Formation and Dissociation of Complex between Alkaline Protease Inhibitor (API-2c) and Subtilisin BPN'[†]

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API-2c, one of the alkaline protease inhibitors produced by *Streptomyces griseoincarnatus* strain No. KTo-250, interacts with subtilisin BPN'. The API-2c-subtilisin BPN' complex was isolated by gel filtration on Sephadex G-100. Its homogeneity was determined by disc electrophoresis. From the results of molecular weight determination of the complex (MW 86,000) and of inhibitory assay of the API-2c released from the complex, it was shown that the complex was composed of one mole (two subunits) of API-2c and two moles of subtilisin BPN'.

The inhibitory activity was gradually released from the complex by dialysis against 0.05 N HCl at 4°C. The inhibitor released showed a different mobility from virgin API-2c. These results suggest that API-2c was modified to API-2c* which peptide linkage in the reactive site was specifically cleaved.

In the previous papers,^{1,2)} we reported that a strain (KTo-250) of *Streptomyces griseoincarnatus* produced three kinds of protease inhibitors designated as API-2a, API-2b and API-2c in the culture filtrate. Purification procedures and some properties of API-2b and API-2c have also been described. The strain produced API-2b→API-2c converting protease³⁾ and API-2 degrading protease⁴⁾ which were thought to be concerned in the fate of API-2 in the growing system. Purification procedures and some properties of these enzymes have also been described.

In the present paper, the interaction of API-2c with subtilisin BPN' was examined, and formation and dissociation of the complex between API-2c and subtilisin BPN' are mainly described.

MATERIALS AND METHODS

Materials. API-2c used in this work was prepared from the culture filtrate of *Streptomyces griseoincarnatus* strain No. KTo-250 as described in the previous paper.²⁾ Its homogeneity was determined by disc electrophoresis.

Subtilisin BPN' (Nagase Biochemicals, Ltd.), Sephadex G-100 (Pharmacia Fine Chemicals), sodium laurylsulfate, acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine (these four reagents from Nakarai Chemicals, Ltd.) were purchased from the respective companies.

Determination of protein concentration. The concentrations of API-2c and subtilisin BPN' were determined with a Hitachi 124 spectrophotometer taking the extinction coefficients as $E_{1\%}^{1\text{cm}, 280\text{nm}} = 8.7$ and 11.7, respectively.

Polyacrylamide gel disc electrophoresis. Polyacrylamide gel disc electrophoresis was carried out according to the method of Davis⁵⁾ at pH 8.0 using a 7% gel. Sodium dodecyl sulfate (SDS)-polyacrylamide gel disc electrophoresis was performed using a 14.5% gel. Samples were incubated, before electrophoresis, at room temperature for 24 hr in 0.01 M phosphate buffer (pH 7.2) containing 1% SDS in the presence of 2-mercaptoethanol. The gels were stained with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (5:1:5, v/v), and destained with 7.5% acetic acid in 5% methanol.

Assay for the inhibitory activity. Assay procedure for the inhibitory activity was the same as that described in the previous paper.¹⁾ Other methods were also the same as those described in the previous papers.^{1~4)}

[†] Studies on Alkaline Protease Inhibitor (API-2) Produced by *Streptomyces griseoincarnatus* Strain No. KTo-250. Part V. For Parts I~IV, see refs. 1~4.

RESULTS AND DISCUSSION

Molecular weight determination of API-2c

The molecular weight of API-2c in its native state was estimated by gel filtration on Sephadex G-100 as about 32,000.²⁾ To determine the molecular weight of its subunit, SDS-polyacrylamide gel disc electrophoresis was performed at 7 mA per gel column for 6 hr at room temperature in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. In Fig. 1, relative mobilities were plotted against the logarithm of molecular weight for each reference protein and API-2c. From the results, an approximate molecular weight of 11,000 was obtained for the subunit of API-2c. This value was virtually the same as the minimal molecular weight calculated from the amino acid composition.²⁾

FIG. 1. Molecular Weight Determination of API-2c by SDS-Polyacrylamide Gel Disc Electrophoresis.

●, insulin (MW 5,730); ▲, cytochrome *c* (MW 12,400); ■, trypsin (MW 24,000); ○, ovalbumin (MW 46,000); ⊙, API-2c.

Determination of inhibitory equivalent of API-2c to subtilisin BPN'

Inhibitory equivalent of API-2c to subtilisin BPN' was determined by measuring enzyme inhibition at various inhibitor concentrations.

API-2c was dissolved in 0.01 M boric acid-borax buffer (pH 9.0). One ml of each solution was mixed with 1 ml of a subtilisin BPN' solution containing 10.7 μ g. After preincubation for 5 min at 37°C, each 1 ml of 2% casein and 0.2 M borax-NaOH buffer (pH 9.5) was added. After incubation for 15 min, the

FIG. 2. Determination of Inhibitory Equivalent of API-2c to Subtilisin BPN'.

See the text for experimental details.

residual proteolytic activity was determined. For these experiments, crystalline subtilisin BPN' was dialyzed against distilled water to be free from the denatured enzyme before use. As shown in Fig. 2, 1 μ g of API-2c inhibited 2.6 μ g of the enzyme. Thus, considering the molecular weights of API-2c subunit (MW 11,000) and subtilisin BPN' (MW 27,700), one subunit of API-2c inhibits one molecule of the enzyme.

Isolation of API-2c-subtilisin BPN' complex

API-2c (5 mg) was dissolved in 0.02 M boric acid-borax buffer (pH 7.5) containing 0.1 M KCl. To this solution was added an enzyme solution containing 20 mg of subtilisin BPN' in the same buffer. The reaction mixture was allowed to stand for 1 hr at 37°C, and then applied to a Sephadex G-100 column previously equilibrated with 0.02 M boric acid-borax buffer (pH 7.5) containing 0.1 M KCl. The column was eluted with the same buffer. Figure 3 shows the elution pattern. The main peak showed almost neither proteolytic activity nor inhibitory activity. Even after being heated in a boiling water bath for 10 min, the main peak showed little inhibitory activity. However, after treatment with 0.05 N HCl, as described in the subsequent section, the peak recovered almost all the original inhibitory activity. Thus, the main peak was regarded as to contain an API-2c-subtilisin BPN' complex. Fractions corresponding to the main peak were pooled and added solid ammonium sulfate to 80% saturation. After standing

FIG. 3. Gel Filtration of the Reaction Mixture of API-2c and Subtilisin BPN' on Sephadex G-100.

Buffer, 0.02 M boric acid-borax buffer (pH 7.5) containing 0.1 M KCl; flow rate, 10 ml/hr; column size, 1.8×105 cm; fraction size, 3 ml. Amounts of enzyme and inhibitor were 20 mg and 5 mg, respectively.

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

FIG. 5. Molecular Weight Determination of API-2c-Subtilisin BPN' Complex on Sephadex G-100 Column.

●, cytochrome *c* (MW 12,400); ▲, AP-I (MW 27,500); ■, ovalbumin (MW 46,000); ○, the complex.

FIG. 4. Polyacrylamide Gel Disc Electrophoresis of API-2c and API-2c-Subtilisin BPN' Complex.

overnight at 4°C, the precipitate was collected by centrifugation, dissolved in a minimal volume of distilled water, and dialyzed against water to remove any salt, and then lyophilized.

Homogeneity of the lyophilized API-2c-subtilisin BPN' complex was examined by polyacrylamide gel disc electrophoresis. As shown in Fig. 4, the complex migrated as a single, clear band.

Molecular weight of API-2c-subtilisin BPN' complex

The molecular weight of the API-2c-

subtilisin BPN' complex was estimated according to the method of Andrews⁶ with a Sephadex G-100 column. Gel filtration was carried out at 4°C in 0.02 M boric acid-borax buffer (pH 7.5) containing 0.1 M KCl. In Fig. 5, the ratio of elution volume to void volume is plotted against the logarithm of molecular weight for each reference protein and the complex. From the results, an approximate molecular weight of 86,000 was obtained for the complex. It was thus suggested that the complex was composed of two mol of subtilisin BPN' and one mol (two subunits) of

API-2c.

Dissociation of API-2c-subtilisin BPN' complex

To find the best conditions for dissociation of the API-2c-subtilisin BPN' complex, it was treated under various conditions. One half ml of the complex solution was incubated at various temperatures, and then mixed with 0.5 ml of 0.1 N HCl. After neutralization with 1 ml of 0.2 M borax-NaOH buffer (pH 9.5), inhibitory activity was assayed. As Table I shows, more API-2c was released from the complex with 0.05 N HCl at 28°C than at other temperatures examined.

TABLE I. DISSOCIATION OF API-2c-SUBTILISIN BPN' COMPLEX

	Temp. (°C)	pH	Time (min)	Recovery of inhibitory activity (%)
0.05 N HCl	0	1.5	60	85
0.05 N HCl	21	1.5	60	85
0.05 N HCl	28	1.5	60	94
0.05 N HCl	37	1.5	60	58
No HCl	100	7.5	10	12

API-2c-subtilisin BPN' complex (1 mg) was dissolved in 0.5 ml of distilled water. The solution was incubated at 28°C and mixed with 0.5 ml of preincubated 0.1 N HCl. At the indicated incubation times at 28°C, inhibitory activity of the mixture was assayed after neutralization. As shown in Fig. 6, dis-

FIG. 6. Release of API-2c from the Complex in 0.05 N HCl at 28°C.

One half ml of the aqueous complex solution was mixed with 0.5 ml of 0.1 N HCl and incubated at 28°C for indicated period of time. Then 1 ml of 0.2 M borax-NaOH buffer (pH 9.5) was added to the reaction mixture and the quantity of API-2c released from the complex was assayed by inhibitory activity.

○—○, 1,000 μg of the complex; ●—●, 400 μg of API-2c (control).

sociation completed within 30 min, and 385 μg (1.2×10^{-8} mol) of API-2c was released from 1,000 μg of the complex. If the remainder is regarded as subtilisin BPN', it amounted to 615 μg (2.2×10^{-8} mol). These results support the view that API-2c-subtilisin BPN' complex is composed of one mol (two subunits) of API-2c and two mol of subtilisin BPN'.

Electrophoresis of API-2c released from API-2c-subtilisin BPN' complex

The API-2c released from the API-2c-subtilisin BPN' complex under the conditions described above was compared with virgin API-2c by polyacrylamide gel disc electro-

FIG. 7. Polyacrylamide Gel Disc Electrophoresis of Virgin and Modified API-2c at pH 8.0.

phoresis. In every case, they showed the same mobility. The API-2c gradually released from the complex by dialysis against 0.05 N HCl at 4°C, however, showed a different mobility from that of virgin API-2c, as shown in Fig. 7. It is thought that API-2c is modified by specific cleavage of peptide linkage in its reactive site.⁷⁾

We will described in detail on this modification and the reactive site of API-2c in the next paper.⁸⁾

REFERENCES

- 1) M. Uyeda, K. Suzuki, H. Uwatoko and M. Shibata, *Agric. Biol. Chem.*, **42**, 49 (1978).
 - 2) K. Suzuki, M. Uyeda and M. Shibata, *ibid.*, **42**, 1539 (1978).
 - 3) M. Uyeda, K. Suzuki, M. Sugiyama and M. Shibata, *ibid.*, **43**, 1849 (1979).
 - 4) K. Suzuki, M. Uyeda, M. Sugiyama and M. Shibata, *ibid.*, **44**, 113 (1980).
 - 5) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
 - 6) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
 - 7) M. Uyeda and K. Suzuki, *Agric. Biol. Chem.*, **44**, 699 (1980).
 - 8) K. Suzuki, M. Uyeda, K. Ookubo and M. Shibata, *ibid.*, **44**, 2555 (1980).
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