Purification and Properties of API-2b→API-2c Converting Protease from *Streptomyces griseoincarnatus* Strain No. KTo-250, an API-2 Producer[†]

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Among three alkaline protease inhibitors (API-2a, -2b, -2c) produced by *Streptomyces griseoincarnatus* strain No. KTo-250, API-2b was converted to API-2c in the growing system.

The cultural conditions were examined exclusively for the production of API-2b→API-2c converting protease in the culture filtrate. The protease was purified about 1080-folds by salting-out with ammonium sulfate, column chromatography on DEAE-cellulose and gel filtration on Sephadex G-100.

The optimal and maximal caseinolytic activities of the protease were around pH 9.0 and at 28°C, respectively. The protease activity was inhibited by EDTA and DFP, but not by PCMB, *o*-phenanthroline, TPCK, TLCK, AP-I and S-SI. The protease was a DFP and EDTA-sensitive alkaline protease, and it required Ca²⁺ ion for its activity and stability.

In the previous papers,^{1,2)} we reported that a strain (KTo-250), identified as *Streptomyces griseoincarnatus* was found to produce three kinds of inhibitors designated as API-2a, API-2b and API-2c in culture filtrate, and the purification procedures of API-2b and API-2c and some differences among API-2b, API-2c, S-SI³⁾ and plasminostreptin⁴⁾ were described. Further, it was found that some amino acid residues from the amino-terminal part in API-2b were removed to form API-2c in the growing system. The enzyme which converts API-2b to API-2c is now designated as "API-2b→API-2c converting protease."

In the present paper, the purification procedure and some properties of the converting

protease in culture filtrate of the strain are described.

MATERIALS AND METHODS

Materials. API-2b and API-2c used in this work were prepared from the culture filtrate of *S. griseoincarnatus* strain No. KTo-250 according to the procedures described in the previous papers,^{1,2)} and its homogeneity was determined by disc electrophoresis. S-SI was kindly donated by Professor S. Murao of the University of Osaka Prefecture. Sephadex G-100 and DEAE-cellulose (Pharmacia Fine Chemicals), EDTA, DFP and MIA (these three reagents from Katayama Chemical Industries Co., Ltd.), TLCK, TPCK, PCMB and *o*-phenanthroline (these four reagents from Nakarai Chemicals, Ltd.), and SBTI (Sigma Chemical Co.) were purchased. Other reagents were guaranteed grade.

Assay for the protease activity. A mixture of 2 ml of enzyme solution and 1 ml of Tris-HCl buffer (0.2 M, pH 9.0) was incubated at 37°C for 3 min, then to the solution 1 ml of 2% casein solution was added. After 60 min incubation at 37°C, 2 ml of 12% perchloric acid solution was added, and the mixture was filtered after standing at room temperature for 30 min. The filtrate was assayed for enzyme activity by measuring extinction at 660 nm according to the method of Lowry's colorimetry.⁵⁾

[†] Studies on Alkaline Protease Inhibitor (API-2) Produced by *Streptomyces griseoincarnatus* Strain No. KTo-250. Part III. (See ref. 1, 2.)

Abbreviations: DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetic acid; MIA, monoiodoacetate; PCMB, *p*-chloromercuribenzoate; TLCK, *N*-tosyl-L-lysylchloromethane hydrochloride; TPCK, *N*-tosyl-L-phenylalanylchloromethylketone; AP-I, alkaline protease inhibitor produced by *S. pseudogriseolus* strain No. KTo-332 (See ref. 6~8); S-SI, *Streptomyces subtilisin* inhibitor; SBTI, soybean trypsin inhibitor.

As will be described in the text, the enzyme requires Ca^{2+} ion for stability, so the assay was carried out in the presence of 1 mM CaCl_2 unless otherwise described.

Disc electrophoresis. Polyacrylamide gel disc electrophoresis was carried out according to the reported method.¹⁾

RESULTS AND DISCUSSION

Time course of strain No. KTo-250 culture in S-medium

The cultural conditions, including the composition of medium, were examined for obtaining the highest production of API-2b→API-2c converting protease. The optimal conditions are summarized in Table I. Cultivation of this strain was carried out based on these cultural conditions. Time course changes

TABLE I. CULTURAL CONDITIONS FOR THE PRODUCTION OF API-2b→API-2c CONVERTING PROTEASE

Strain used:	<i>Streptomyces griseoincarnatus</i> strain No. KTo-250
Culture medium:	S-medium glucose 2.0, starch 3.0, C.S.L. 1.0, S.B.F. 1.0, peptone 0.5, NaCl 0.3, CaCO_3 0.5 (%)
Initial pH:	7.0
Culture volume:	40 ml-medium/200 ml-Erlenmeyer flask
Inoculum size:	2 ml/40 ml-medium (5% seed)
Culture temperature:	28°C
Agitation:	180 rpm

FIG. 1. Time Course of *Streptomyces griseoincarnatus* Strain No. KTo-250 Culture in S-medium.

○—○, protease activity; △—△, inhibitory activity; ●—●, pH; ●—●, absorbance at 280 nm.

in protease and inhibitory activity, protein and pH of culture filtrate are shown in Fig. 1. The protease activity reached maximum after 72 to 120 hr cultivation, in late phase of culture, whereas the inhibitory activity reached maximum in 24 hr, in early phase of culture.

Production and purification of API-2b→API-2c converting protease

The strain was cultivated for 96 hr, and the mycelium and other residues were removed by filtration. Overall purification procedure of the protease is summarized in Fig. 2. At first, to the clear filtrate was added solid ammonium sulfate to give 80% saturation. After standing overnight at 4°C, the precipitate was collected by centrifugation, dissolved in a minimal volume of 0.02 M Tris-HCl buffer (pH 7.5) containing 5 mM CaCl_2 and 0.1 M KCl and then dialysed against the same buffer for 2 days to remove ammonium sulfate. This dialysate solution was applied on DEAE-cellulose column which had been equilibrated with the above dialysing buffer. The elution pattern is shown in Fig. 3. The protease was eluted with the same buffer. The active fractions (Tube Nos. 12 to 17) were combined and to the solution four volumes of cold acetone was added. After standing for 60 min at 0°C, the resulting precipitate was dialysed against 0.02 M Tris-HCl buffer (pH 7.5) containing 5 mM CaCl_2 and 0.1 M KCl at 4°C for 1 day. Then the crude solution was applied on a Sephadex G-100 column previously equilibrated with the same buffer. The elution pattern is shown in Fig. 4. The protease ac-

FIG. 2. Purification Steps of API-2b→API-2c Converting Protease from Culture Filtrate of *S. griseoincarnatus* Strain No. KTo-250.

FIG. 3. Column Chromatography of Dialysate on DEAE-cellulose.

Column size, 2.5×13 cm; flow rate, 8 ml/hr; fraction size, 5 ml; buffer system, Tris-HCl (0.02 M, pH 7.5) contg. 0.1 M KCl and 5 mM CaCl_2 and same buffer contg. linear concentrations of KCl from 0.1 to 0.5 M (started at the point marked by arrow). ○—○, protease activity; ●—●, absorbancy at 280 nm; -----, KCl concentration.

FIG. 4. Gel Filtration of Crude Solution on Sephadex G-100.

Buffer, Tris-HCl (0.02 M, pH 7.5) contg. 0.1 M KCl and 5 mM CaCl_2 ; column size, 1.8×100 cm; flow rate, 12 ml/hr; fraction size, 5 ml. ○—○, protease activity; ●—●, absorbancy at 280 nm.

tivity was separated into two fractions. The API-2b→API-2c converting activity was found mainly in the first peak. Therefore, fractions of the first peak (Tube Nos. 22 to 28) were combined, and to the solution four volumes of cold acetone was added. After standing for 60 min at 0°C , the resulting precipitate was dialysed against the same buffer at 4°C for

FIG. 5. Gel Filtration of Active Solution on Sephadex G-100.

Buffer, Tris-HCl (0.02 M, pH 7.5) contg. 0.1 M KCl and 5 mM CaCl_2 ; column size, 1.8×100 cm; flow rate, 12 ml/hr; fraction size, 5 ml. ○—○, protease activity; ●—●, absorbancy at 280 nm.

1 day. Then the active solution was refiltered through a Sephadex G-100 column previously equilibrated with the above dialysing buffer. The elution pattern is shown in Fig. 5. The active fractions were combined and purified protease was collected by precipitation with acetone.

Homogeneity of the preparation was examined by polyacrylamide gel disc electrophoresis at pH 8.0 and 2.3. As shown in Fig. 6, the protease migrated as a single, clear band, so it was regarded to be homogeneous. Recovery of the enzyme from the culture filtrate is shown in Table II.

FIG. 6. Polyacrylamide Gel Disc Electrophoresis of API-2b→API-2c Converting Protease.

Polyacrylamide gel disc electrophoresis was carried out at pH 8.0 and 2.3, at 3 mA per gel column.

TABLE II. SUMMARY OF PURIFICATION STEPS OF
API-2b→API-2c CONVERTING PROTEASE
FROM CULTURE FILTRATE

	Total activity (OD at 660 nm)	Recovery (%)	Purification rate
Culture filtrate	22231	100	1.0
Dialysate	30581	137.6	42.0
Crude solution	27795	125.0	463.0
Active solution	14216	63.9	664.5
Purified protease	10435	46.9	1080.0

Effect of pH on the converting activity

Optimal pH for the converting activity was determined by polyacrylamide gel disc electrophoresis at pH 8.0. The protease and API-2b were incubated at 37°C for 20 hr in buffer solutions of various pH's. After incubation, disc electrophoresis was carried out using each of the reaction mixtures. As shown in Fig. 7, API-2b was converted to API-2c at pH 9.0 much more than at other examined pH.

FIG. 7. Effect of pH on API-2b→API-2c Converting Activity.

b and c in the figure are API-2b and API-2c, respectively.

Effect of pH on the protease activity

Optimal pH for the protease activity of the enzyme was determined by caseinolytic activity with casein as substrate. Figure 8 shows the pH-activity curve of the protease. The pH optimum was 9.0, the same as that of the converting activity, suggesting that the protease is an alkaline protease.

Effect of temperature on the protease activity

Optimal temperature for the protease activity was examined in incubation temperature range from 5°C to 60°C at pH 9.0. Figure 9 shows that the protease activity was maximum at around 28°C under the conditions described in the figure.

FIG. 8. Effect of pH on Protease Activity of API-2b→API-2c Converting Protease.

Enzyme activity was expressed as caseinolytic activity.

FIG. 9. Effect of Temperature on Protease Activity of API-2b→API-2c Converting Protease.

The protease activity was assayed at each temperature for 60 min. Relative activity was expressed at 28°C as 100.

Effect of pH on stability

As shown in Fig. 10, the protease was stable in the pH range of 6 to 9 for 60 min at 37°C; however, rapid inactivation was observed at other pH.

Effect of temperature on stability

After treatment at various temperatures for 60 min at pH 9.0, each remaining protease ac-

FIG. 10. Effect of pH on Stability.

After incubation at 37°C for 60 min, residual activity was measured at pH 9.0.

tivity was assayed at 37°C. As shown in Fig. 11, the protease was stable up to 40°C, but above 40°C gradual inactivation occurred, then the protease was completely inactivated at 60°C.

FIG. 11. Effect of Temperature on Stability.

Effect of various inhibitors

Effect of various enzyme inhibitors on the protease was investigated by determining the remaining protease activity after incubating the protease with inhibitors at pH 9.0, 37°C for 30 min. The results are summarized in Table III. The protease was completely inhibited by EDTA, DFP, API-2b and API-2c, but was not affected by PCMB, SBTI, TLCK and TPCK (the latter two inhibitors are known as a specific inhibitor for trypsin and chymotrypsin, respectively). The protease was inhibited by DFP and was most active at pH 9.0, and it was thus classified as an alkaline protease in the serine protease group. It should be noted that the protease was not inhibited by AP-I⁶⁻⁸⁾ and S-SI,³⁾ known as specific inhibitors for microbial serine proteases. This

TABLE III. EFFECT OF VARIOUS INHIBITORS

Inhibitor	Conc. (M)	Remaining activity (%)	Inhibitor	Conc. (μg)	Remaining activity (%)
Control		100	Control		100
EDTA	1×10^{-3}	4	AP-I	100	100
<i>o</i> -phenanthroline	1×10^{-3}	84	API-2b	100	16
DFP	2×10^{-2}	8	API-2c	100	11
PCMB	1×10^{-3}	84	S-SI	100	70
MIA	1×10^{-3}	95	SBTI	100	100
SDS	1×10^{-3}	100			
TPCK	1×10^{-3}	100			
TLCK	1×10^{-3}	100			

is interesting in connection with some reports^{9,10)} on S-SI-resistant alkaline protease.

Reactivation of EDTA-treated protease with Ca^{2+} ion

The stabilizing effect of Ca^{2+} ion has been reported with many bacterial proteases,¹¹⁻¹⁵⁾ therefore, reactivation of EDTA-treated protease with Ca^{2+} ion was examined. After the enzyme was incubated with or without (control) final 5 mM EDTA at 0°C or 37°C for 10 min, CaCl_2 solution was added to make a final concentration of 25 mM to the mixture which was assayed for the protease activity. As shown in Table IV, Ca^{2+} -free enzyme completely lost its activity by incubation with EDTA at 0°C for 10 min, but followed by addition of a sufficient amount of Ca^{2+} ion at 0°C, the enzyme regained activity. The fact suggested that calcium atoms, which are essential for its activity, might be contained in the enzyme molecule. The irreversible inactivation of EDTA-treated enzyme at 37°C was perhaps due to autolysis of unstable Ca^{2+} -free enzyme.

TABLE IV. REACTIVATION OF EDTA-TREATED PROTEASE WITH Ca^{2+}

The activity is shown as a relative value to that obtained from the buffer without EDTA.

	EDTA at 0°C	EDTA at 37°C	Buffer at 0°C or 37°C
Ca^{2+} added	86	3	100
Ca^{2+} not added	5	7	100

Time course changes of API-2b→API-2c conversion by the purified protease

Time course changes of API-2b→API-2c conversion by the purified converting protease were demonstrated. As shown in Fig. 12, API-2b was converted to API-2c perfectly in 16 hr under the conditions described in the figure.

During this conversion, a band of EI complex (converting protease-API-2b complex) was observed in the gel, which is marked by an asterisk.

There have been some reports¹⁶⁻¹⁸⁾ on

FIG. 12. API-2b→API-2c Conversion by the Purified Protease.

One half mg of API-2b was added to 2 ml of 0.02 M Tris-HCl buffer (pH 9.0) contg. 5 mM CaCl_2 with 2 mg of the converting protease. Reaction mixture was incubated at 28°C. Using the reaction mixture after incubation for the indicated time, disc electrophoresis was carried out at pH 8.0.

enzymes which do not always follow the classification by Hartley.¹⁹⁾ Among them, Sfericase, an alkaline protease from *Bacillus sphaericus* is reported to be inhibited by DFP and EDTA. The API-2b→API-2c converting protease shows similar behavior in the sensitivity to DFP and EDTA; however, it is clearly different from Sfericase in the optimal temperature on both activity and stability. All data reported above suggest that the converting protease from *S. griseoincarnatus* strain No. KTo-250, an API-2 producer, is a unique alkaline protease.

Examination of amino acid freed from the amino-terminal part in API-2b by the enzyme is now in progress. Also amino acid composition, metal contents and substrate specificity of the protease will be described in the

next paper.

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