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Subsite Affinity of a Glucoamylase from *Aspergillus saitoi*

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In order to investigate the subsite structure of the glucoamylase from *Asp. saitoi*, kinetic parameters (K_m and k_0 values) of the enzyme for various sizes of maltooligosaccharide substrates were measured. The subsite affinities of this enzyme were calculated from the kinetic parameters and compared with those of the *Rhizopus* sp. glucoamylase, which differs from the *Asp. saitoi* glucoamylase in molecular weight, p*I* and of course, origin. The characteristic features of the subsite affinities of glucoamylase from *Rhizopus*, reported by Hiromi and his colleagues [*Agric. Biol. Chem.*, **47**, 573 (1983); *Biochim. Biophys. Acta*, **302**, 362 (1973)], were retained in the glucoamylase from *Asp. saitoi*. However, the k_{int} value for the glucoamylase from *Asp. saitoi* was about half of that for the enzyme from *Rhizopus* sp.

Keywords——glucoamylase; subsite affinity; kinetic parameter; *Rhizopus*; *Aspergillus saitoi*; maltooligosaccharide

Glucoamylase [EC 3.2.1.3, α-D-(1-4)-glucanglucohydrolase] is an exoenzyme which cleaves glucose units from the non-reducing end of starch, and it has been purified from various microorganisms. Some glucoamylases have been obtained in pure states.[1-5] The molecular weights of these glucoamylases are distributed in the range of 38000—110000[1e,1m] and the isoelectric points are in the range of pH 3.6[1j,1l] to 8.8.[1g,2,5]

Based on kinetic studies of the enzymes from *Rhizopus delemar*[6] and *Rhizopus niveus*,[2] Hiromi and his colleagues proposed a typical subsite structure of glucoamylase. According to their proposal, (i) glucoamylase has 7 subsites, (ii) glycosidic linkages of maltooligomer are cleaved between subsite 1 and subsite 2, (iii) subsite affinities are greatest at subsite 2, (iv) the affinity of individual site decreases from subsite 3 to subsite 7, (v) the subsite affinity at site 1 is very low.

We have isolated three forms of glucoamylase from *Rhizopus sp.*[5] and two forms of the enzyme from *Aspergillus saitoi*,[3] and reported some enzymatic properties of these enzymes. The isoelectric points of the former group were 8.7—8.8 and those of the latter group were 3.86. The apparent molecular weight of the major glycoamylase of *Rhizopus sp.* was 74000 and that of *Asp. saitoi* was 90000. Thus, the glucoamylase from *Asp. saitoi* seemed to be quite different from that of the *Rhizopus sp.* In order to investigate the subsite structure of *Asp. saitoi* glucoamylase, kinetic parameters of the enzyme with maltooligomers of various chain lengths were measured and the subsite affinities were calculated. For comparison, subsite affinities of the major glucoamylase from *Rhizopus sp.* isolated in this laboratory were also measured.

Materials and Methods

Enzyme——The major glucoamylase from *Rhizopus sp.* (Gluc 1) was purified from the commercial digestive,

"Gluczyme" (*Rhizopus sp.*, Amano Pharm. Co.), according to the method of Takahashi *et al.*[5] The major glucoamylase from *Asp. saitoi* (Gluc M_1) was purified as reported previously[3] from the commercial digestive, "Molsin" (*Asp. saitoi*, Seishin Pharm. Co.).

Substrates——Maltooligosaccharides from maltose to maltohexaose were purchased from Wako Pure Chem., and maltoheptaose from Boehringer Mannheim.

Measurement of Kinetic Parameters——The kinetic parameters (K_m and k_o) for maltooligosaccharides were determined at pH 5.0 and 25 °C. Enzyme solution (*ca.* 20 μl) was added to 1 ml of acetate buffer (10 mM) containing 0.1 M NaCl and substrate (0.02—10 mM) to start the reaction. The reaction was terminated by heating at 100 °C for 1 min. The amount of glucose released was measured by use of the "Glucose autotest" (Wako Pure Chem.). Kinetic parameters were determined from double reciprocal plots.[7]

Calculation of the Subsite Affinities——Subsite affinities of glucoamylases were calculated according to Hiromi *et al.*[6] from the kinetic parameters for maltooligosaccharides of various degrees of polymerization. The calculation procedures reported by Hiromi *et al.* can be summarized as follows. Kinetic parameters of the hydrolysis of maltooligosaccharide by glucoamylase were expressed in terms of the subsite affinity (Fig. 1) at each subsite and the sole rate constant for glycosidic bond cleavage, k_{int} as follows.

$$(1/K_m)_n = 0.018 \sum_j \exp\left(\sum_i^{cov} A_i/\boldsymbol{RT}\right)_{n,j} \tag{1}$$

$$(k_o)_n = k_{int} \cdot \exp\left(\sum_{i=1}^{n} A_i/\boldsymbol{RT}\right) \Big/ \sum_j \exp\left(\sum_i^{cov} A_i/\boldsymbol{RT}\right)_{n,j} \tag{2}$$

$$(k_o/K_m)_n = 0.018\, k_{int} \cdot \exp\left(\sum_{i=1}^{n} A_i/\boldsymbol{RT}\right) \tag{3}$$

where n is the degree of polymerization of glucose units; k_m and k_o are the Michaelis constant and molecular activity of n-mer substrate; i, subsite number counted from the non-reducing end; A_i, affinity of the i-th subsite; $\boldsymbol{R}$, gas constant; $\sum_j$, the sum taken over every mode of binding (j), and $\sum_i^{cov}{}_{n,j}$, the sum taken over all the subsites occupied in the j-th binding mode of the n-mer substrate. From Eq. (3), the subsite affinity of the $(n+1)$-th site can be calculated, see Eq. (4).

$$A_{n+1} = \sum_{i=1}^{n+1} A_i - \sum_{i=1}^{n} A_i = \boldsymbol{RT}[\ln(k_o/K_m)_{n+1} - \ln(k_o/K_m)_n] \tag{4}$$

Evaluation of A_i and k_{int}——Even the largest subsite affinity among A_3—A_7 was much smaller than that of maltose with each glucoamylase calculated from the equation $\boldsymbol{RT}\ln(1/K_m)_2 + 2.4$ kcal (6.7 kcal for Gluc 1, and 6.4 kcal for Gluc M_1). Thus, the contribution of A_1, A_2 or A_1+A_2 to the substrate binding seems to be larger than that of any other site. Therefore, it is reasonable to assume that site 2 always contributes to the binding of an oligomeric substrate. In this case, Eq. (2) can be simplified to give Eqs. (5) and (6).

$$(k_o)_n = k_{int} \cdot \exp\left(\sum_{i=1}^{n} A_i/\boldsymbol{RT}\right) \Big/ \left[\exp\left(\sum_{i=1}^{n} A_i/\boldsymbol{RT}\right) + \exp\left(\sum_{i=2}^{n+1} A_i/\boldsymbol{RT}\right)\right] \tag{5}$$

$$\exp(A_{n+1}/\boldsymbol{RT}) = [k_{int}/(k_o)_n - 1]\exp(A_1/\boldsymbol{RT}) \tag{6}$$

Based on this equation and the A_3—A_7 values observed, k_{int} and A_1 were evaluated from a plot of the $\exp(A_{n+1}/\boldsymbol{RT})$—$1/(k_o)_n$ relation.

Evaluation of A_2——According to the assumption described above, Eq. (7) was derived from Eq. (1).

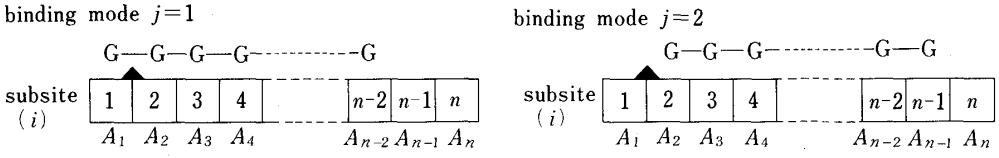

Fig. 1. Schematic Representation of the Active Site of Glucoamylase and the Mode of Binding of n-Mer Substrate

G represents a glucose residue in n-mer substrate; wedge, catalytic site at which cleavage of the glycosidic bond occurs; A_1—A_n, A_i is the subsite affinity at the i-th subsite. The binding mode $j=1$ refers to a productive mode of binding and $j=2$ to a non-productive mode of binding. For details, see reference 6.

$$(1/K_m)_n = 0.018\left[\exp\left(\sum_{i=1}^{n} A_i/RT\right)+\exp\left(\sum_{i=2}^{n+1} A_i/RT\right)\right] \quad (7)$$

From A_1 and A_3—A_7 thus obtained and the K_m values for the substrates (maltose to maltoheptaose), six A_2 values were obtained, then averaged.

Results and Discussion

Kinetic parameters (K_m and k_o) for Gluc 1 and Gluc M_1 with various maltooligosaccharides are listed in Table I. The dependences of $\log(1/K_m)$, $\log k_o$ and $\log(k_o/K_m)$ on the degree of polymerization (n) are illustrated in Fig. 2. For both glucoamylases, the kinetic parameters k_o, K_m and k_o/K_m depend largely on the n value up to $n=4$. However, for the substrates of $n=5$—7, the n-dependency of the kinetic parameters was very small. The A_3—A_7 values for both glucoamylases, obtained from the k_o/K_m values in Table I, are listed in Table II. From the exp (A_{n+1}/RT)—$1/k_o$ plot, k_{int} and A_1 values were calculated as described in "Materials and Methods" (Fig. 3, Table II). The A_2 values for both glucoamylases were

TABLE I. Kinetic Parameters of the Hydrolysis of Maltooligosaccharides Catalyzed by Gluc M_1 and Gluc 1 at pH 5.0 and 25 °C

Enzyme	Gluc M_1		Gluc 1	
Substrate	K_m (mM)	k_0 (s^{-1})	K_m (mM)	k_0 (s^{-1})
Maltose	1.15	2.20	1.54	3.32
Maltotriose	0.264	9.36	0.350	14.7
Maltotetraose	0.174	20.1	0.188	35.6
Maltopentaose	0.156	21.9	0.141	35.9
Maltohexaose	0.139	22.5	0.107	34.5
Maltoheptaose	0.129	22.4	0.098	36.2

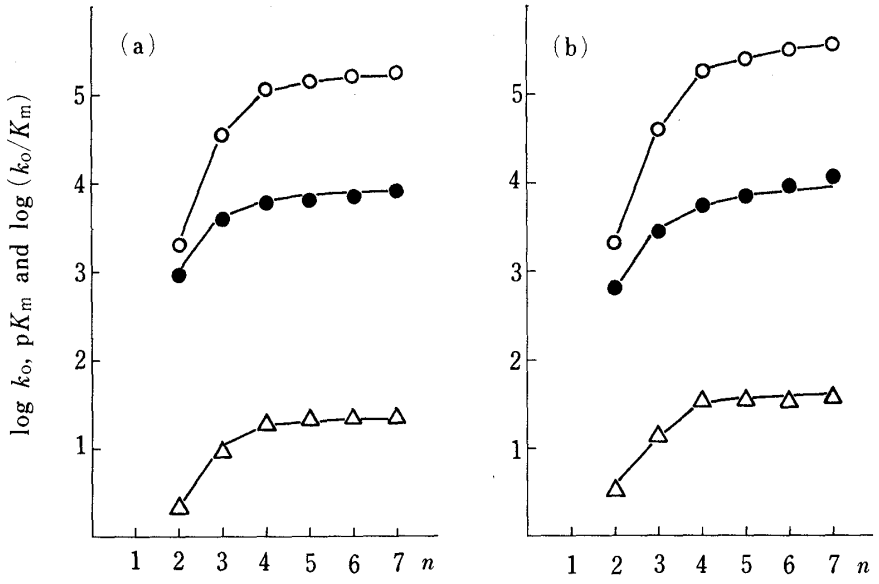

Fig. 2. Dependence of K_m, k_0 and k_0/K_m Values for the Hydrolysis of Maltooligomers Catalyzed by Glucoamylases from *Asp. saitoi* and *Rhizopus* sp. on the Degree of Polymerization (n) of the Substrates

(a) Gluc M_1, (b) Gluc 1.
○, $\log(k_o/K_m)$; ●, pK_m; △, $\log k_o$.

TABLE II. Subsite Structures of Glucoamylases from *Asp. saitoi* and *Rhizopus sp.* at pH 5.0 and 25 °C

	Subsite number (i)	1	2	3	4	5	6	7
Glucoamylase from *Asp. saitoi* (Gluc M_1)	Subsite affinity (A_i) (kcal/mol)	0	4.63	1.73	0.70	0.12	0.08	0.04
	k_{int} (s^{-1})				43.5			
Glucoamylase from *Rhizopus sp.* (Gluc 1)	Subsite affinity (A_i) (kcal/mol)	−0.18	4.39	1.76	0.89	0.18	0.14	0.08
	k_{int} (s^{-1})				96.0			

Subsites are numbered starting from the site at which the non-reducing end glucose of maltooligomer is bound in the productive mode.

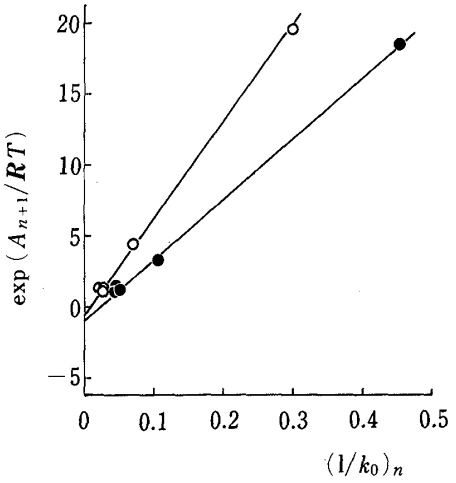

Fig. 3. Evaluation of k_{int} and A_1 from the exp (A_n+1/RT) − $(1/k_0)_n$ relation
●, Gluc M_1; ○, Gluc 1.

calculated from Eq. (7) and are also listed in Table II.

The results for Gluc 1 were very similar to those for the two *Rhizopus* enzymes reported by Hiromi *et al.*[6] and Tanaka *et al.*[2] That is, (i) the subsite affinity at site 1 is nearly zero, (ii) the subsite affinity at site 2 is about 4.5 kcal (the highest among the seven subsites), (iii) the subsite affinities of the other subsites decrease in the order of 3,4,5,6 and 7.

The affinity of each subsite for Gluc M_1 was very similar to that of Gluc 1, indicating that the characteristic features of *Rhizopus* glucoamylases found by Hiromi and his colleagues[2,6] are retained in Gluc M_1 which differs from Gluc 1 in molecular weight, p*I* and of course, origin. It should now be possible to deduce the common structure of the subsites of glucoamylases by comparing the structure of the two different glucoamylases from *Asp. saitoi* and *Rhizopus sp.*

Although Gluc M_1 has a subsite structure similar to that of Gluc 1, a major difference between these two glucoamylases was observed in their k_{int} values. The k_{int} for Gluc M_1 was only about 50% of that of Gluc 1. Thus, further comparative studies on the structures of these two glucoamylases having quite different k_{int} and similar subsite affinities may clarify the factors which influence the catalytic process of glucoamylase action.

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Added in Proof (September 27, 1983) Recently, Avelyev *et al.*[8] reported the subsite structure of a glucoamylase from *Asp. awamori* and obtained results very similar to those for *Asp. saitoi*. The k_{int} value for this enzyme was 47.9 (s^{-1}) at 37 °C, very similar to that of *Asp. saitoi*.

References

1) *a*) D. R. Lineback, I. J. Russell and C. Rasmussen, *Arch. Biochem. Biophys.*, **134**, 539 (1969); *b*) J. H. Pazur, H. R. Knull and A. Cepure, *Carbohydr. Res.*, **20**, 83 (1971); *c*) S. Hayashida, *Agric. Biol. Chem.*, **39**, 2093 (1975); *d*) S. Ueda and S. Kano, *Starch*, **27**, 123 (1975); *e*) M. N. N. Miah and S. Ueda, *ibid.*, **29**, 235 (1977); *f*) Y. Yamasaki, Y. Suzuki and J. Ozawa, *Agric. Biol. Chem.*, **41**, 1443 (1977); *g*) Y. Yamasaki, K. Tsuboi and Y. Suzuki, *ibid.*, **41**, 2139 (1977); *h*) Y. Yamasaki, Y. Suzuki and J. Ozawa, *ibid.*,**41**, 2149 (1977); *i*) Y. Yamasaki and Y. Suzuki, *ibid.*, **42**, 971 (1978); *j*)) A. Razzaque and S. Ueda, *J. Ferment. Technol.*, **56**, 296 (1978); *k*) H. Iizuka and S. Mineki, *J. Gen. Appl. Microbiol.*, **23**, 217 (1977); *l*) B. C. Saha, T. Mitsue and S. Ueda, *Starch*, **31**, 307 (1979); *m*) Rao V. Basaveswara, N. V. S. Sastri and P. V. SubbaRao, *Biochem. J.*, **193**, 379 (1981); *n*) S. Medda, B. C. Saha and S. Ueda, *J. Fac. Agric. Kyushu Univ.*, **26**, 139 (1982).
2) A. Tanaka, Y. Fukuchi, M. Ohnishi, K. Hiromi, S. Aihara and Y. Morita, *Agric. Biol. Chem.*, **47**, 573 (1983).
3) T. Takahashi, N. Inokuchi and M. Irie, *J. Biochem.* (Tokyo), **89**, 125 (1981).
4) N. Inokuchi, T. Takahashi and M. Irie, *J. Biochem.* (Tokyo), **90**, 1055 (1981).
5) T. Takahashi, Y. Tsuchida and M. Irie, *J. Biochem.* (Tokyo), **84**, 1183 (1978).
6) K. Hiromi, Y. Nitta, C. Numata and S. Ono, *Biochim. Biophys. Acta*, **302**, 362 (1973).
7) H. Lineweaver and B. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
8) A. N. Savelyev, V. R. Sergeev and L. M. Firsov, *Biokhimiya*, **47**, 390 (1982).