Evaluation of Subsite Affinities of Soybean β -Amylase by Product Analysis

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Subsite affinities of soybean β -amylase were evaluated by a new method to elucidate the effect of multiple attack. Product analysis of the reducing end-labeled maltooligosaccharides showed that G_6^* and G_7^* were degraded through the multiple attack pathway, in 0.02 M acetate buffer, pH 5.4 at 25°C. The apparent first-order rate constants, $(k_0/K_m)e_0$, for n -mer substrates ($n = 3 - 7$) were obtained from the decrease of substrate followed by paper chromatography under the condition of $[S] \ll K_m$. Using the constants and the cleavage distribution of G_3^* , the subsite affinities (A_1 , A_4 and A_5) of β -amylase were evaluated. It was revealed that the first subsite of soybean β -amylase had a larger subsite affinity than any subsites of other amylases so far evaluated. Thus the first subsite seems to play an important role for the characteristic action pattern of this enzyme, namely exclusive maltose-forming action. The frequency of multiple attack decreased as either or both pH and temperature become unfavorable for enzymic activity.

β -Amylase is found mainly in higher plants. This enzyme has a characteristic exo-type action pattern such that the enzyme attacks the nonreducing terminus of starch or its related substrates with the scission of the penultimate linkage with exclusive formation of β -maltose.^{1,2)} Many investigators have paid attention to its degradative pattern, including multiple (repetitive) attack^{3~11)}. After the initial catalytic reaction, the product remains on the enzyme surface to give a new productive complex, and such subsequent attack may be repeated several times before the enzyme and remaining substrate dissociate. This type of mechanism is found in the action of other amylases on amylose.^{12~18)}

Recently a unified theory was developed to analyze quantitatively the action patterns of various amylases in terms of "subsite affi-

nities." Subsite affinities are represented as the unitary free energy decrements upon binding of the glucose residue of substrate to individual subsites constituting the active site of an amylase.^{19~26)} The subsite theory is essentially applicable to all amylase reactions, but slight modification is necessary for enzyme reactions accompanied with the multiple attack and/or the bi-substrate processes such as transglycosylation and condensation, since these factors complicate the meaning of experimentally obtainable rate parameters. In the preceding paper,²⁶⁾ we showed the effect of the bi-substrate processes could be eliminated by using the apparent first order rate constant, $(k_0/K_m)e_0$, obtainable at sufficiently low substrate concentrations ($[S] \ll K_m$).

In this paper, we evaluate the subsite affinities of soybean β -amylase, using the apparent first order rate constants, $(k_0/K_m)e_0$, obtained from the measurement of the decrease in the starting substrates (G_3 - G_7^{*1}), so that the effect of the multiple attack may be eliminated.

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*1 Abbreviations: G_3 , maltotriose; G_4 , maltotetraose; G_5 , maltopentaose; G_6 , maltohexaose; G_7 , maltoheptaose.

MATERIALS

Crystalline soybean β -amylase was prepared from defatted soybean flour (supplied by Honen Seiyu Co. Ltd., Tokyo) according to the method of Morita *et al.*²⁷⁾ Two components (components I and II) were obtained.²⁷⁻²⁹⁾ The concentration of the enzyme was estimated spectrophotometrically, using the value of absorption coefficient, $E_{280\text{ nm}}^{1\%}$, of 17.0 cm^{-1} and the molecular weight of 57,000, for both components I and II.²⁹⁾ No contamination of α -glucosidase was confirmed by using the radioactive maltose as substrate; maltose was not hydrolyzed by sufficiently high concentration of the enzyme preparations to hydrolyze 96.7% of G_3 in a reaction period.

Maltotriose and other maltooligosaccharides labeled in the reducing end glucose unit were prepared with ^{14}C -glucose (the Radiochemical Centre, England) and cyclohexaamylose (Hayashibara Biochemical Laboratories, Inc., Okayama) by means of the coupling reaction of *B. macerans* transglycosylase.³⁰⁾ Each labeled oligosaccharide was purified by multi-ascending paper chromatography and re-chromatographed more than once to obtain pure saccharide. The purity of the radioactive maltotriose obtained was 99.76%.

METHODS

The experimental procedure with the reducing end-labeled maltooligosaccharide to determine the apparent first order rate constant, $(k_0/K_m)e_0$, is essentially the same as the one in the preceding paper.²⁶⁾ Component II, which is the major component of soybean β -amylase was used for this study. The enzyme reaction was performed in a microtube in 0.02 M acetate buffer, pH 5.4, at 25°C , and was terminated with 0.05 M NaOH after appropriate time intervals. The reaction products were separated by multiple ascending paper chromatography.³¹⁾ After irrigation twice, the strip of paper was cut into segments and their radioactivities were counted for 5 min with a Packard 3320 liquid scintillation spectrophotometer. The counts were corrected for background and substrate blank.

RESULTS AND DISCUSSION

Estimation of the rate parameter (k_0/K_m) by the product analysis

Figure 1 shows typical examples of the time courses of change in substrate and product saccharides in the degradation of the reducing end-labeled maltohexaose G_6^* and maltoheptaose G_7^* . Both products G_2^* and G_4^* increased simply in progress of the degradation of G_6^* . The degradation of G_7^* produced G_3^* and G_5^* .

The enzyme cleaved the nonreducing ter-

FIG. 1. Time Courses of Degradation of the Reducing End-labeled Maltohexaose (G_6^*) (Left) and Maltoheptaose (G_7^*) (Right) Catalyzed by Soybean β -Amylase.

The reaction was performed in 0.02 M acetate buffer, pH 5.4, at 25°C . $e_0 = 3.65 \times 10^{-10}\text{ M}$, $[G_6]_0 = 25\text{ }\mu\text{M}$, $[G_7]_0 = 12\text{ }\mu\text{M}$. The radioactivity of each section of paper cut-off is represented as a fraction of the total radioactivity in the reaction mixture taken for analysis.

minus of substrate to produce maltose, as illustrated in Fig. 1. Unless multiple attack (repetitive attack on the same substrate molecule) happened, G_2^* would not be produced from G_6^* before the primary product G_4^* (produced by the initial attack on G_6^*) accumulates in the reaction system. Since G_4^* was hydrolyzed more slowly than G_6^* , time course of G_2^* production was thought to have a lag phase. The lag phase, however, was not observed in the time course of G_2^* production as shown Fig. 1. Thus, the multiple attack is an essential property of action of β -amylase. The results also indicated that G_4^* , the primary product from G_6^* , was produced faster than G_2^* , the secondary product, whereas G_3^* , the secondary product from G_7^* , overwhelmed G_5^* , the primary product (Fig. 1). Smaller substrates than G_5^* produced single labeled products, respectively. Therefore, the frequency of multiple attack was considered to depend on the degree of polymerization (n) of the substrate.

When the multiple attack mechanism is operating, the following equation should hold;

$$d[P]/dt = -(d[S]/dt) \times F \quad (1)$$

where $[P]$ and $[S]$ represent molar concentration of maltose produced and that of the

starting substrate, respectively. F is the frequency of the multiple attack ($F > 1$). Thus the rate measured by the reducing power, which refers to the total molar concentration of the products, $[P]$, is not equal to the rate of decrease of the starting substrate, $[S]$, that is, $d[P]/dt \neq -d[S]/dt$.

We estimated the real reaction rate by means of measurement of the remaining substrate which was free from the effect of multiple attack. The hydrolytic reaction of the enzyme followed the first-order kinetics, since the reaction was performed under the condition of $[S] \ll K_m$. The value of K_m estimated by Kato *et al.*^{32a)} was tentatively used. Under this condition, bi-substrate reaction such as transglycosylation or condensation is eliminated. Thus,

$$\begin{aligned} -\frac{d[S]}{dt} &= \frac{V_{\max}[S]}{K_m + [S]} \cong (V_{\max}/K_m)[S] \\ &= (k_0/K_m)e_0[S] \end{aligned} \quad (2)$$

The first-order plot for a starting substrate G_7^* , where the plot was obtained by the procedure reported elsewhere,²⁶⁾ is shown in Fig. 2. The slope provides us with an apparent first-order rate constant, $(k_0/K_m)e_0$ for the substrate. The rate parameters of k_0/K_m for several kinds of substrate obtained by the procedure are summarized in Table I together with the results obtained by Kato *et al.*^{32a)} Using partially purified enzyme, they estimated

FIG. 2. First-order Plot of Time Course of Hydrolysis of Maltoheptaose by Soybean β -Amylase.

$e_0 = 3.65 \times 10^{-10}$ M, $[G_7]_0 = 12 \mu\text{M}$, 0.02 M acetate buffer, pH 5.4, at 25°C. The ordinate shows change in the amount of substrate as the logarithm of the ratio of the total radioactivity to the radioactivity of maltoheptaose in the aliquot taken for analysis.

TABLE I. DEPENDENCY OF THE RATE PARAMETER (k_0/K_m) ON THE DEGREE OF POLYMERIZATION OF SUBSTRATE

	(a)	(b)
G_3	2.44×10^3	5.33×10^2
G_4	1.70×10^7	7.30×10^6
G_5	9.24×10^7	2.07×10^7
G_6	7.85×10^7	1.96×10^7
G_7	8.36×10^7	7.47×10^7
$G_{12.5}$	—	7.47×10^7

(a) present data; (b) data by M. Kato (32a); dimension, $\text{min}^{-1} \text{M}^{-1}$.

the reaction rates for those substrates by measuring the reducing power, which represents the total products; thus their values may have included the influence of multiple attack. In the present study, the value of k_0/K_m for G_7 was nearly equal to that for G_5 , while in the case by Kato *et al.*, a significantly big difference was observed between those for G_5 and G_7 . The difference in tendency between Kato and the present studies seems to be due to difference in the method of estimating k_0/K_m .

Analysis of cleavage pattern on the reducing end-labeled maltotriose, G_3^* as the substrate

The cleavage of the end-labeled maltotriose by β -amylase was performed at the substrate concentration of 0.11 mM. The paper chromatogram of the hydrolysate is shown in Fig. 3, indicating that the radioactive glucose, G_1^* , was the major product from the substrate G_3^* , while only a small amount of radioactive maltose, G_2^* , was also produced. The ratio of the radioactive glucose to the radioactive maltose produced was determined to be about 240 ($[G_1^*]/[G_2^*] = 240/1$). Maltotriose must be bound at the active site of β -amylase in two productive binding modes, I and II (Fig. 4); G_1^* and G_2^* are formed corresponding to the mode I and II, respectively. Thus, the present results show that the main productive binding mode of maltotriose is the mode I.

The relative rate of G_1^* and G_2^* productions, $v_{G_1^*}/v_{G_2^*}$, is proportional to the ratio of the probabilities of the mode I and II, P_I/P_{II} .

$$\frac{v_{G_1^*}}{v_{G_2^*}} = \frac{d[G_1^*]/dt}{d[G_2^*]/dt} = \frac{P_I}{P_{II}} \quad (3)$$

According to the subsite theory,^{19,24)} the ratio P_I/P_{II} is related to the difference between the subsite affinities (A_i) of the two subsites which are not common to the two modes (A_I and A_4 in this case),

$$P_I/P_{II} = \exp [(A_I - A_4)/RT] \quad (4)$$

where R is the gas constant and T is the absolute temperature. Both the productions of G_1^* and G_2^* obey the first-order kinetics under the condition of $[S] \ll K_m$. Since the multiple attack is not observed in the case of maltotriose, the following equation is obtained from Eq. 2.

FIG. 3. Chromatogram of β -Amylase Hydrolysate of the Reducing End-labeled Maltotriose (G_3^*).

Radioactive maltotriose (5.7×10^5 cpm) was used as the substrate. The reaction was performed at 25°C, in 0.02 M acetate buffer, pH 5.4, containing 10 mM mercaptoethanol and 2 mM EDTA. $e_0 = 2.8 \times 10^{-9}$ M, $[S] = 110$ μ M. The ordinate shows the radioactivity of the section of the paper cut off. —, cpm $\times 10^{-4}$; ---, cpm $\times 10^{-2}$.

FIG. 4. Schematic Model for Two Possible Productive Binding Modes (I and II) of Maltotriose with β -Amylase.

G and G* represent unlabeled and labeled glucose residues, respectively. The wedge ($\blacktriangle$) shows the catalytic site of the enzyme. The boxes are the subsites numbered as indicated. The hedge on the left represents the steric hindrance in substrate binding.

$$\frac{v_{G_1^*}}{v_{G_2^*}} = \frac{d[G_1^*]/dt}{d[G_2^*]/dt} = \frac{k[S]}{k'[S]} = \frac{k}{k'} = K \text{ (constant)} \quad (5)$$

where k and k' represent the first-order rate constants for the productions of G_1^* and G_2^* , respectively.

$$d[G_1^*]/d[G_2^*] = K$$

Since both concentrations of $[G_1^*]$ and $[G_2^*]$ are zero at $t=0$,

$$[G_1^*] = K[G_2^*]$$

Therefore,

$$v_{G_1^*}/v_{G_2^*} = [G_1^*]/[G_2^*] \quad (6)$$

Then, ($A_I - A_4$) can be obtained from Eqs. 3 and 4 as follows;

$$\begin{aligned} A_I - A_4 &= RT \ln (v_{G_1^*}/v_{G_2^*}) \\ &= RT \ln ([G_1^*]/[G_2^*]) = RT \ln (240/1) \\ &= 3.2 \text{ kcal/mol} \quad (14 \text{ kJ/mol}) \end{aligned}$$

The subsite affinity of the subsite 1, A_1 , was estimated to be larger than that of the subsite 4, A_4 , by 3.2 kcal/mol.

Evaluation of subsite affinities

As mentioned earlier, β -amylase is regarded practically to have such a single productive binding mode that the first subsites are occupied by a substrate molecule. According to the subsite theory, rate parameter k_0/K_m for an n -mer substrate is proportional to the association constant for the productive binding mode, which is determined by the subsite affinities of subsites that are covered by the substrate molecule as follows¹⁹⁾:

$$\begin{aligned} (k_0/K_m)_n &= k_{int} K_{n,p} \\ &= 0.018 k_{int} \exp \left(\sum_i^{\text{cov}} A_i/RT \right)_{n,p} \quad (7) \end{aligned}$$

where k_{int} is the intrinsic rate constant for the hydrolysis of the substrate linkage in a productive complex, which is assumed to be constant irrespective of the degree of polymerization of substrate, n , and the mode of productive binding, p (Similar to glucoamylase, β -

amylase can be practically regarded to have single productive binding mode). $K_{n,p}$ represents the association constant of n -mer substrate to form the productive ES complex. The summation $\sum_i^{\text{cov}} A_i$ means that the sum is taken for the subsites occupied by the n -mer substrate in the specified binding mode p . The factor 0.018 arises from the correction for the change in free energy of mixing upon the binding of E and S, which is common for a bi-molecular association equilibrium (2.4 kcal/mol or 10 kJ/mol at 25°C in aqueous solution³³).

The rate parameter k_0/K_m increased with n , when $n < 5$, and it became almost constant when $n \geq 5$ (see Table I). These facts lead to the conclusion that soybean β -amylase has 5 subsites.

Then, in the manner similar to that used for glucoamylase,¹⁹ the fourth and the fifth subsite affinities of β -amylase can be evaluated using the k_0/K_m values for maltooligosaccharides, as follows;

$$\frac{(k_0/K_m)_5}{(k_0/K_m)_4} = \frac{0.018 k_{\text{int}} \exp [(A_1 + A_2 + A_3 + A_4 + A_5)/RT]}{0.018 k_{\text{int}} \exp [(A_1 + A_2 + A_3 + A_4)/RT]} = \exp (A_5/RT) \quad (8)$$

5.2 kcal/mol (21.8 kJ/mol), the value of A_1 is estimated to be 8.4 kcal/mol (35.8 kJ/mol). The subsite affinities thus obtained are shown in a histogram in Fig. 5. β -Amylase has 5 subsites as described above, and the catalytic site is located between the second and the third subsites as expected from its action pattern of exclusive

FIG. 5. Histogram of Subsite Structure of Soybean β -Amylase.

The wedge (▲) shows the catalytic site of the enzyme. The boxes represent the subsites numbered as indicated. The hedge on the left shows the steric hindrance in substrate binding.

Therefore,

$$A_5 = RT \ln \frac{(k_0/K_m)_5}{(k_0/K_m)_4} = RT \ln \frac{1.26 \times 10^8}{2.32 \times 10^7} \\ = 1.0 \text{ kcal/mol} \\ (= 4.2 \text{ kJ/mol})$$

$$A_4 = RT \ln \frac{(k_0/K_m)_4}{(k_0/K_m)_3} = RT \ln \frac{2.32 \times 10^7}{3.33 \times 10^3} \\ = 5.2 \text{ kcal/mol} \\ (= 21.8 \text{ kJ/mol})$$

Thus, the ratio of k_0/K_m value for n -mer to that of $(n-1)$ -mer substrate permits us to evaluate the subsite affinity of the n -th subsite which is occupied by the longer subsite alone.

From the cleavage distribution of G_3^* , i.e., the probabilities of cleavage at respective glycosidic bond, the relative value of the first subsite affinity to the fourth subsite affinity was estimated to be 3.2 kcal/mol (14 kJ/mol) as described in the preceding section. Since A_4 is

TABLE II. SUBSITE AFFINITIES OF SOYBEAN β -AMYLASE

A_1	$A_2 + A_3$	A_4	A_5	
8.4	—	5.2	1.0	kcal/mol
35.8	—	21.8	4.2	kJ/mol

formation of maltose. The two subsites adjacent to the catalytic site cannot be evaluated for these affinities by this method. One of the noticeable characteristics of β -amylase is that the first subsite with subsite affinity of 8.4 kcal/mol (35.8 kJ/mol) must be responsible for the exclusive maltose-forming ability of β -amylase (the binding mode I should occur much easier than the binding mode II; see Figs. 3 and 4).

Effect of pH and temperature on multiple attack

The influence of some effectors on the frequency of multiple attack was investigated. The

enzyme reaction were performed at various values of pH and temperature. Experiments for pH as variable were performed at 25°C, and those for temperature at pH 5.4. For this purpose, the reducing end-labeled maltoheptaose was used as substrate, which was hydrolyzed easily into labeled maltotriose by multiple attack of β -amylase. As seen from Fig. 6, the frequency of multiple attack, which is expressed in the ratio of labeled maltotriose to labeled maltopentaose, G_3^*/G_5^* , decreases with decrease in temperature and with departure from the optimum pH. Thus the frequency of the multiple attack markedly depended on temperature and pH of the reaction medium, in addition to the degree of polymerization of substrate.

Many reserch groups have so far estimated the frequency of multiple attack for the reactions of β -amylase,^{3~11,16)} α -amylases from porcine pancreas,^{12,13)} human saliva,^{12,16)} *Aspergillus oryzae*,^{12,18)} *Bacillus subtilis* (liquefying),¹⁶⁾ malted rye,¹⁴⁾ oats,¹⁴⁾ wheat¹⁴⁾ and soybean Z-enzyme.¹⁵⁾ Methods employed are as follows: (i) product analysis,^{9~11,13,18)} (ii) shift in maximum wavelength of the blue value,¹⁰⁾ (iii) the relationship between the blue value and the reducing value,¹²⁾ (iv) the relationship between fluorescence of 6-*p*-toluidinylnaphthalene-2-sulfonate (TNS) and the reducing value,¹⁷⁾ and (v) the relationship between $\overline{DP}_w^{*2}$ estimated by viscosity and $\overline{DP}_n^{*2}$ from enzymic assay.¹⁶⁾ The degree of multiple attack of various amylases have been found to decrease at unfavorable pH or temperature,^{6,11~13,17)} by chemical modification of the enzyme,⁷⁾ in the presence of glycerol¹⁶⁾ and with an uncompetitive inhibitor, methyl α -glucoside,⁷⁾ but not be affected by the addition of maltose,⁷⁾ cyclodextrin,⁷⁾ and by the elimination of Cl^- ion (for porcine pancreatic α -amylase.¹⁷⁾

The present study has revealed that soybean β -amylase exhibits multiple attack on G_7 and G_6 from the nonreducing termini. On the other

FIG. 6. Dependency of Multiple Attack on Temperature and pH in the Reaction of Soybean β -Amylase with the Reducing End-labeled Maltoheptaose (G_7^*).

The reactions were performed in 0.1 M acetate buffer (pH 4.0 and 5.4), in 0.1 M Tris-HCl buffer (pH 8.0) and 0.1 M Glycine-NaOH buffer (pH 9.0). The ordinate shows the ratio of the secondary product (G_3^*) to the primary product (G_5^*) as a measure for the degree of multiple attack. $e_0 = 6.9 \times 10^{-10}$ M, $[S] = 9 \mu\text{M}$.

hand, with G_5 and shorter maltooligosaccharides, the enzyme performs multi-chain attack, in which the enzyme removes single units (maltose) in completely random fashion from all available polymer ends.⁹⁾ The ratio of the secondary product to the primary product was 2.6 ($=G_3^*/G_5^*$) and 0.3 ($=G_2^*/G_4^*$) for G_7^* and G_6^* , respectively (pH 5.4, 25°C). Our result for maltoheptaose was not consistent with that reported by French *et al.*; they reported that maltopentaose did not accumulate in significant amount during the enzyme digests of maltoheptaose.⁵⁾ The disagreement might be due, in part at least, to the methods of product analysis used; their chromatographic determination may have been not sensitive enough. However, other results that the frequency of multiple attack decreased at unfavorable pH or temperature⁶⁾ were reconfirmed by the present study.

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*2 $\overline{DP}_w$: the weight average degree of polymerization, $\overline{DP}_n$: the number average degree of polymerization.

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