is concerned with the taxonomical study of *Streptomyces peptidofaciens* and some enzymatic properties of both aminopeptidase and carboxypeptidase.

MATERIALS AND METHODS

Microorganisms. Microorganisms tested in the present study were about 300 type cultures of various species in our culture collections and about 1000 cultures of *Streptomyces* which were newly isolated from soil samples.

Screening and media. An agar medium with the following composition was used for the isolation of *Streptomyces*: 0.3% soluble starch, 0.5% soy bean meal, 0.1% corn steep liquor, 0.05% K₂HPO₄, 0.01% chloramphenicol and 2.0% agar (pH 7.6). Soil suspensions were streaked on the agar plates. After incubation at 27°C for 5 days, the *Streptomyces* grown on the plates were transferred to agar slants of the same medium. These cultures were inoculated into 50 ml of the SBM-medium composed of 3% soluble starch, 5% soy bean meal, 0.5% corn steep liquor and 0.1% Na₂HPO₄(pH 7.6), in 250 ml Erlenmeyer flasks and cultivated on a rotary shaker at 30°C for 48 hr. After the cultivation, aminopeptidase and carboxypeptidase

[†] Studies on Peptidase from *Streptomyces*. Part I. Abbreviations used are; Leu- β -naphthylamide: L-Leucyl- β -naphthylamide, Leu-Gly-Gly: L-Leucyl-glycyl-glycine, Cbz-Gly-Leu: Carbobenzoxyl-glycyl-L-leucine.

activities of the culture filtrates were measured by using Leu- β -naphthylamide and Cbz-Gly-Leu as substrates, respectively.

Taxonomical studies. *Streptomyces* sp. KY 2389 was inoculated on various agar media conventionally used for the classification of *Streptomyces* and incubated for 4 weeks at 27°C. The structure of spore was observed with an electron microscope. Utilization of carbon sources was determined by cultivation on Pridham and Gottlieb's media¹⁰⁾ which contain 1% of various carbon compounds.

Enzyme assays. The synthetic substrates used in these experiments were obtained from the Institute for Protein Research, Osaka University, Osaka.

The aminopeptidase activity was determined by a modified method of Goldberg and Rutenburg,¹¹⁾ using Leu- β -naphthylamide as substrate; 0.5 ml of 6.8×10^{-4} M substrate and 0.5 ml of the enzyme solution in 0.05 M Veronal buffer (pH 8.0) containing 10^{-3} M calcium acetate were mixed and incubated at 37°C for 20 min. One aminopeptidase unit was defined as an amount of the enzyme that hydrolyzed 1 μ mole of Leu- β -naphthylamide per minute under the conditions described.

The carboxypeptidase activity was determined by measuring the amounts of leucine liberated from Cbz-Gly-Leu after incubation at 40°C for 30 min. The reaction mixture was composed of 0.5 ml of 0.1 M substrate and 0.5 ml of the enzyme solution in 0.05 M Veronal buffer (pH 8.0) containing 10^{-3} M CoCl_2 . Leucine was measured by the method of Yemm and Cocking.¹²⁾ One carboxypeptidase unit was defined as an amount of the enzyme that hydrolyzed 1 μ mole of Cbz-Gly-Leu per minute under the conditions described.

The proteinase activity was assayed at pH 7.0 by the method of Hagihara *et al.*¹³⁾ using milk casein as substrate and indicated by the absorbancy at 280 m μ .

Protein determination. Protein concentration was determined spectrophotometrically by measuring the absorbancy at 280 m μ . $E_{1\text{cm}}^{1\%}$ values of 16.4 and 15.0 for aminopeptidase and carboxypeptidase, respectively, were used throughout this work. The former was obtained by measurement of absorbance and by dry weight determinations. Specific activity was expressed in units of activity per mg of protein.

Sugar determination. Residual sugar in culture medium was determined by an improved Anthrone method of Loewus.¹⁴⁾

Cell growth determination. Cell growth was determined by measuring solid volume: Ten ml of culture broth was centrifuged in a graduated tube at

3000 rpm for 10 min and solid volume was defined as the percent of the volume of sedimentary solid per 100 ml of culture broth.

Purification procedures. Crystalline aminopeptidase was prepared from the culture filtrate of *Streptomyces peptidofaciens* KY 2389 grown on the SBM-medium, by procedures involving acetone precipitation, DEAE-cellulose and hydroxyapatite column chromatographies, Sephadex G-75 gel filtration and addition of ammonium sulfate, as described previously.⁹⁾ The crystalline aminopeptidase was homogeneous on ultracentrifugation and electrophoresis.

Purified carboxypeptidase was prepared from the culture filtrate of *S. peptidofaciens* KY 2389, as follows. The fermentation broth (30 liters) was centrifuged with a continuous-flow separator and the supernatant fluid (27 liters), which contained about 45 g of protein and 5,100 units of carboxypeptidase activity, was concentrated 5-fold *in vacuo* in a flash evaporator (40°C). To 1 volume of the concentrated solution were added 2 volumes of acetone (−15°C) and the resulting precipitate was collected by filtration and dissolved in the minimum volume of 0.05 M acetate buffer (pH 5.0). This solution was dialyzed against 10 liters of the same buffer for 48 hr. The dialyzed enzyme solution (350 ml), which contained 15.1 g of protein and 3,600 units of activity, was applied on Doullite A-7 resin column (7.5 $\times$ 65 cm) equilibrated with 0.05 M acetate buffer, (pH 5.0). The enzyme was not adsorbed on the column under the conditions employed, but many inert materials including most of the black pigment were adsorbed and separated from the enzyme. The active fractions were combined and concentrated *in vacuo* in a rotary evaporator (40°C). The concentrated carboxypeptidase fraction (26 ml), which contained 980 mg of protein and 1,100 units of activity, was subjected to gel filtration on a Sephadex G-75 column (2 $\times$ 100 cm) equilibrated with 0.01 M Veronal buffer (pH 7.6) and separated into two protein fractions. The first fraction is colorless and the second is yellow. The enzyme activity was found only in the colorless protein fraction. The purified carboxypeptidase (93 ml, protein 270 mg, total units 770) obtained in this manner could be preserved at 5°C in 0.01 M Veronal buffer (pH 7.6). These procedures resulted in a 25-fold purification with an over-all recovery of activity of 15%. Purified carboxypeptidase was homogeneous on ultracentrifugation.

RESULTS

Screening of peptidase-producing microorganisms

Among about 1300 cultures of microorganisms examined, *Streptomyces* sp. KY

TABLE I. AMINOPEPTIDASE AND CARBOXYPEPTIDASE ACTIVITIES OF CULTURE FILTRATES OF VARIOUS MICROORGANISMS TESTED

The cultivation was carried out at 30°C for 60 hr on a rotary shaker, in 50 ml of the SBM-medium. The aminopeptidase and carboxypeptidase activities of the culture broth were measured under the standard assay conditions.

Strain	Amino peptidase activity	Carboxy peptidase activity
	(u/ml)	(u/ml)
<i>Streptomyces</i> <i>flavochromogenes</i>	0.045	0
<i>S. halstedii</i>	0.072	0
<i>S. phaeochromogenes</i>	0.095	0
<i>S. oidiosporus</i>	0.056	0
<i>S. citreus</i>	0.14	0
<i>S. erythreus</i>	0.041	0
<i>S. flavoviridis</i>	0.049	0
<i>S. globisporus</i>	0.24	0
<i>S. fradiae</i>	0.20	0.13
<i>S. filamentus</i>	0	0.13
<i>S. griseolus</i>	0	0.10
<i>S. griseoflavus</i>	0	0.11
<i>S. peptidofaciens</i>	0.85	1.80
KY 2389		
<i>S. sp.</i> KY 1510	0.48	0.38
<i>S. sp.</i> KY 1065	0.75	0
<i>S. sp.</i> KY 1340	0.25	0
<i>Aspergillus melleus</i>	0.54	0
<i>A. flavus</i>	0.21	0
<i>A. oryzae</i>	0.06	0
<i>A. effusus</i>	0.45	0
<i>A. ochraceus</i>	0.06	0
<i>A. sclerotium</i>	0.07	0
<i>Ustilago tritici</i>	0.08	0
<i>Penicillium lilacinum</i>	0	0
<i>P. nigricans</i>	0	0
<i>P. brunneum</i>	0	0
<i>P. casei</i>	0	0
<i>Rhizopus acetoinus</i>	0	0
<i>R. chinensis</i>	0	0
<i>R. tritici</i>	0	0
<i>Bacillus subtilis</i>	0.03	0
<i>B. stearothermophilus</i>	0.03	0
<i>B. natto</i>	0.01	0
<i>B. licheniformis</i>	0	0

2389 exhibited the most remarkable activities of aminopeptidase and carboxypeptidase, but almost no activity of proteinase in the culture fluid (Table I). Therefore, this *Streptomyces* sp. KY 2389 was selected for further investigation.

FIG. 1. Microscopic Photograph of Aerial Mycelia of *S. peptidofaciens*. ($\times 600$)

FIG. 2. Electronmicrograph of Conidia of *S. peptidofaciens*. ($\times 5,000$)

Taxonomical studies on *Streptomyces* sp. KY 2389

An aerial mycelium of *Streptomyces* sp. KY 2389 on the synthetic media, exhibits a linear, monopodial branching structure, sometimes with a slight curvature (Fig. 1). The conidia are formed in chains of more than ten spores. The spores are cylindrical, $0.7 \sim 0.8 \mu \times 1.0 \mu$, with smooth surface (Fig. 2).

The cultural and physiological characteristics of *Streptomyces* sp. KY 2389 are listed in Tables II and III. The utilizations of carbon sources of the strain are shown in Table IV.

Among many species of *Streptomyces* described in "Bergey's Manual of Determinative Bacteriology,"¹⁵⁾ and "The Actinomycetes," Vol. 2,¹⁶⁾ *Streptomyces* sp. KY 2389 appeared to be somewhat related to *Streptomyces*

TABLE II. CULTURAL PROPERTIES OF *S. peptidofaciens*

S. peptidofaciens was inoculated on each agar medium as indicated and incubated at 27°C for 4 weeks.

Medium	Aerial mycelium	Vegetative mycelium	Soluble pigment
Synthetic agar	Thick, dry, grey	Brown	Brownish yellow
Glucose-asparagine agar	Thick, grey	Brownish grey	Pale brown
Glycerine-asparagine agar	Pinkish white	Yellowish grey	Brown
Starch agar	Thick, greyish or pinkish white	Yellowish grey	Pale yellowish brown
Tyrosine agar	Greyish white	Yellowish grey	Black
Bouillon agar	Pinkish white	Yellowish white	Brown
Yeast-malt agar	Thick, dry, greyish or pinkish white	Yellowish brown	Dark brown
Oat meal agar	Greyish or pinkish white	Yellowish grey	Dark brown

TABLE III. PHYSIOLOGICAL PROPERTIES OF *S. peptidofaciens*

Exopeptidase activity	+
Endopeptidase activity	-
Milk coagulation	-
Milk peptonization	+
Melanine formation	+
Tyrosinase activity	+
Nitrate reduction	+
Gelatin liquefaction	+
Catalase activity	+
Starch hydrolysis	+
Cellulolytic activity	-
Temperature for growth	20°C ~ 37°C

melanogenes from morphological and physiological characteristics, but some differences were found among them, described as follows.

1) *Streptomyces* sp. KY 2389 utilized rhamnose, although *S. melanogenes* does not.

2) Tyrosinase production by *Streptomyces* sp. KY 2389 is positive, but that by *S. melanogenes* is negative.

TABLE IV. UTILIZATION OF CARBOHYDRATES BY *S. peptidofaciens*

Utilization of carbon sources by *S. peptidofaciens* was determined by cultivation on Pridham and Gottlieb's media¹⁰⁾ which contain 1% of various carbon compounds as indicated.

Carbohydrates	Utilization
D-Arabinose	+
D-Xylose	+
D-Glucose	+
Sucrose	+
Inositol	+
D-Rhamnose	+
Raffinose	+
D-Mannitol	+

3) *Streptomyces* sp. KY 2389 produces aminopeptidase and carboxypeptidase, although *S. melanogenes* does not.

4) *Streptomyces* sp. KY 2389 does not produce proteinase, although *S. melanogenes* produces it.

From these definite differences, it was concluded that *Streptomyces* sp. KY 2389 should be assigned to a new strain and designated as *Streptomyces peptidofaciens*.

Time course of peptidase production

FIG. 3. Time Course of Cell Growth and Peptidase Production during Cultivation of *S. peptidofaciens* with SBM-medium.

S. peptidofaciens was cultivated in 15 liters of the SBM-medium at 30°C in a jar fermenter. The aminopeptidase and carboxypeptidase activities of the culture broth were measured under the standard assay conditions.

FIG. 4. pH-activity Curves of Aminopeptidase and Carboxypeptidase.

Crystalline aminopeptidase with a specific activity of 69.7 and purified carboxypeptidase with a specific activity of 2.5 were used. The enzyme activity was determined under the standard assay conditions except that pH of the buffer was changed as indicated. The buffer solutions used were 0.05 M phosphate buffer for pH 6~7, 0.05 M Veronal buffer for pH 7~9 and 0.05 M phosphate-NaOH buffer for pH 9~10. ●—●, Aminopeptidase; ○---○, Carboxypeptidase.

FIG. 5. pH-stability Curves of Aminopeptidase and Carboxypeptidase.

The peptidase (crystalline aminopeptidase containing 24 μ g of protein and 1.7 units or purified carboxypeptidase containing 225 μ g of protein and 0.56 units) was kept for 1 hr at 37°C in 1.0 ml of 0.05 M buffer solution of various pH values. The remaining enzyme activity was measured under the standard assay conditions. The buffer solutions used were acetate-HCl buffer for pH 2~5, phosphate buffer for pH 6~7, Veronal buffer for pH 8~9 and phosphate-NaOH buffer for pH 10~12.

●—●, Aminopeptidase; ○---○, Carboxypeptidase

FIG. 6. Heat Stability Curves of Aminopeptidase and Carboxypeptidase.

The peptidase (crystalline aminopeptidase containing 24 μ g of protein and 1.7 units, or purified carboxypeptidase containing 225 μ g of protein and 0.56 units) in 1.0 ml of 0.01 M Veronal buffer, pH 8.0, was incubated for 1 hr at various temperatures as indicated. In the case of aminopeptidase, the incubation was performed in the presence or absence of 10^{-3} M Ca^{2+} . The remaining enzyme activity was assayed under the standard assay conditions.

○---○, Aminopeptidase; ●—●, Aminopeptidase + 10^{-3} M Ca-acetate; Δ --- Δ , Carboxypeptidase.

The chemical changes in the fermentation in relation to the peptidase production were investigated with *S. peptidofaciens*. *S. peptidofaciens* was inoculated in 15 liters of the SBM-medium. Cultivation was carried out at 30°C for 80 hr. At every 5 hr intervals, the cell growth, the residual sugar and peptidase activity were determined. The maximum production of both peptidases was observed coincidentally at the stationary phase of the cell growth (Fig. 3).

Some properties of peptidase from S. peptidofaciens

Optimum pH. The pH-activity curves of aminopeptidase and carboxypeptidase are shown in Fig. 4. Both enzymes exhibit the highest activities at pH 8.0.

pH-stability. The pH-stability curves of the enzymes are shown in Fig. 5. The enzymes were kept for 1 hr at various pH values. Aminopeptidase was stable from 5.0 to 9.0,

TABLE V. SUBSTRATE SPECIFICITIES OF AMINOPEPTIDASE AND CARBOXYPEPTIDASE

One ml of reaction mixture containing 25 μ mole of substrate, 50 μ moles of Veronal buffer, pH 8.0, 48 μ g of crystalline aminopeptidase (specific activity 69.7) or 450 μ g of purified carboxypeptidase (specific activity 2.5) was kept at 37°C for 30 min. Ca-acetate (1 μ mole) was added to the former reaction mixture, and CoCl₂ (1 μ mole) to the latter. The rate of hydrolysis was measured by the ninhydrin method of S. Moore and W. H. Stein.¹⁷⁾ The arrows show the peptide bond hydrolyzed.

Aminopeptidase Substrate	Rate of hydrolysis	Carboxypeptidase Substrate	Rate of hydrolysis
Leu-Gly-Gly	100 ^{a)}	Cbz-Gly-Leu	100 ^{b)}
Leu-Gly	10	Cbz-Phe-Leu	98
Leu-Tyr	29	Cbz-Gly-Phe	98
Leu-Phe	42	Cbz-Gly-Val	65
Leu-NH ₂	47	Bz-Gly-Lys	60
Phe-Gly	43	Cbz-Phe-Tyr	34
Phe-Leu	17	Cbz-Gly-Pro	0
Gly-Leu	0	Cbz-Gly-Trp	0
Gly-Gly	0	Cbz-Lys-Ser	0
Gly-Gly-Gly	0	Cbz-Met-Gly	0
Ala-Gly	0		

^{a), b)}: The specific activities were 62.5 and 2.8, respectively.

whereas carboxypeptidase was stable from 3.0 to 11.0.

Thermostability. Figure 6 shows the heat stability of each peptidase. Aminopeptidase was stable below 60°C by treatment for 1 hr and became stable up to 70°C in the presence of 10⁻³ M Ca²⁺. On the other hand, carboxypeptidase was heat-labile and inactivated over 50% by incubation at 60°C for 1 hr.

Substrate specificity. Substrate specificity of each peptidase was determined (Table V). Among numerous synthetic peptides tested, Leu-Gly-Gly and Cbz-Gly-Leu were the best substrates for aminopeptidase and carboxypeptidase, respectively.

DISCUSSION

Narahashi *et al.*⁶⁾ reported that the enzyme preparation of *S. griseus* K-1, "Pronase,"

was a mixture of several proteolytic enzymes including seven proteinases, two aminopeptidases and carboxypeptidase. Morihara *et al.*⁷⁾ also reported that *S. fradiae* produced multiple proteolytic enzymes such as keratinase, leucine aminopeptidase and carboxypeptidase.

In the present paper, the production of aminopeptidase and carboxypeptidase by *S. peptidofaciens* was reported. And special mention must be made of the fact that *S. peptidofaciens* excreted substantially no proteinase in the culture medium.

S. peptidofaciens appeared to resemble somewhat *S. melanogenes* from morphological and physiological characteristics. The former was however found not to be identical with the latter in some physiological properties, such as rhamnose utilization, tyrosinase activity and protease productivity.

Aminopeptidase from *S. peptidofaciens* was thermostable and showed a wide range of pH stability. The enzyme most rapidly hydrolyzed substrates which possess N-terminal amino acid residues containing aliphatic side chains, less rapidly those with aromatic side-chains, while did not hydrolyze those with no side-chains.

On the other hand, carboxypeptidase from *S. peptidofaciens* was heat-labile, although it had a wider range of pH stability. The enzyme most rapidly hydrolyzed substrates of which C-terminal residues are aliphatic and aromatic, and less rapidly hydrolyzed those of which the terminal are basic.

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