Carbohydrate Research, 212 (1991) 201–212
Elsevier Science Publishers B.V., Amsterdam

Analysis of the characteristic action of D-enzyme from sweet potato in terms of subsite theory

Toshihiko Suganuma*, Shinji Setoguchi, Shigeo Fujimoto, and Tomonori Nagahama
Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Korimoto, Kagoshima 890 (Japan)

(Received July 9th, 1990; accepted for publication, October 23rd, 1990)

ABSTRACT

Disproportionating enzyme (D-enzyme, EC 2.4.1.25) was purified chromatographically from a β-amylase-deficient variety of sweet potatoes, and obtained free of amylases and glucosidases. Some heavy metal ions, particularly Hg^{2+} and Ag^{+}, inactivated the enzyme. The K_m value for maltotriose was found to be 7.3mM. Analysis by h.p.l.c. of digests of malto-oligosaccharides (G_3–G_7) showed that, as with other D-enzymes, maltose is not formed in any case. The products from all substrates except G_4 are those resulting from maltosyl transfer as the predominant reaction, wherein G_{n-2} and G_{n+2} are produced from G_n. The various malto-oligosaccharides undergo reaction at almost the same rates. The frequency of cleavage of the various bonds in malto-oligosaccharides was estimated from the observed molar ratios of products smaller than the substrates. A possible subsite structure for the D-enzyme is proposed on the basis of computer modelling of data describing the cleavage pattern etc.

INTRODUCTION

Disproportionating enzyme (D-enzyme, EC 2.4.1.25) is a transglycosylase that degrades malto-oligosaccharides (G_n) into both smaller saccharides (G_{n-i}) and larger saccharides (G_{n+i}) through glycosyl (*i*-mer) transfer. The enzyme was first found in potato tubers by Peat *et al.*[1,2]. Thereafter, several studies on D-enzymes from plant sources were reported[3–6]. Recently, the barley enzyme was obtained in enzymatically pure form by chromatography[7]. Amylomaltase from microorganisms is known as an enzyme possessing marked transfer activity similar to that of D-enzyme[8–11], and this enzyme, unlike D-enzyme, acts on maltose as a donor substrate.

Jones and Whelan studied the characteristic action of D-enzyme in terms of the "forbidden linkages" that Peat *et al.* had proposed, using radioactive substrates. They reached the following conclusions[12]: *i*) maltose does not serve as a donor substrate; *ii*) only one bond (on the reducing end) in maltotriose is susceptible; *iii*) there are two forbidden linkages (the first bond on the nonreducing end and the penultimate bond at the reducing end) in the longer saccharides.

There has been a quantitative theory for analyzing the action of amylases, namely "subsite theory", proposed independently by Hiromi *et al.*[13,14] and Thoma *et al.*[15,16]

* To whom correspondence should be addressed.

0008-6215/91/$ 03.50

According to this theory, the binding site of an amylase is composed of a row of several subsites, and the "subsite affinity" governs the interaction of each subsite with a glucose residue of the substrate. The notion of multiple binding modes, productive and nonproductive, reasonably explains the observed action patterns, *i.e.*, the cleavage pattern and the dependence of hydrolytic rate on the d.p. of the substrate.

A new variety of sweet potato, Satsuma Hikari, lacks β-amylase, which is a major enzyme in ordinary varieties of this vegetable[17]. In the present study, D-enzyme was isolated from sweet potatoes of the Satsuma Hikari variety in enzymatically pure form (free from amylases and α-glucosidase). The malto-oligosaccharides produced by the enzyme were determined by h.p.l.c. We then analyzed the characteristic transfer action of D-enzyme in terms of subsite theory, to make it clear why forbidden linkages appear at the certain locations in its substrates.

EXPERIMENTAL

Materials. — Sweet potatoes of the Satsuma Hikari variety were donated by the Kagoshima Prefectural Agricultural Experiment Station. Maltotriose (G_3) for D-enzyme assay, a product of Hayashibara Biochemical Laboratories, Inc., was obtained from Nacalai Tesque Inc. For action-pattern experiments it was used after purification on a carbon column. Malto-oligosaccharides G_4 to G_7 were prepared from acid hydrolyzates of cyclomaltoheptaose by cellulose column chromatography[18]. Each oligosaccharide was pure (more than 99%) by h.p.l.c. Glucose assay kits were purchased from Wako Pure Chemical Industries, Ltd.

Assay. — The activity of D-enzyme was assayed by measuring the glucose produced from maltotriose (G_3) by a glucose oxidase (GO) method[18]. A mixture of 0.1 mL of 1% G_3 (to give a final concentration of 9.9mM) and 0.1 mL of the enzyme solution in sodium Tris–maleate (0.075M, 0.075M) buffer, pH 7.0, was incubated for 30 min at 30°. The reaction was terminated by heating the mixture for 3 min in a boiling water bath, and the product glucose was determined. One unit of enzyme is defined as the amount that can release 1 μmol of glucose per min under the conditions just described.

Product analysis of the digests by h.p.l.c. — A mixture of a malto-oligosaccharide (4mM) and the partially purified D-enzyme (8.3×10^{-2} unit) in 0.2 mL of sodium Tris–maleate (0.05M, 0.05M) buffer, pH 7.0, was incubated for 1 h or 3 h at 30°. The reaction was terminated by boiling for 3 min. To remove the buffer salts the reaction mixture was passed through mini-columns (8×30 mm) of cation-exchange resin (Amberlite CG-120), and anion-exchange resin (Amberlite IRA-45X). The eluate was lyophilized, the residue was dissolved in 0.2 mL of distilled water, and the solution was subjected to h.p.l.c. analysis. A packed column of SSC-NH171 (Senshu Kagaku Co.) was eluted at a flow rate of 1.0 mL/min with 7:3 acetonitrile–water. The eluate was monitored by an RI detector (Knauer Nr. 98.00) equipped with a data processor (SIC Chromatocorder 11). The products (G_1–G_{12}) were quantified from the respective peak areas on the chromatograms, after correction for trace amounts of saccharide impurities.

Assay for relative activity after the addition of metal salts and other chemicals. — In experiments on the addition of metal salts and other chemicals, glucose release from G_3 was determined by h.p.l.c. instead of the GO method. A mixture of the chemical to be tested (metal ion 5mM, usually as the chloride salt, plus 0.5% bovine serum albumin or 0.04% Triton X-100) and the partially purified D-enzyme (4.9×10^{-1} unit) in 0.1 mL of sodium Tris–maleate (0.075M, 0.075M) buffer, pH 7.0, was preincubated at 25°. After 30 min, 0.4 mL of 0.1M Tris–HCl buffer, pH 7.0, was added to the mixture. An aliquot (0.1 mL) of the mixture and 0.1 mL of 1% G_3 was incubated for 1 h at 30°. The mixture was heated to boiling for 3 min, then desalted by ion exchange as mentioned above. The deionized solution was chromatographed on a packed column of Shodex S-801 (Showa Denko), suitable for the analysis of monosaccharides and disaccharides. It was eluted with distilled water at a flow rate of 0.8 mL/min.

Purification of D-*enzyme from sweet potato.* — All procedures were carried out at 4°. Sweet potato cubes were homogenized with two volumes of deionized water. The homogenate was squeezed through four-layered gauze and centrifuged (4500*g*, 20 min). The extract was treated with ammonium sulfate, and the active fraction, precipitating at 30–70% saturation, was redissolved in sodium Tris–maleate buffer and passed through a column of Sephadex G-10 (2 × 39 cm), previously equilibrated with 0.1M citrate buffer, pH 6.0. Then, it was applied to a DEAE-Toyopearl 650M column (1.8 × 12.5 cm), which was eluted with a gradient of 0 to 0.5M NaCl in 0.05M citrate buffer, pH 6.2. The fractions comprising the single active peak were pooled and concentrated by ammonium sulfate precipitation (40% satn.). The enzyme, redissolved in sodium Tris–maleate (0.1M, 0.1M), pH 7.0, was then subjected to gel filtration on a column (1.8 × 72 cm) of Cellulofine GCL-2000-sf (Seikagaku Kogyo Co., Ltd.; exclusion limit >3 000 000 mol. wt.), equilibrated with sodium Tris–maleate (0.1M, 0.1M) buffer, pH 7.0. The column was eluted with the same buffer, and the active fractions (partially purified enzyme) were pooled and stored.

RESULTS

Purification and some physical properties of D-*enzyme.* — Sweet potato D-enzyme was purified until free of carbohydrolases. On DEAE-Toyopearl chromatography it gave a single active peak, and subsequently gel chromatography on Cellulofine GCL-2000-sf was the only procedure that effected an increase in the specific activity of the enzyme. The molecular weight was estimated to be more than 200 000 from the elution volume of the single active peak on this column.

In this gel-filtration chromatography a minor peak of activity emerging after the major peak was detected by the GO method, but not by the "phosphorylase method" we devised. The principle of the latter method is as follows: maltotriose is a poor substrate for the synthetic action of phosphorylase, whereas G_5, produced from G_3 by the transferase action of D-enzyme, is a good substrate. Thus, when the D-enzyme digest of G_3 and glucose 1-P are subjected to phosphorylase, the reaction proceeds at a rate proportional to the amount of G_5. This method was much more specific for D-enzyme

than the procedure based on glucose determination. The minor peak from which the enzyme was separated might be attributable to maltase; the enzyme was also separated effectively from a small amount of amylase.

D-Enzyme obtained by gel-filtration chromatography showed a specific activity of 51.1 unit/mg, which was about 100-fold higher than in the crude extract. The yield was poor, less than 5%, which is attributable mainly to the lability of the enzyme. The activity decreased sharply when the enzyme was transferred to a solvent (buffer) of lower ionic strength: the activity in 5mM Tris–maleate buffer was about one third of that in 50mM buffer.

The enzyme preparation was found to be free from maltase or other (1→4)-α-glucanohydrolases on long-term incubations with *p*-nitrophenyl α-glucoside or soluble starch as substrates. The K_m value for maltotriose was determined to be 7.3mM from an s/V *vs.* s plot obtained by the GO method.

pH-Dependence of activity and stability. — The optimum pH for activity of the enzyme was about 7 in both 0.075M McIlvain buffers (pH 3–8) and in sodium Tris–maleate (0.075M, 0.075M) (pH 6–10) at 30°, and half-maximum activity was found at pH 5.5 and 9. The enzyme was stable at 30° only around pH 7, thus its stability behavior was similar to the pH dependence of its activity. Thermal stability was maintained up to 35° in 75mM Tris–maleate buffer at pH 7.0.

Effect of metal ions and chemicals. — Preincubation with heavy-metal ions such as Ag^+ and Hg^{2+} inactivated the enzyme almost completely. Other metal ions such as Ca^{2+}, Mg^{2+}, and Pb^{2+} did not affect the activity so significantly. Addition of a chelating

TABLE I

Effect of metal ions and chemicals on D-enzyme activity[a]

Additive[b]	*Residual activity (%)*
None	100
Ca^{2+}	94
Mg^{2+}	85
Al^{3+}	28
Mn^{2+}	90
Fe^{3+}	53
Co^{2+}	77
Cu^{2+}	19
Zn^{2+}	76
Ag^+	0
Hg^{2+}	0
Pb^{2+}	77
EDTA·2Na	87
2-Mercaptoethanol	93
Cysteine	93
Bovine serum albumin	160
Triton X-100	150

[a] See Experimental section for details. [b] Ag^+ added as the nitrate, Pb^{2+} as the acetate, other metals as chlorides.

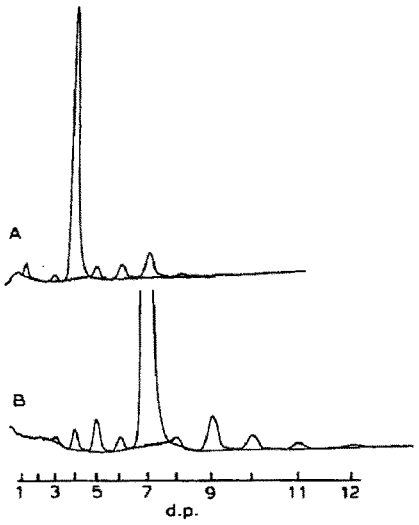


Fig. 1. H.p.l.c. profiles of the products formed by the action of sweet potato D-enzyme: *A*, digest of G_4 at 3 h; *B*, digest of G_7 at 3 h. See text for details.

agent or thiol compounds did not increase the activity, whereas bovine serum albumin and Triton X-100 effected a significant amplification. In the presence of cyclomaltohexaose or cyclomaltoheptaose (5mM each), the activity was inhibited by 13% and 50%, respectively (not shown in Table I).

TABLE II

Analysis of products of the action of D-enzyme on malto-oligosaccharides

Product	*Distribution[a] in the digest of the indicated substrate*				
	G_3	G_4	G_5	G_6	G_7
G_1	11.9(100)[b]	8.5(83)	3.6(20)	1.4(6)	0.1(1)
G_2	0	0	0	0	0
G_3	78.0	1.8(17)	11.5(64)	3.0(13)	0.7(6)
G_4	0	77.8	2.9(16)	14.7(64)	3.6(31)
G_5	9.2	2.4	65.1	3.9(17)	5.4(46)
G_6	0	3.4	2.4	56.4	1.9(16)
G_7	0.9	5.6	9.3	4.0	78.9
G_8	0	0.4	1.9	10.1	0.5
G_9	0	0	2.3	3.4	5.1
G_{10}	0	0	0.7	1.7	2.1
G_{11}	0	0	0.3	1.3	1.0
G_{12}	0	0	0	0	0.6
Degrad. ratio[c]	22	22	35	44	21

[a] Mole percent of each *i*-mer (G_i) appearing in the digest. [b] The values in parentheses are distributions for products smaller than the substrate, expressed as a percentage (molar basis) of the sum of the smaller products. [c] See text.

Products from malto-oligosaccharides. — The chromatograms of the digests of G_3 to G_7 at three hours of reaction are shown in Fig. 1. Table II presents the product distributions revealed in the digests. The values are molar percentages, calculated from molar ratios obtained by dividing each respective peak area by the d.p. of the saccharide represented. Using the notation ΣP_i for the sum of all products on a molar basis, a degradation ratio may be defined as $(\Sigma P_i/\Sigma G_i) \times 100$, where ΣG_i is the sum of all saccharides (products plus starting substrate) in the digest. Similar results were obtained in the digests at one hour of reaction.

No formation of maltose was observed in any of the D-enzyme digests of malto-oligosaccharides (see Table II). This result was confirmed by the use of a different column (packed with Shodex S-801). The analyses also showed no significant contamination by amylases, especially β-amylase, in this preparation of D-enzyme.

In the digest of G_3 at 3 h, similar amounts of G_1 and G_5 were determined as major products and G_7 was found as a minor product. In the digest of G_4 the major products were G_1 and G_7, and several minor products were found. Among the higher malto-oligosaccharides ($> G_4$), G_5 was converted mainly to G_3 and G_7, G_6 gave mainly G_4 and G_8, and G_7 gave mainly G_5 and G_9.

The rate of the D-enzyme reaction did not increase markedly with increase in the d.p. of the substrate over the range G_3–G_7.

DISCUSSION

Product analysis of the D*-enzyme digests.* — The action pattern of D-enzyme from potato towards malto-oligosaccharides (G_3–G_6) has been studied by Jones and Whelan[12], using radioactive substrates. The product saccharides were analyzed quantitatively by paper chromatography. In the current study we have quantified all the saccharides of d.p. < 13 produced from the malto-oligosaccharides G_3–G_7 by the action of sweet potato D-enzyme. The use of an h.p.l.c. column of NH_2-silica enabled us to determine the respective peak areas for products of d.p. 1–12 (see Fig. 1). Analysis of the D-enzyme digests revealed that the oligosaccharide G_n was converted predominantly into G_{n-2} and G_{n+2} by the sweet potato enzyme. The only exception was maltotetraose, which was degraded mainly into G_{n-3} (G_1) and G_{n+3} (G_7). These observations, including the failure to produce maltose, seem to be generally characteristic of D-enzyme action[1,2,7,12].

For studying the action pattern of an enzyme through product analysis, it is important to check whether or not the enzyme preparation is free from contamination by other enzymes, especially those that act on the same substrate(s). β-Amylase, contained in a significant amount in the common variety of sweet potato, can rapidly degrade malto-oligosaccharides larger than the triose. With the Satsuma Hikari variety however, this was not a problem, and our enzyme preparation was shown to be free of other significant contaminants. It may be noted that saccharides larger than the substrate were produced in an amount almost equimolar with that of smaller saccharides in each digest (see Table II). This phenomenon of symmetrical disproportionation suggests that sweet potato D-enzyme has no significant hydrolytic activity. In this

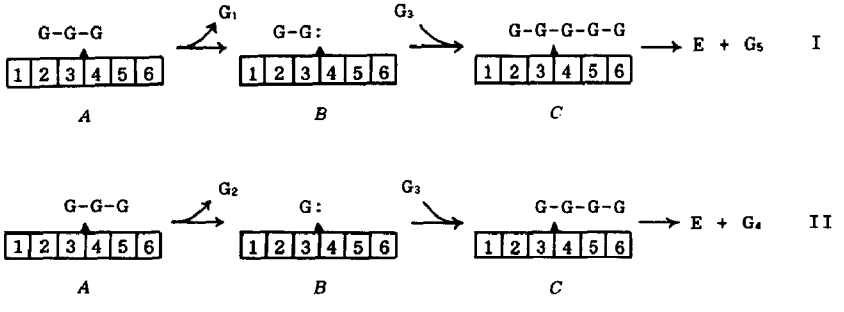
respect it differs from cyclodextrin glucanotransferase (CGTase), which catalyzes some hydrolysis in addition to its primary reaction of disproportionation under the usual conditions[20,21]. Our determinations of amounts of the products G_{n-i} smaller than substrate allow us to elucidate the pattern of cleavage of the malto-oligosaccharides by the enzyme, on the basis of the following discussion.

In the reaction of sweet potato D-enzyme with maltotriose, a small amount of G_7 was detected in the digests at 1 h and 3 h, in addition to the major products, G_1 and G_5. This may be due to successive transfer reactions, whereby G_7 is produced from the primary product G_5.

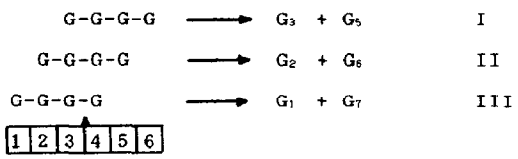
We can picture the maltosyl transfer reaction from maltotriose as follows. The first molecule of G_3 binds to the active site of the enzyme in such a way that the second glycosidic bond in G_3 is at the catalytic site (see I-*A* in Scheme 1). The glycosidic bond is cleaved, with the formation of a maltosyl-enzyme complex and the release of glucose (I-*B*). Then, a second molecule of G_3 binds to the complex as an acceptor to form a new α-(1→4) linkage[2,22], and producing G_5 (I-*C*). In the overall reaction, two molecules of G_3 are converted into one molecule of G_1 and one molecule of G_5.

With G_3 there is another possible binding mode leading to the formation of a glucosyl-enzyme complex (see II, Scheme 1). However, G_2 and G_4, which would be formed as a result, were not observed in the digest of G_3. Thus, this transfer pathway can be excluded in the reaction of D-enzyme. The molecule of G_3 binds, at first, exclusively in such a way as to give maltosyl transfer. Only the bond at the reducing end in maltotriose is susceptible to cleavage.

In the digest of G_4, almost equal amounts (molar basis) of G_1 and G_7 were found as predominant products. In this case, the first molecule of G_4 would bind so that the catalytic site is located at the third glycosidic bond, releasing glucose at the reducing end (III, Scheme 2). This leads to the transient formation of maltotriosyl-enzyme, instead of the maltosyl-enzyme complex. In the overall reaction, two molecules of G_4 are converted into one molecule of G_1 and one molecule of G_7. Small amounts of G_3, G_5, and G_6, and a trace amount of G_8, were also detected in the digest (Fig. 1 and Table II). A glucosyl transfer, to produce G_3 and G_5, could occur in part (I, Scheme 2). Although



Scheme 1. Possible pathways for the transglycosylation of G_3 by sweet potato D-enzyme, depending on the cleavage point. Boxes represent the subsites, numbered from the non-reducing end, and the wedge shows the catalytic site of the enzyme. The symbols G-G: and G: indicate glycosyl-enzyme intermediates.



Scheme 2. Possible pathways for the transglycosylation of G_4.

equimolar amounts of G_1 and G_7 should result as coproducts, G_7 was produced in a little smaller amount than G_1. Therefore, there is probably also some second-stage transglycosylation between G_7 and G_4, to give G_5 (G_{7-2}) and G_6 (G_{2+4}).

The results with substrates larger than maltotetraose indicate that in the reaction with G_5 two molecules of substrate are converted into one molecule of G_3 and one molecule of G_7 *via* a maltosyl-enzyme complex. And the situation is evidently the same for the saccharides higher than G_5; the primary products G_{n-2} and G_{n+2} arise through maltosyl transfer. In other words, the penultimate bond at the nonreducing end of the substrate is cleaved preferentially. However, several minor products were seen in all digests, suggesting the existence of alternative reaction paths: glucosyl transfer to give G_{n-1} and G_{n+1}, maltotriosyl transfer to give G_{n-3} and G_{n+3}, and *i*-mer transfer to give G_{n-i} and G_{n+i}. It may be noted that the coproducts of glucosyl transfer, G_{n-1} and G_{n+1}, were found in all digests of malto-oligosaccharides except that from G_3. This observation is contrary to the finding of Jones and Whelan[12] on potato D-enzyme. They reported that the first bond is also a forbidden linkage, since the products of glucosyl transfer were not detected.

The formation of maltose in digests of malto-oligosaccharides (G_3–G_7) by sweet potato D-enzyme, as well as other D-enzymes[2,16], has not been observed. This characterizes the penultimate bond at the reducing end as a forbidden linkage.

It seems curious that a possible coproduct of maltose, for instance G_6 in the G_4 reaction, G_8 in the G_5 reaction, G_{10} in the G_6 reaction, and so on, could be detected in all digests of substrates larger than G_3, in spite of the failure of maltose formation. The two coproducts, G_{n-i} and G_{n+i}, should be produced simultaneously in equimolar amounts through G_i-transfer. However, the phenomenon can be explained reasonably by postulating a secondary transfer reaction on the primary products, as has already been mentioned. For example, in the reaction with G_5, the primary product G_7 would be converted into G_8 (G_{3+5}).

Explanation of the cleavage pattern of the D*-enzyme in terms of subsite theory.* — Peat *et al.* referred to nonsusceptible linkages in explaining the characteristic action of potato D-enzyme[2]. Jones and Whelan[12] termed these linkages "the forbidden linkages", and demonstrated them in radioactively labeled malto-oligosaccharides. They discussed the action pattern of potato D-enzyme in terms of the forbidden linkages, but did not explain why the specific bonds of malto-oligosaccharides are in this category.

For the analysis of the action pattern of sweet potato D-enzyme we used a series of unlabeled malto-oligosaccharides (G_3–G_7). We noted the products smaller than the substrate (G_{n-i}), produced as leaving groups in each transglycosylation. Any possible

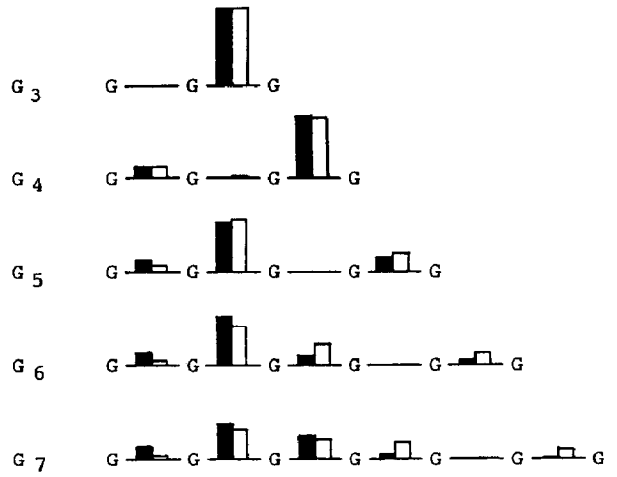


Fig. 2. Histograms showing the pattern of cleavage of malto-oligosaccharides by sweet potato D-enzyme. The black columns indicate the fractional cleavage distributions, calculated from the proportions of the corresponding products smaller than the substrate (Table II). The white columns represent values calculated on the basis of the proposed subsite structure.

hydrolytic action of the D-enzyme could be neglected, because the total amounts of the smaller saccharides were observed to be almost equal (molar basis) to those of the larger saccharides in all digests, as already mentioned. Therefore, the amount of any smaller saccharide (G_{n-i}) produced should be proportional to the frequency of cleavage of i-th bond. On the basis of the above considerations, we could describe the pattern of cleavage of malto-oligosaccharides by the D-enzyme as shown in Fig. 2.

According to the subsite theory[13,23], the probability of cleavage at a certain bond is proportional to the probability of formation of the corresponding enzyme-substrate complex, in which the saccharide is bound with the susceptible bond at the catalytic site of the enzyme. This is based on the assumption that the intrinsic rate constant k_{int} for the cleavage process stays the same, irrespective of the d.p. of the substrate and the cleavage position. For the binding of G_n to the enzyme in a "productive" binding mode a probability p of formation of the complex can be calculated from the affinities of the subsites that are occupied by the substrate, according to the following equation[13,14]:

$$K_{n,p} = 0.018 \exp\left[\frac{\sum^{cov} A_i}{RT}\right] \qquad (I)$$

For instance, for a complex of enzyme and G_3 as shown I-A in Scheme 1, the probability of formation should be proportional to the sum of the affinities ($A_2 + A_3 + A_4$) of subsites 2–4. Similarly, the probability of the complex shown in II-A should be proportional to the sum of the subsite affinities ($A_3 + A_4 + A_5$).

By comparing the probability of formation of complex I-A in Scheme 1 with that of II-A, we can obtain information about the relationship between the two subsites that are not common to the two complexes, *i.e.*, the difference $A_2 - A_5$ (refs. 15, 16). Product analysis showed that maltosyl transfer *via* the complex I-A occurs exclusively in the

reaction of D-enzyme with G_3. Therefore, we can say that $A_2 \gg A_5$.

A subsite affinity can be evaluated on the basis of kinetic data for the two saccharides, G_n and G_{n-1} (refs. 13, 14). By comparing the rate of production of G_1 from G_3 (I-*A* in Scheme 1) with that of G_2 from G_4 (II in Scheme 2), we could obtain the value of the subsite affinity A_5, if the respective rates were obtained as follows[23]:

$$\text{(the rate of production of } G_1 \text{ from } G_3) \propto \exp[(A_2 + A_3 + A_4)/RT] \quad (2)$$

$$\text{(the rate of production of } G_2 \text{ from } G_4) \propto \exp[(A_2 + A_3 + A_4 + A_5)/RT] \quad (3)$$

By dividing Eq. *3* by Eq. *2* and taking logarithms, we get

$$A_5 = RT_{\ln}\left[\frac{\text{(rate of production of } G_2 \text{ from } G_4)}{\text{(rate of production of } G_1 \text{ from } G_3)}\right] \quad (4)$$

The formation of maltose was not observed in the reaction with G_4. Thus, we can estimate the value of A_5 to be negative.

By comparing the rate of formation of G_3 from G_5 with that of G_1 from G_3, we can estimate the value of the sum of $(A_5 + A_6)$ to be nearly zero, in spite of a negative value of A_5.

It has been reported that glucose serves as an acceptor in the reaction of D-enzyme[2,12], which suggests that the possible acceptor site, subsite 4, would have a significantly large positive affinity.

In this way, the possible subsite structure was estimated tentatively on the basis of data of the cleavage pattern, the dependency of the rate on the d.p. of substrate, and the K_m value for G_3. Then, the fit to the cleavage pattern was refined by calculations on a

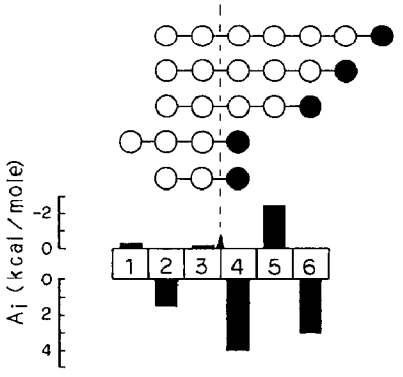


Fig. 3. A possible subsite structure for sweet potato D-enzyme, and its preferred binding modes. The wedge shows the catalytic site of the enzyme, the numbered boxes indicate the subsites, and the vertical bars shown subsite affinity. Glucose residues of malto-oligosaccharides are represented as open circles or (at the reducing end) a closed circle. The dotted line indicates the cleavage position.

personal computer, using a trial-and-error method. The subsite structure we propose is shown in Fig. 3.

The number of subsites of sweet potato D-enzyme was estimated to be six; in other words, the minimum number necessary to fit the action pattern by computer was six. The catalytic site of the enzyme is considered to lie between the third and the fourth subsites (see Fig. 3). Estimated values for subsite affinities are -0.3, 1.5, -0.2, 4.0, -2.5, and 2.8 kcal/mole for subsites 1–6, respectively.

The attribution of a small negative affinity to subsite 1 was found to be necessary to fit the action pattern for saccharides higher than G_5, for which maltosyl transfer is the predominant reaction.

The fifth subsite, with a negative affinity of -2.5 kcal/mole, is responsible for the lack of maltose formation in any digestions of malto-oligosaccharides, that is, the occurrence of forbidden linkages penultimate to the reducing end. This subsite becomes a strong barrier to the binding of the first molecule of substrate to the enzyme in such a way as to produce maltose. It is also responsible for maltotriosyl transfer as the predominant mode of reaction with G_4, which is distinct from the reactions with other substrates. The probability of binding mode III in Scheme 2 is proportional to the sum of the affinities $A_1 + A_2 + A_3 + A_4 (= 5.0)$, and is larger than the probabilities for the other two modes ($A_2 + A_3 + A_4 + A_5 = 2.8$ or $A_3 + A_4 + A_5 + A_6 = 4.1$).

With G_3, the probability of binding mode I-A is proportional to the sum of the affinities ($A_2 + A_3 + A_4 = 5.3$), and is much larger than that of II-A, ($A_3 + A_4 + A_5$) = -1.3). With G_5, the probability of a complex to produce maltosyl transfer is proportional to the sum $A_2 + A_3 + A_4 + A_5 + A_6 (= 5.6)$, which is larger than the sums for any other possible complexes of G_5. With substrates larger than G_5, the situation is the same as for G_5; the probability of the binding mode leading to maltosyl transfer is larger than the probabilities of any other modes.

The subsite structure we propose in the present study may still be insufficient to explain completely the frequency of the minor cleavages (see Fig. 2). But, the structure provides a very reasonable explanation of the major, important characteristics of D-enzyme action: the forbidden linkages, maltosyl transfer as the predominant reaction with the exception only of G_4, and almost uniform degradation rates irrespective of the d.p. of substrate. Such an approach in terms of subsite theory is applicable to D-enzymes other than that of sweet potato, and we believe a very useful tool to analyze their actions.

ACKNOWLEDGMENTS

The authors wish to express thanks to the Kagoshima Prefectural Agricultural Experiment Station for their kind donation of sweet potatoes of the new variety, Satsuma Hikari.

REFERENCES

1 S. Peat, W. J. Whelan, and W. R. Rees, *Nature*, 172 (1953) 158–158.
2 S. Peat, W. J. Whelan, and W. R. Rees, *J. Chem. Soc.*, (1954) 44–53.

3 D. J. Manners and K. L. Rowe, *Carbohydr. Res.*, 9 (1969) 441–450.
4 E. Y. C. Lee, J. J. Marshall, and W. J. Whelan, *Arch. Biochem. Biophys.*, 143 (1971) 365–374.
5 T. W. Okita, E. Greenberg, D. N. Kuhn, and J. Preiss, *Plant Physiol.*, 64 (1979) 187–192.
6 T. Suganawa, K. Yasutake, S. Fujimoto, and T. Nagahama, *J. Jpn. Soc. Starch Sci.*, 31 (1984) 191–191.
7 N. Yoshio, I. Maeda, H. Taniguchi, and M. Nakamura, *J. Jpn. Soc. Starch Sci.*, 33 (1986) 244–252.
8 H. Wiesmeyer and M. Cohn, *Biochim. Biophys. Acta*, 39 (1960) 427–439.
9 M. Doudoroff, W. Z. Hassid, E. W. Putman, A. L. Potter, and J. Lederberg, *J. Biol. Chem.*, 179 (1949) 921–934.
10 S. Kitahata, in the Amylase Research Society of Japan (Eds.), *Handbook of Amylases and Related Enzymes*, Pergamon Press, Oxford, 1988, pp. 169–172.
11 S. Kitahata, H. Ishibashi, and S. Okada, *J. Jpn. Soc. Starch Sci.*, 33 (1986) 217–217.
12 G. Jones and W. J. Whelan, *Carbohydr. Res.*, 9 (1969) 483–490.
13 K. Hiromi, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1–6.
14 K. Hiromi, Y. Nitta, C. Numata, and S. Ono, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
15 J. A. Thoma, C. Brothers, and J. Spradlin, *Biochemistry*, 9 (1970) 1768–1775.
16 J. A. Thoma, G. V. K. Rao, C. Brothers, J. Spradlin, and L. H. Li, *J. Biol. Chem.*, 246 (1971) 5621–5635.
17 T. Baba, H. Nakama, Y. Tamaru, and T. Kono, *Nippon Shokuhin Kogyo Gakkaishi*, 34 (1987) 249–253.
18 J. A. Thoma, H. B. Wright, and D. French, *Arch. Biochem. Biophys.*, 85 (1959), 452–460.
19 H. U. Bergmeyer and E. Bernt, in H. U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Vol. 3, Verlag Chemie GmbH, Weinheim, Germany, 1974, pp. 1205–1215.
20 S. Kobayashi, K. Kainuma, and S. Suzuki, *Carbohydr. Res.*, 61 (1978) 229–238.
21 S. Kitahata and S. Okada, *J. Jpn. Soc. Starch Sci.*, 29 (1982) 13–18.
22 S. Peat, W. J. Whelan, and G. W. F. Kroll, *J. Chem. Soc.*, (1956) 53–55.
23 T. Suganuma, R. Matsuno, M. Ohnishi, and K. Hiromi, *J. Biochem. (Tokyo)*, 84 (1978) 293–316.