Complex Formation of Alkaline Protease Inhibitor (AP-I) with Subtilisin BPN’ and Its Properties

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El complex formation of AP-I, existing as dimer, with subtilisin BPN’ was investigated in detail.

By changing the E/I2 ratio in the reaction mixture, two types of El complex were recognized; E2I2 complex which was composed of two moles of subtilisin BPN’ and one mole (two subunits) of AP-I, and EI2 complex which was composed of one mole of the enzyme and one mole of AP-I. Their existence was demonstrated by gel filtration, inhibitory equivalent, disc electrophoresis and isoleucine content in the complexes.

The former complex is thought to be a final and stable complex, and the latter to be an intermediate or transient type of complex.

In the course of screening for new enzyme affectors, Streptomyces pseudogriseolus strain No. KTo-332 was found to produce alkaline protease inhibitor (abbrev. as AP-I) in the culture filtrate. The purification and crystallization procedures, inhibitory spectrum and some general properties of AP-I were reported in the preceding papers.1,2

To find out the differences between AP-I and S-SI, Streptomyces albogriseolus subtilisin inhibitor reported by Murao et al.,2-7) EI complex formation of AP-I with subtilisin BPN’ was investigated in detail. In this paper, the results of this investigation are mainly described.

MATERIALS AND METHODS

Materials. AP-I used in this work was prepared from the culture filtrate of Streptomyces pseudogriseolus strain No. KTo-332 in accordance with the procedures described in the previous paper,2) and its homogeneity was determined by disc electrophoresis.

Subtilisin BPN’ (Nagase & Co., Ltd.), Sephadex G-100 and G-25 (Pharmacia Fine Chemicals), acrylamide, N, N’-methylenebisacrylamide and N, N, N’, N’-tetramethylenediamine (these three reagents from Nakarai Chemicals, Ltd.) were purchased.

Assay for the inhibitory activity. Assay procedure was the same as that described in the previous paper.1) Other methods were also the same as those described in the previous paper.1-5

RESULTS AND DISCUSSION

Isolation of AP-I-subtilisin BPN’ complex

As already described,2) AP-I was considered to exist as dimer (form of I2) in usual conditions. From the preliminary experiments for AP-I-subtilisin BPN’ complex formation, the E/I2 ratio in the reaction mixture proved to be an important factor; therefore, gel filtration was carried out with respect to mainly three different reaction mixtures: (a) E>I2, (b) E=I2, and (c) E<I2.

General procedures for the formation of EI complex were as follows: Indicated amount of AP-I was dissolved in 0.5 ml of 0.02 M boric acid-borax buffer (pH 7.5) containing 0.1 M KCl. To this solution, 0.5 ml of enzyme solution in the same buffer was added. The reaction mixture was incubated at 37°C for 10 min.

For the isolation of AP-I-subtilisin BPN’ complex, the reaction mixture was applied on a Sephadex G-100 column (1.9 x 90 cm) previously equilibrated, and then eluted with the

† Studies on Alkaline Protease Inhibitor (AP-I) Produced by Streptomyces pseudogriseolus Strain No. KTo-332. Part II. (See Refs. 1), 2)).
same buffer. Amounts of enzyme and inhibitor used in the three cases were 16 mg and 4 mg for E > I₂, 5 mg and 5 mg for E = I₂ or 4 mg and 8 mg for E < I₂, respectively. Figures 1a to 1c show the gel filtration patterns of these reaction mixtures.

In the E > I₂ reaction mixture (Fig. 1a), the main peak, which was eluted first, had no proteolytic activity or inhibitory activity. But, after treatment with 0.05 N HCl, as described in the subsequent section, the main peak recovered almost all inhibitory activity. The second peak in this case was attributed to excess enzyme.

In case of E = I₂ (Fig. 1b), three peaks were recognized. The first peak had the same properties and elution position as the main peak in Fig. 1a, whereas the second peak showed some degree of inhibitory activity, though inhibitory activity which might be due to excess AP-I was detected in the third peak.

As the amount of AP-I (I₂) increased (in case of E < I₂, Fig. 1c), the first peak which was observed in Fig. 1a or 1b decreased, the second peak turned out larger, and the third peak due to excess AP-I showed strong absorbance at 280 nm. The pattern of inhibitory activity showed the same tendency as recorded in Fig. 1b.

By the estimation of the molecular weight carried out in accordance with the method of Andrews,8-9) the first peak in Fig. 1b was determined to be 83,000 and the second, 56,000, and the third, 28,000 (Fig. 2). Thus, considering the molecular weights of AP-I (MW 27,500, by gel filtration) and subtilisin BPN' (MW 27,700), the first peak was attributed to E₂I₂ complex which was composed of two moles of subtilisin BPN' and one mole...
(two subunits) of AP-I, and the second peak, to EI<sub>1</sub> complex which was composed of one mole of the enzyme and one mole of AP-I and therefore retained half inhibitory activity. This type of complex (EI<sub>1</sub>) was not reported by Murao et al. The third peak was ascribed to residual AP-I or subtilisin BPN' depending on the amount added to the reaction mixture.

Fractions corresponding to EI<sub>1</sub>I<sub>2</sub> complex or EI<sub>2</sub> complex were combined and concentrated with collodion bag. Each complex solution was applied on a Sephadex G-25 column to be free from salts, and lyophilized.

**Determination of inhibitory equivalent of AP-I to subtilisin BPN'**

Various concentrations of AP-I were prepared by dissolving in 0.01 M boric acid-borax buffer (pH 9.0). One ml of each solution was mixed with 1 ml of subtilisin BPN' solution containing 14.75 µg. After preincubation for 5 min at 37°C, each 1 ml of 2% casein solution and 0.2 M boric acid-NaOH buffer (pH 9.5) was added. After incubation for 15 min, the residual proteolytic activity was determined. For these experiments, crystalline BPN' was purified by gel filtration with Sephadex G-50 to be free from denatured enzyme before use. As shown in Fig. 3, 1 µg of AP-I proved to inhibit 1.93 µg of the enzyme. Thus, considering the molecular weights of AP-I (MW 27,500) and subtilisin BPN' (MW 27,700), one mole (two subunits) of AP-I inhibited two moles of the enzyme. These results are in good accordance with those reported and discussed in the preceding paper<sup>2</sup> using alkaline protease from Taka-diaistase.

**Transition of EI<sub>1</sub> complex to E<sub>2</sub>I<sub>2</sub> complex**

To obtain more EI<sub>2</sub> complex, gel filtration was carried out after the E<sub>1</sub>I<sub>2</sub> reaction (Fig. 4a). Fractions corresponding to EI<sub>2</sub> (Fraction Nos. 36~41) were combined and concentrated to 2 ml. The solution was refiltered through the same column of Sephadex G-100 under the same conditions. As recorded in Fig. 4b, EI<sub>2</sub> complex showed a tendency to change E<sub>2</sub>I<sub>2</sub> complex and residual I<sub>2</sub>. The result suggested that EI<sub>1</sub> complex was not stable in the solution and that this was an intermediate or transient form to E<sub>2</sub>I<sub>2</sub> complex.

Furthermore, the phenomenon described above was demonstrated by polyacrylamide gel disc electrophoresis. As shown in Fig. 5, isolated EI<sub>1</sub> complex changed to E<sub>2</sub>I<sub>2</sub> and I<sub>2</sub> during electrophoresis, though it was still
remaining as obscure band in the gel. Considering the factor of reaction equilibrium, excess I₂ was added to EI complex immediately before electrophoresis. In this case, a clear band of EI was recognized.

**Dissociation of E₂I₂ complex or EI₂ complex**

Dissociation test was carried out in accordance with the method of Murao et al. After treatment with 0.05 N HCl, E₂I₂ complex recovered 100% of inhibitory activity and EI₂ complex which originally retained 50% of activity recovered 100% of activity as shown in Table I. Moreover, the same result was obtained in the same test using top peak fraction in the fractions corresponding to EI₂ complex immediately after gel filtration.

**Isoleucine content**

As reported in the preceding paper, AP-I contained no isoleucine, and therefore isoleucine detected in the complex was considered to have originated not from AP-I but from subtilisin BPN'. The amino acid composition of subtilisin BPN' was determined by Matsubara et al., and the isoleucine content was decided to be 13 residues in a molecule. From amino acid analysis, isoleucine residues in E₂I₂ or EI₂ complex were determined to be 20 or 10 residues, respectively, though they may be calculated at 26 or 13 theoretically (Table I). More detailed analysis will be carried out to clarify the disaccordance of the numbers of isoleucine residues. As far as we suppose, isoleucine content in E₂I₂ complex is twice that in EI₂ complex; that means really two moles of enzyme exist in the former complex and one mole in the latter.

**Hypothetical state of EI complex formation between AP-I and subtilisin BPN’**

From these results obtained above, we propose the hypothetical state of EI complex formation between AP-I existing as dimer (I₂) and subtilisin BPN' as shown in Fig. 6.

\[
E + I₂ \rightarrow E₂I₂ \quad (1)
\]
\[
E₂I₂ + E \rightarrow E₂I₂ \quad (2)
\]

One mole of subtilisin BPN' combines with AP-I (I₂) to form EI₂ complex (reaction (1)), and EI₂ complex combines with another mole of the enzyme (reaction (2)). In these reactions, E₂I₂ complex is thought to be a stable complex, and EI₂ complex to be an intermediate or transient type of complex.

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<thead>
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<th>Table I. Comparison of EI Complexes with AP-I (I₂)</th>
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<tbody>
<tr>
<td>E₂I₂</td>
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<tr>
<td>Molecular weight</td>
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<tr>
<td>Relative inhibitory activity (%)</td>
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<tr>
<td>Relative inhibitory activity (%) after treatment with 0.05 N HCl</td>
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<td>Isoleucine residue per mole</td>
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E: Subtilisin BPN'

¹ Numbers in parenthesis refer to theoretical numbers of residues based on the data by Matsubara et al.
Studies on Microbial Alkaline Protease Inhibitor (AP-I)

REFERENCES

3) S. Murao, S. Sato and N. Muto, ibid., 36, 1737 (1972).