Short Communication

Formation of Complex of Proteinous
Animal α -Amylase Inhibitor
(Haim) with Hog Pancreatic
 α -Amylase

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Novel proteinous inhibitors (Haim I, II) of *Streptomyces griseosporus* YM-25, inhibit animal α -amylase specifically.¹⁾ As Haim is a protein containing no sugar moiety, the inhibition of Haim against the amylase was considered to be attributed to the result of protein-protein interaction. On the other hand, S-AI,²⁾ an amylase inhibitor produced by *St. diastaticus* subsp. *amylostaticus*, inhibits various α -amylases of animal, plant, and microbial origin. S-AI consists of 6 mol of glucose with unknown moiety tentatively named as S-AI-X.³⁾ S-AI may be classified as a substrate analog inhibitor.

In this paper, we described the interaction among hog pancreatic α -amylase (HPA), proteinous inhibitor (Haim), and XG* derived from S-AI, as a substrate analog.

First of all, the inhibitory equivalence of Haim to HPA was investigated by measuring the extent of enzyme inhibition as a function of the inhibitor concentration. A mixture of 0.5 ml of HPA ($2 \times 10^{-4}\%$; Sigma, twice cryst.; liberating 1 g maltose from soluble starch/mg/min at 37°C) solution in 10 mM Tris-HCl-5 mM CaCl_2 buffer (pH 7.0) and 0.5 ml of various concentrations of Haim I solution was incubated at 37°C. After 5 min of incubation, the residual α -amylase activity was

determined by the iodine staining method reported previously.²⁾ A good linear relationship was observed between the inhibitory ratio and the inhibitor concentration until the inhibitory ratio showed about 80%. Even if the inhibitor concentration increased more than ten-fold, the inhibitory ratio did not reach to 100%. But extrapolation to 100% inhibition of the amylase activity gave a rough estimation of a quantity of Haim corresponding to the theoretical point of complete inhibition. The result indicated that 1 μg of HPA was inhibited by 0.16 μg of Haim. In other words, 1.9×10^{-11} mol of the inhibitor inactivated 2×10^{-11} mol of the α -amylase, on the basis of the molecular weight of the α -amylase as 51,000⁵⁾ and that of the inhibitor as 8,500.¹⁾

The formation of Haim-HPA complex was demonstrated as follows: A mixture of Haim of 1.18×10^{-7} mol and HPA of 5.6×10^{-8} mol in 10 mM Tris-HCl-5 mM CaCl_2 buffer (pH 7.0) was incubated at 37°C for 1 hr, and the mixture was applied to a column of Bio-Gel P-100 (2.5×41 cm) equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. This chromatogram is shown in Fig. 1. The first peak fraction was eluted between the void volume and the eluting point of HPA, associating with the amylolytic activity, but it showed no inhibitory effect on HPA activity. However, heating the first peak fraction in boiling water for 2 min after the pH was adjusted to 3.5 with HCl, it produced the inhibitory activity against HPA. The first peak fraction was thus found to be a Haim-HPA complex. On the other hand, the second peak fraction which had the inhibitory activity, was found to be Haim remaining as free form. The amount of this free form of Haim was estimated to be about 5×10^{-8} mol. The stoichiometry of inhibition of HPA by Haim was calculated to be 1:1 of each molecule.

The interaction among HPA, Haim, and XG was also investigated. XG inhibited various glucoside hydrolases but with different degrees. The inhibitory activity of Haim was distinguishable from XG, by the different specificity toward various glucosidases. Thus

* XG: XG consisted of 1 mol of S-AI-X³⁾ and 1 mol of glucose and the reducing end of XG was glucose. XG was obtained from S-AI²⁾ by enzymatic and/or chemical procedures.⁴⁾ The molecular weight of XG was about 500.

FIG. 1. Bio-Gel P-100 Gel Filtration Elution Profile of HPA Incubated with Haim.

The mixture of HPA and Haim was applied to a column (2.5 × 41 cm) of Bio-Gel P-100 equilibrated 25 mM phosphate-0.1 M NaCl buffer (pH 7.0). Fraction volume was 3.6 ml. The arrow shows the position of elution of HPA.

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

the experiments to elucidate formation of a complex between HPA and XG, and between HPA-XG and Haim were performed (Fig. 2). One mg of XG and 3.2 mg of HPA were dissolved in 2 ml of 10 mM Tris-HCl-5 mM CaCl_2 buffer (pH 7.0), and incubated for 1 hr at 37°C. The mixture was subjected to gel filtration on a Bio-Gel P-100 column equilibrated with 25 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, and eluted with the same buffer. Figure 2(a) shows the gel filtration pattern of the mixture. The protein was eluted at a identical position with that of HPA, associating with amylolytic activity. When the pH of this fraction was adjusted to 1 with 1 N HCl and heated in boiling water for 10 min, the inhibitory activity of XG was recovered. This fact indicated the formation of HPA-XG complex. the HPA-XG complex isolated was concentrated *in vacuo*, mixed with 1 mg of Haim, and incubated at 37°C for 1 hr. The mixture was then subjected to gel filtration on a column of Bio-Gel P-100 under the same conditions as above. As shown in Fig. 2(b), the first peak fraction appeared associating with amylolytic activity, which was eluted between the void volume of the column and the elution position for HPA. Heating the protein fraction obtained as described above, it resulted in

FIG. 2. Gel Filtration for Identification of Triple Complex Formation.

The conditions of the gel filtration were the same as in Fig. 1. (a), elution pattern of the complex, XG-HPA; (b), elution pattern of the triple complex, XG-HPA-Haim.

○—○, absorbance at 280 nm; ●—●, inhibitory activity of Haim; ○---○, inhibitory activity of XG.

recovery of the inhibitory activities of both Haim and XG. The second peak fraction in Fig. 2(b) was Haim of a free form. The XG activity due to free XG was not detected in any fraction. These results clearly indicated that the first peak fraction was a triple complex of XG-HPA-Haim. However, the results also showed that XG did not bind the Haim-HPA complex.

Inferring the results from the triple interaction among Haim, XG, and HPA, Haim should join at a neighborhood of a catalytic site or at a situation like a allosteric site of HPA.

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