Temperature-dependence of the Reconstitution of an Adzuki Bean Subtilisin Inhibitor from Its Inactive Two Fragments

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Adzuki bean subtilisin inhibitor ASI-II is classified into “potato inhibitor I family” based on sequence homology. The members of this family have been confirmed to require no intramolecular disulfide linkage to retain a suitable conformation for enzyme inhibition. ASI-II consists of a single polypeptide chain with 73 amino acid residues, and its reactive site peptide bond is Ala(49)-Asp(50). The reactive site peptide bond of ASI-II can be readily cleaved with subtilisin during a reversed-phase HPLC of the enzyme-inhibitor complex using a trifluoroacetic acid-acetonitrile elution system. Since ASI-II have no intramolecular disulfide linkage, the inhibitor is converted into two fragments after the limited cleavage at its reactive site. One of the fragments corresponds to the region from the amino-terminal Lys to the reactive site P, Ala, and the other fragment, to the region from the reactive site P, Asp to the carboxyl-terminal Gly. Although each fragment has no effect on subtilisin activity, an equimolar mixture of both inactive fragments inhibits the target enzyme to the same extent as the intact ASI-II.

In this paper, we describe the temperature-dependence of the reconstitution of the active subtilisin inhibitor from the two inactive fragments. The amino- and carboxyl-terminal side fragments of the reactive site-modified ASI-II (N- and C-fragments, respectively) were prepared as described in our previous paper. The amounts of intact ASI-II and both fragments were measured by amino acid analysis after 24-h hydrolysis of these peptides with redistilled HCl in evacuated tubes. Inhibitory activity for subtilisin BPN' was measured by the remaining enzyme activity by the casein–Folin method, on a 1/10 scale at 30°C in 0.1 M Na-phosphate buffer (pH 7.6) using 0.5% Hammarsten's casein as a substrate.

Figure 1 shows the increase with time of the anti-subtilisin activity of an equimolar mixture of the fragments incubated at different temperatures. At 2°C, full inhibitory activity was restored after 5 h incubation, and at 15°C after 50 min, under the experimental conditions. With elevation of temperature, the rate of recovery of inhibitory activity became faster, but the extent was reduced. At 30°C, where the activity of intact ASI was taken as a control, the final level of inhibitory activity recovered decreased to 68%.

When an equimolar mixture of the N- and C-fragments with full

![](image)

**Fig. 2. Temperature-dependence of the Anti-subtilisin Activity of the Reconstituted Modified ASI-II.

After an equimolar mixture of the N- and C-fragments with full inhibitory activity, which had been prepared by incubation of both fragments for 7 h at 2°C, was further incubated at different temperatures, up to 50°C for 30 min, the anti-subtilisin activity was measured as described in the legend to Fig. 1 (●). The inhibitory activity of the intact ASI-II, incubated under the same conditions, was also measured (○).

![Graph]

**Fig. 3. The Effects of Incubation Time with Subtilisin on the Inhibitory Activity of the Equimolar Mixture of the N- and C-Fragments.

Both fragments were mixed in 0.1 M Na-phosphate buffer of pH 7.6 (50 pmol each). After incubation at 2°C, for either 7 h (○), or 1 min (●), the mixture was added to subtilisin at the molar ratio of 1.0 (enzyme/inhibitor). After incubation with the enzyme at 30°C for the indicated times, the remaining enzyme activity was measured. The inhibitory activity of intact ASI-II measured under the same conditions was referred to as 100% inhibition.

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*Abbreviations: ASI, adzuki bean subtilisin inhibitor; N-fragment, amino-terminal side fragment of reactive site-modified ASI-II; C-fragment, carboxyl-terminal side fragment of reactive site-modified ASI-II.*
inhibitory activity, which had been prepared by incubation at 2°C, was further incubated at different temperatures for 30 min, its anti-subtilisin activity was reduced proportionally to the elevation of incubation temperature (Fig. 2). This fact might be ascribed either to the shifting of equilibrium between the dissociated inactive and the associated active forms of the N- and C-fragments, or to the increased formation of some associated but inactive forms of the fragments. In contrast, intact ASI-II showed constant and full activity when incubated under the same conditions. Thus, it was indicated that the reconstitution of the active inhibitor was temperature-dependent.

We also examined the effects of subtilisin on the reconstitution of the active inhibitor from the two inactive fragments (Fig. 3). The fully activated mixture of the fragments, prepared by an incubation at 2°C for 7 h, had 100% anti-subtilisin activity even after incubation for only 1 min at 30°C with the enzyme, in the same way as the intact ASI. The complex of the fully-activated mixture of the fragments with subtilisin was stable under the experimental conditions. On the other hand, we prepared a 10%-activated mixture of the fragments by incubation at 2°C for 1 min. When this mixture was incubated with the target enzyme at 30°C, there was no increase with time in the enzyme inhibition. The anti-subtilisin activity of the equimolar mixture of the two inactive fragments could be restored up to 68% by incubation of the mixture at 30°C, in the absence of subtilisin (Fig. 1). These results indicated that subtilisin prevented the N- and C-fragments from associating to form an active inhibitor. It is possible that the dissociated inactive fragments were digested into small peptides by the enzyme.

In many cases, the inhibitory activity of the reactive site-modified serine proteinase inhibitors can be restored almost completely when they are incubated with their target enzymes for a long time.11 As for ASI-II, however, this study indicated that the N- and C-fragments of the inhibitor could be reconstituted into an active form by incubation without the target enzyme. Both fragments associated with each other to form a fully-active inhibitor when they were incubated below 15°C at a neutral pH; in these conditions, they inhibited subtilisin as strongly as did the intact ASI-II.

References