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Carbohydrate Research 257 (1994) 249–268

CARBOHYDRATE RESEARCH

Models for the action of barley alpha-amylase isozymes on linear substrates †

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(Received July 15th, 1993; accepted November 8th, 1993)

Abstract

The formation of maltodextrins, G_1 to G_{12}, during the hydrolysis of amylose by alpha-amylases 1 and 2 from barley malt was followed by HPLC. Similar, but not identical, distributions of products were obtained with the two alpha-amylase components. Maltose, G_6, and G_7 were major products, but G_7 was degraded as hydrolysis proceeded. alpha-Amylase 1 produced more G_1 and G_3 than did alpha-amylase 2 at all stages of hydrolysis. Products formed during the hydrolysis of G_9, G_{10}, G_{11}, and G_{12} by the two alpha-amylases were also determined. A different spectrum of products was observed with each substrate and small differences were observed in the action pattern of the two alpha-amylases, e.g., G_3 and G_7 were the major products formed during the hydrolysis of G_{10} by alpha-amylase 1, whereas G_2 and G_8 were the major products formed by alpha-amylase 2 on the same substrate. These results were used to develop a model of the active site of barley malt alpha-amylases. This site contains ten contiguous subsites with the catalytic site situated between subsites 7 and 8. The model can be used to predict hydrolysis patterns of amylose and maltodextrins by cereal alpha-amylases.

1. Introduction

Two major groups of isozymes of alpha-amylase have been identified in germinated barley [1]. The groups are designated alpha-amylase 1 and alpha-amylase 2, and differ in physical properties such as isoelectric point, with the proteins of group 1 having lower isoelectric points [2] than those of group 2. In addition, these

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SSDI 0008-6215(93)E0417-Y

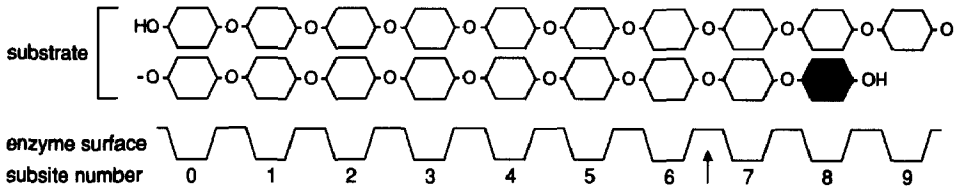

Fig. 1. Active centre of barley malt alpha-amylase showing 10 subsites (0–9), each of which is capable of interacting with a glucose residue of the substrate: ↑, catalytic site of the enzyme; ⌄, an enzyme subsite; •○•, a glucose residue of the substrate; •●, reducing end of the substrate.

alpha-amylase groups differ slightly in their action on starch granules [3,4], linear maltodextrins [5], and *p*-nitrophenyl glycosides of malto-oligosaccharides [5,6].

Models to explain the action of barley alpha-amylase on linear substrates have been proposed [6,7]. In these models, the enzyme active site is considered to consist of a number of contiguous subsites, where each subsite can interact with a glucose residue of a (1 → 4)-α-D-glucan substrate. Differences in interaction energy between a glucose residue and amino acid side-chains at each subsite are believed to account for aspects of the hydrolytic action of the alpha-amylases, such as the distribution of oligosaccharide products obtained from amylose.

Here we examine the hydrolysis of amylose and oligosaccharides catalysed by alpha-amylase 1 and compare this to degradation by alpha-amylase 2. In the light of these results and a previous study of the hydrolysis of small maltodextrins [5], we propose revisions to the earlier subsite models [6,7] for barley alpha-amylases.

2. Theory

The theory used is an extension of that described earlier [7] for barley alpha-amylase 2. It is postulated that there are 10 contiguous subsites at the active centre of a cereal alpha-amylase (Fig. 1), with the catalytic centre situated between subsites 6 and 7. For hydrolysis to take place, it is not necessary for all subsites to be filled, but subsites 6 and 7 must be occupied by glucose residues. Subsites are assumed to act independently, so that the free-energy of interaction between a glucose residue and a subsite does not depend on binding of substrate at another subsite. In earlier work [7], each subsite was considered to have one characteristic free-energy of interaction with a glucose residue. It is not known, however, whether this is true for interaction of any one binding site with an internal glucose residue, a nonreducing-end residue, and a reducing-end residue, since the three differ slightly, but significantly, in structure. In this study, therefore, we consider the possible effects of differences in interaction energy between enzyme and substrate, for three types of glucose residue.

Production of an oligosaccharide by hydrolysis of a longer substrate depends on the positioning of the substrate at the enzyme active site, the total free-energy of interaction between enzyme and substrate for the appropriate complex, and the

rate of hydrolysis of a C-1–O bond in the complex. The position of the lower substrate molecule in Fig. 1 shows the enzyme–substrate complex required for release of maltose from the reducing end of a substrate. In order to simplify the calculations, it was assumed that the rate of C-1–O bond hydrolysis is independent of substrate size and position at the enzyme active site.

The binding and hydrolysis of substrate A_n, of length n, by enzyme E can be represented as

$$E + A_n \underset{k_{-1,r,n}}{\overset{k_{+1,r,n}}{\rightleftharpoons}} EA_n \xrightarrow[k_2]{} E + P_{r,n-m} + P_{r,m}$$

where $P_{r,n-m}$ and $P_{r,m}$ are products of length $(n-m)$ and m formed from the nonreducing end and reducing end, respectively, of the substrate, $k_{+1,r,n}$, $k_{-1,r,n}$, and k_2 are the respective rate constants for binding of substrate to enzyme, dissociation of enzyme–substrate complex, and hydrolysis of the C-1–O bond in the substrate. The subscript r denotes the subsite occupied by the reducing-end glucose residue of the substrate—for the lower substrate molecule of Fig. 1, for example, r = 8.

The rate of production of product, $P_{r,m}$, is given by [8,9]

$$\frac{d[P_{r,m}]}{dt} = \frac{[E][A_n]k_2/K_{r,n}}{1 + [A_n]/K_{m,n}},$$

where $K_{r,n}$ is the Michaelis constant for the formation of $P_{r,m}$ from the reducing end of the substrate i.e., $K_{r,n} = (k_{-1,r,n} + k_2)/k_{1,r,n}$, and $K_{m,n}$ is the macroscopic Michaelis constant for all possible $E–A_n$ complexes. If $k_2 \ll k_{-1,r,n}$, then $K_{r,n} \approx k_{-1,r,n}/k_{1,r,n} = 1/K'_{r,n}$ where $K'_{r,n}$ is the association constant for the formation of the enzyme–substrate complex with the substrate reducing-end glucose residue at subsite r.

From any substrate, the ratio of rates of production of glucose and maltose, for example, from the substrate reducing end should thus be given by

$$\frac{d[G_1^*]}{dt} \Big/ \frac{d[G_2]}{dt} = \frac{[E][A_n]k_2K'_{7,n}}{1 + [A_n]/K_{m,n}} \cdot \frac{1 + [A_n]/K_{m,n}}{[E][A_n]k_2K'_{8,n}} = \frac{K'_{7,n}}{K'_{8,n}} \quad (1)$$

Cereal alpha-amylases hydrolyse maltodextrins, smaller than maltooctaose, slowly [5], and in the presence of longer substrates, these oligosaccharides may be considered to be resistant to attack. Then Eq. *1* gives not only the ratio of production rates but also of yields of glucose and maltose from a long substrate $(n > 8)$.

The association constant, $K'_{r,n}$, for a complex can be related to free-energies of interaction of occupied subsites with glucose residues by [10]

$$-RT \ln K'_{r,n} = \sum_{i=r-n+1}^{r} \Delta G_i + 10\,000 \quad (2)$$

* Abbreviations G_1, G_2, G_3, ...etc., are used for glucose, maltose, maltotriose,...etc.

where R is the gas constant, T is the absolute temperature, ΔG_i is the free energy of binding a glucose residue at subsite i, and the 10000 J mol^{-1} is the cratic free-energy contribution to binding [11].

On rearrangement of Eq. *2*,

$$K'_{r,n} = \exp\left[-\frac{1}{RT}\left(\sum_{i=r-n+1}^{r}\Delta G_i + 10000\right)\right]$$

and substitution for association constants in Eq. *1* gives

$$\frac{\text{Yield of } G_1}{\text{Yield of } G_2} = \frac{\exp\left[-\frac{1}{RT}\left(\sum_0^7 \Delta G_i + 10000\right)\right]}{\exp\left[-\frac{1}{RT}\left(\sum_0^8 \Delta G_i + 10000\right)\right]} = \exp\left(\frac{\Delta G_8}{RT}\right) \tag{3}$$

Equivalent expressions can be derived for yields of other small oligosaccharides, and from these, information can be obtained on subsite interaction energies.

If, however, the interaction energy between a glucose residue and a subsite depends on whether the glucose is an internal residue or occurs at the end of a substrate chain, then we must consider three possible values for ΔG_i, i.e., $\Delta G_{nr,i}$ for binding a nonreducing-end glucose residue, ΔG_i for an "internal" glucose residue, and $\Delta G_{re,i}$ for a reducing-end glucose. If a long substrate is bound at an alpha-amylase active centre with the reducing-end glucose residue at subsite 7, for example, the contribution to binding of glucose–enzyme interaction energies would be $\Sigma_0^6 \Delta G_i + \Delta G_{re,7}$, and the association constant for the complex $K'_{7,n}$ would be given, from Eq. *2*, by

$$K'_{7,n} = \exp\left[-\frac{1}{RT}\left(\sum_0^6 \Delta G_i + \Delta G_{re,7} + 10000\right)\right]$$

Substitution in Eq. *1* gives

$$\frac{\text{Yield of } G_1}{\text{Yield of } G_2} = \frac{\exp\left[-\frac{1}{RT}\left(\sum_0^6 \Delta G_i + \Delta G_{re,7} + 10000\right)\right]}{\exp\left[-\frac{1}{RT}\left(\sum_0^7 \Delta G_i + \Delta G_{re,8} + 10000\right)\right]}$$

$$= \exp\left[-\frac{1}{RT}(\Delta G_{re,8} + \Delta G_7 - \Delta G_{re,7})\right] \tag{4}$$

In addition, the Michaelis constant, $K_{m,n}$, for a substrate of length n can be expressed as [12,13]

$$1/K_{m,n} = \sum_{r=0}^{9+n-1} K'_{r,n} \tag{5}$$

i.e., the sum of association constants for all possible complexes of the enzyme with one molecule of n-mer.

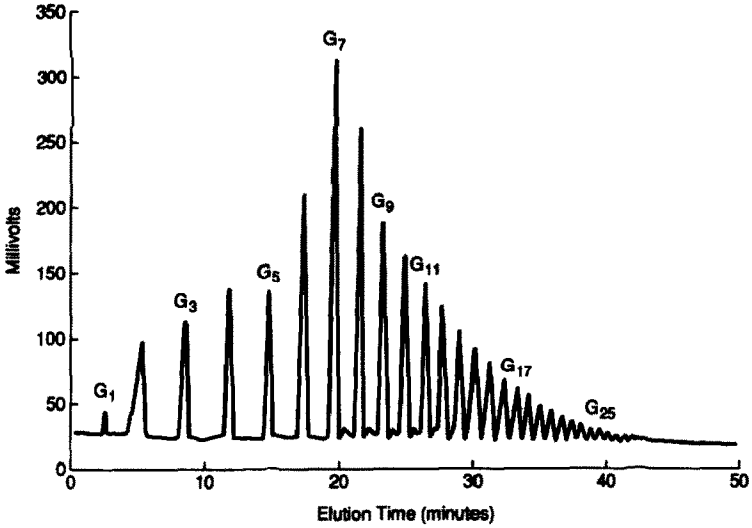

Fig. 2. Maltodextrins formed during the hydrolysis of amylose by alpha-amylase 2. G_1, G_3, G_5, etc., represent glucose, maltotriose, maltopentaose, etc.

The molecular activity, $k_{0,n}$, of the enzyme with the same substrate is given by

$$k_{0,n} = k_2 \sum_{r=7}^{6+n-1} K'_{r,n} / \sum_{r=o}^{r=9+n-1} K'_{r,n} \tag{6}$$

Here $k_{0,n} = V_{max}/[E]$ where V_{max} is the maximum velocity of hydrolysis of n-mer with enzyme at concentration [E], and $\Sigma_{r=7}^{6+n-1} K'_{r,n}$ represents the sum of association constants for productive complexes only, i.e., those complexes in which substrate spans both subsites 6 and 7 and hence the catalytic site of the enzyme.

From Eqs. *5* and *6*,

$$k_{0,n}/K_{m,n} = k_2 \sum_{r=7}^{6+n-1} K'_{r,n} \tag{7}$$

and thus this ratio depends on productive complexes only.

3. Results

Oligosaccharide products (up to G_{25}) of amylose hydrolysis were well separated by HPLC (Fig. 2) and quantities of each maltodextrin up to G_{12} could readily be measured. Yields of dextrins G_1 to G_{12} were determined for four trials. Changes in the amounts of products as hydrolysis proceeded are shown in Tables 1 and 2 and Fig. 3. It can be seen that the oligosaccharide distributions are similar, but not identical, for both isozyme groups of barley alpha-amylase. In all digests, maltose, maltohexaose, and maltoheptaose were important products, while only small

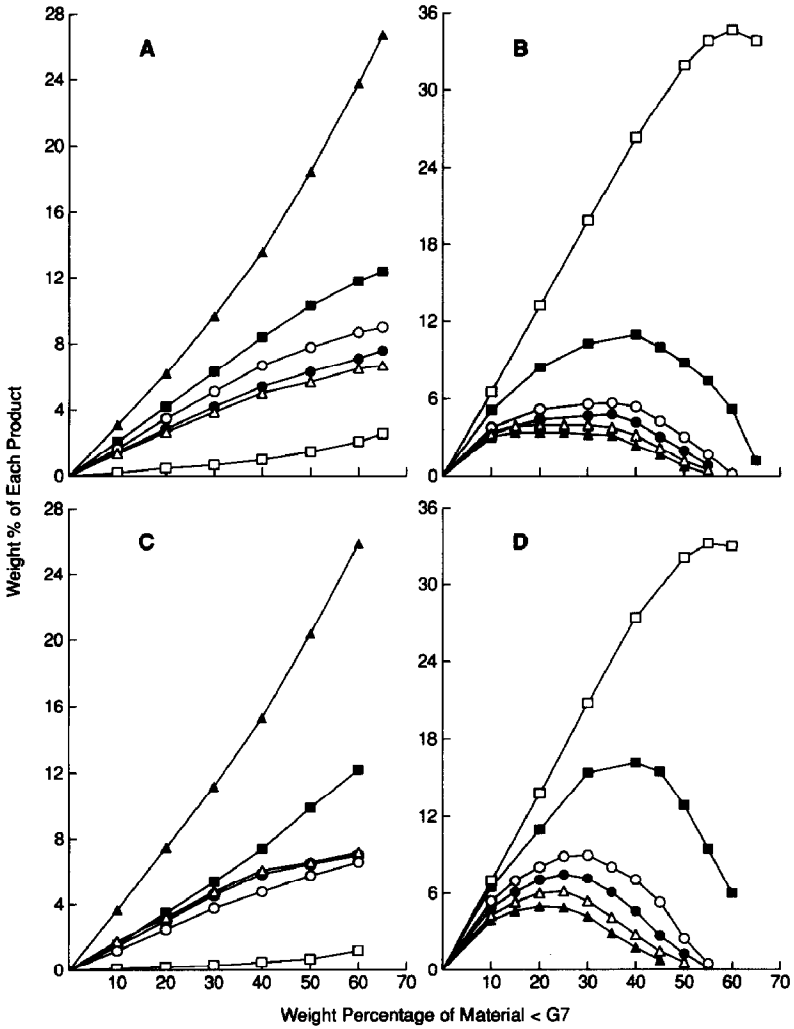

Fig. 3. Products formed during the hydrolysis of amylose by malted barley alpha-amylases: A, formation of G_1–G_6 in alpha-amylase 1 digest; B, formation of G_7–G_{12} in alpha-amylase 1 digest: C, formation of G_1–G_6 in alpha-amylase 2 digest; D, formation of G_7–G_{12} in alpha-amylase 2 digest. For A and C: □, G_1; ■, G_2; ○, G_3; ●, G_4; △, G_5; ▲, G_6. For B and D: □, G_7; ■, G_8; ○, G_9; ●, G_{10}; △, G_{11}; ▲, G_{12}. Curves represent values averaged over four trials.

amounts of glucose were released. The high yield of maltoheptaose is also obvious in Fig. 2. Throughout the hydrolyses, the amounts of glucose to maltohexaose increased, and only in late stages of amylolysis is there evidence of breakdown of maltoheptaose. Higher oligosaccharides, G_8–G_{12}, in contrast, accumulate in significant amounts but then are degraded as hydrolysis proceeds. Alpha-amylase 1 produces more glucose and maltotriose than alpha-amylase 2 at all stages of the reaction. Further, in the alpha-amylase 1 digests, smaller amounts of G_8 and G_9 accumulate (see Tables 1 and 2, and Fig. 3).

Table 1
Yields of oligosaccharides at different stages of the hydrolysis of amylose by barley alpha-amylase 1

Conditions	Extent of hydrolysis (% of products < G_7)	Wt fraction of total carbohydrate (%)											
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}	G_{11}	G_{12}
Experimental	30	0.7 [a]	6.3	5.1	4.2	3.9	9.7	19.9	10.3	5.6	4.7	4.0	3.3
Predicted [b]		0.3	5.4	4.5	4.0	4.2	10.8	20.4	10.3 [c]	5.6	4.7	4.0	3.3
Predicted [d]		0.3	5.5	4.5	4.1	4.2	10.8	20.5	10.3 [c]	5.6	4.7	4.0	3.3
Experimental	40	1.0	8.4	6.7	5.4	5.0	13.6	26.3	11.0	5.4	4.2	3.2	2.4
Predicted [b]		0.4	7.4	5.9	5.3	5.4	14.9	26.8	11.0 [c]	5.4	4.2	3.2	2.4
Predicted [d]		0.5	7.4	5.9	5.3	5.4	15.0	26.9	11.0 [c]	5.4	4.2	3.2	2.4
Experimental	50	1.5	10.3	7.8	6.3	5.7	18.5	31.9	8.8	3.1	2.0	1.2	0.8
Predicted [b]		0.6	9.7	7.1	6.1	6.0	19.3	32.7	8.8 [c]	3.1	2.0	1.2	0.8
Predicted [d]		0.6	9.7	7.1	6.2	6.1	19.4	32.8	8.8 [c]	3.1	2.0	1.2	0.8
Experimental	60	2.1	11.8	8.7	7.1	6.5	23.8	34.6	5.2	0.2			
Predicted [b]		0.8	11.7	7.9	6.6	6.4	23.5	37.5	5.2 [c]	0.2			
Predicted [d]		0.8	11.7	8.0	6.7	6.5	23.5	37.6	5.2 [c]	0.2			

[a] Experimental error is ±5% of values given. [b] Predicted using subsite energies of Table 5. [c] Quantities for G_8 to G_{12} taken to be same as experimental values. [d] Predicted using subsite energies of Table 5, but $\Delta G_2 = \Delta G_3 = \Delta G_4 = \Delta G_5 = 0$.

Distributions of products formed by amylolysis of individual maltodextrins, G_9–G_{12}, are shown in Tables 3 and 4. It can be seen that the barley alpha-amylase isozymes are similar in action, that maltose and maltoheptaose are important hydrolysis products, and that alpha-amylase 1 releases slightly more glucose and

Table 2
Yields of oligosaccharides at different stages of the hydrolysis of amylose by barley alpha-amylase 2

Conditions	Extent of hydrolysis (% of products < G_7)	Wt fraction of total carbohydrate (%)											
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}	G_{11}	G_{12}
Experimental	30	0.3 [a]	5.4	3.8	4.6	4.8	11.2	20.8	15.4	9.0	7.2	5.4	4.2
Predicted [b]		0.2	4.8	4.5	5.0	5.2	10.6	20.4	15.4 [c]	9.0	7.2	5.4	4.2
Predicted [d]		0.2	4.8	4.6	5.0	5.2	10.6	20.4	15.4 [c]	9.0	7.2	5.4	4.2
Experimental	40	0.5	7.4	4.8	5.9	6.1	15.3	27.4	16.2	7.1	4.6	2.8	1.8
Predicted [b]		0.3	7.0	6.0	6.2	6.2	14.6	26.8	16.2 [c]	7.1	4.6	2.8	1.8
Predicted [d]		0.3	7.0	6.0	6.2	6.2	14.6	26.8	16.2 [c]	7.1	4.6	2.8	1.8
Experimental	50	0.8	9.9	5.8	6.5	6.9	20.4	32.1	12.9	2.6	1.3	0.6	0.2
Predicted [b]		0.4	9.6	7.1	6.8	6.6	19.4	32.3	12.9 [c]	2.6	1.3	0.6	0.2
Predicted [d]		0.4	9.5	7.1	6.9	6.6	19.3	32.2	12.9 [c]	2.6	1.3	0.6	0.2
Experimental	60	1.2	12.1	6.6	7.0	7.2	25.9	33.0	7.0				
Predicted [b]		0.5	11.6	7.5	7.0	6.7	24.1	35.2	7.0 [c]				
Predicted [d]		0.6	11.5	7.6	7.1	6.7	23.9	35.2	7.0 [c]				

[a] Experimental error is ±5% of values given. [b] Predicted using subsite energies of Table 5. [c] Quantities for G_8 to G_{12} taken to be same as experimental values. [d] Predicted using subsite energies of Table 5, but $\Delta G_2 = \Delta G_3 = \Delta G_4 = \Delta G_5 = 0$.

Table 3
Yields of products during the hydrolysis of maltooligosaccharides by barley alpha-amylase 1

Substrate	Conditions	Products (mol% of total products)										
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}	G_{11}
G_9	Experimental	3 [a]	36	11	Tr [b]	Tr	10	37	3			
	Predicted [c]	2	37	10	0.2	0.2	10	37	2			
	Predicted [d]	3	37	10	0.2	0.2	10	37	3			
	Predicted [e]	3	19	24	5	5	24	19	3			
G_{10}	Experimental	2	19	20	7	0	8	24	19	2		
	Predicted [c]	2	20	21	7	0.2	7	21	20	2		
	Predicted [d]	2	20	21	7	0.2	7	21	20	2		
	Predicted [e]	1	7	8	32	3	32	8	7	1		
G_{11}	Experimental	2	15	12	14	6	5	19	13	13	2	
	Predicted [c]	2	16	12	15	5	5	15	12	16	2	
	Predicted [d]	2	16	12	15	5	5	15	12	16	2	
	Predicted [e]	1	5	5	19	21	21	19	5	5	1	
G_{12}	Experimental	1	12	10	8	11	9	14	10	12	11	1
	Predicted [c]	1	13	10	8	12	9	12	8	10	13	1
	Predicted [d]	1	13	10	8	12	9	12	8	10	13	1
	Predicted [e]	0.5	3	4	14	14	30	14	14	4	3	0.5

[a] Experimental error is ±1 mol%. [b] Tr = Trace. [c] Predicted from subsite energies of Table 5. [d] Predicted from subsite energies of Table 5, but with $\Delta G_2 = \Delta G_3 = \Delta G_4 = \Delta G_5 = 0$. [e] Predicted using subsite energies of Ajandouz et al. [6].

maltotriose than does alpha-amylase 2. Differences in action of the two isozymes can be seen also in Fig. 4, which shows the hydrolysis of G_{10}. Alpha-amylase 1 produces slightly more $G_3 + G_7$ while alpha-amylase 2 yields more $G_2 + G_8$.

4. Discussion

Results obtained for hydrolysis of amylose by alpha-amylase 2 are similar to those obtained earlier [7], but in the present study yields of G_7 to G_{12} have also been measured. It is obvious from Figs. 2 and 3 and Tables 1–4 that maltoheptaose is a major product of the action of barley alpha-amylases. The subsite model proposed earlier [7] for alpha-amylase 2 is unable, however, to account for the high yields of G_7. This earlier model lacked subsite 0 of the model to be discussed here (Fig. 1), and any predictions of product yield based on the early model would suggest that equal numbers of molecules of G_6 and G_7 should be produced from any long substrate. This would yield a weight ratio of G_7 to G_6 of 1.15, but the values given in Tables 1 and 2 indicate that, experimentally, the weight ratio is always greater. In an attempt to explain the apparently preferential formation of G_7, the original model was modified by addition of subsite 0 (Fig. 1) which can interact with the seventh glucose residue of the substrate on the nonreducing side of the bond to be hydrolysed. Thus, here we consider an enzyme active centre with 10 subsites capable of interacting with substrate glucose residues.

Table 4
Yields of products during the hydrolysis of maltooligosaccharides by barley alpha-amylase 2

Substrate	Conditions	Products (mol% of total products)										
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}	G_{11}
G_9	Experimental	Tr [a]	40 [b]	10	Tr	Tr	8	42	Tr			
	Predicted [c]	2	37	11	0.3	0.3	11	37	2			
	Predicted [d]	2	37	11	0.3	0.3	11	37	2			
	Predicted [e]	3	14	31	2	2	31	14	3			
	Predicted [f]	5	17	27	1	1	27	17	5			
G_{10}	Experimental	1	23	16	8	0	8	19	24	1		
	Predicted [c]	1	21	20	8	0.3	8	20	21	1		
	Predicted [d]	2	21	20	8	0.3	8	20	21	2		
	Predicted [e]	2	8	18	22	1	22	18	8	2		
	Predicted [f]	3	10	16	21	1	21	16	10	3		
G_{11}	Experimental	1	16	11	14	7	7	17	12	15	1	
	Predicted [c]	1	16	12	15	6	6	15	12	16	1	
	Predicted [d]	1	16	12	15	6	6	15	12	16	1	
	Predicted [e]	1	5	12	16	15	15	16	12	5	1	
	Predicted [f]	2	7	11	15	15	15	15	11	7	2	
G_{12}	Experimental	0	13	8	10	12	10	14	12	10	11	0
	Predicted [c]	1	13	10	10	12	9	12	10	10	13	1
	Predicted [d]	1	13	10	10	12	9	12	10	10	13	1
	Predicted [e]	1	4	9	12	12	23	12	12	9	4	1
	Predicted [f]	1	5	9	12	12	23	12	12	9	5	1

[a] Tr = Trace. [b] Experimental error is ±1 mol%. [c] Predicted from subsite energies of Table 5. [d] Predicted from subsite energies of Table 5, but with $\Delta G_2 = \Delta G_3 = \Delta G_4 = \Delta G_5 = 0$. [e] Predicted from subsite energies for AMY2-1 of reference 6. [f] Predicted from subsite energies for AMY2-2 of reference 6.

An active centre of comparable length has been considered for *Bacillus amyloliquefaciens* alpha-amylase, although the catalytic site of this enzyme is situated between subsites 5 and 6 [9] (Fig. 1). It is interesting to note that the bacterial enzyme was found to hydrolyse G_9 to G_{12} at equal rates [9]. A similar phenomenon has been observed for barley alpha-amylase isozymes [14]. We conclude, therefore, that it is likely that the barley and bacterial enzymes have the same number of subsites at the active site. Hydrolysis rates tend to be low for maltodextrins shorter than the active site, but increase and level off with increasing substrate length, as one maltodextrin molecule becomes capable of occupying all subsites at the active centre [9,15]. The fact that G_9 is hydrolysed as fast as G_{12}, under comparable conditions by both barley alpha-amylase isozymes, suggests there must be at least 9 subsites at the enzyme active sites.

A 10-subsite model has already been proposed for barley alpha-amylase, but with the catalytic site [6] between subsites 5 and 6 (Fig. 1). Ajandouz et al. [6] reached their conclusions about the position of the catalytic site relative to the array of substrate-binding subsites from a study of relatively short modified maltodextrins. Of the substrates they used, only *p*-nitrophenyl maltoheptaoside

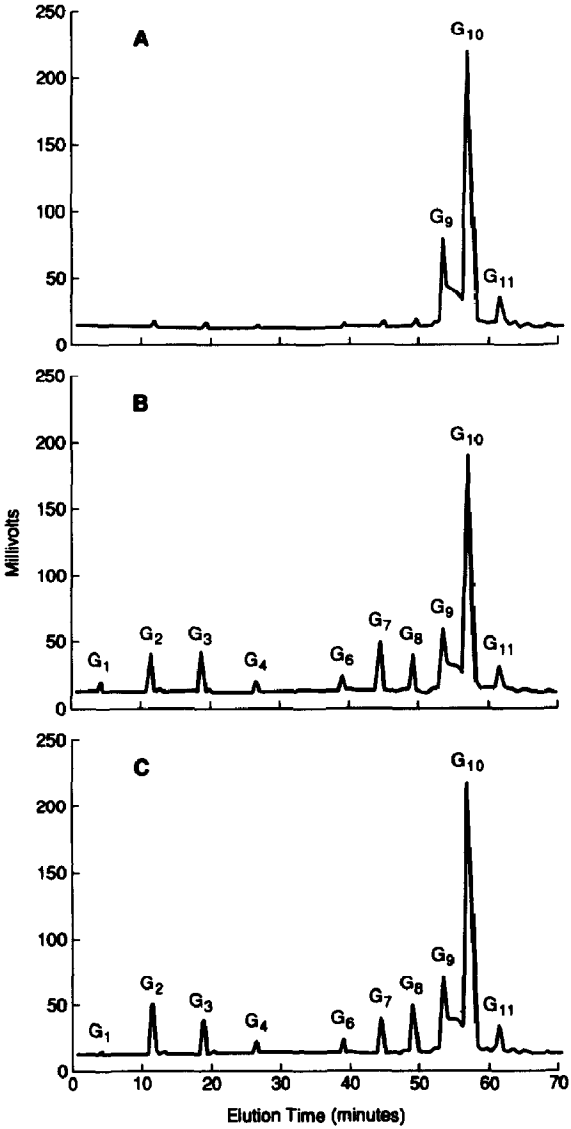

Fig. 4. Hydrolysis of G_{10}: A, chromatogram of G_{10} and alpha-amylase 1 at zero time; B, products formed by alpha-amylase 1 after 25 min; C, products formed by alpha-amylase 2 after 25 min. G_9 and G_{11} present in the zero-time digest represent $< 10\%$ of total carbohydrate.

can span subsites 0–7 and form a productive complex in our model, i.e., can be hydrolysed by the enzyme when a glucose residue occupies subsite 0. We consider it essential to use longer substrates in order to gain information on the importance of the "outer" subsites of the active site.

Estimation of subsite energies for any model of an enzyme catalysing a depolymerisation process can be complicated if the enzyme operates by a multiple attack

mechanism, i.e., cleaves more than one bond in a substrate molecule in a single enzyme–substrate encounter. Repetitive attack is a well-accepted feature of the action of mammalian alpha-amylases [16–25] and the enzyme of *Aspergillus oryzae* [17,22,26–28] but is not considered important during hydrolysis by bacterial liquefying alpha-amylases [19,22,28]. Although an earlier study [7] of the degradation of amylose by barley alpha-amylase 2 was inconclusive concerning multiple attack, later results with both alpha-amylases 1 and 2 indicated that such a mechanism is unlikely for cereal alpha-amylases [5]. This is confirmed by the results in Tables 3 and 4, showing that, for dextrin G_x, the two products of one-bond scission, e.g., G_y and G_{x-y}, are obtained in approximately equal amounts. Results obtained here for G_9 hydrolysis are similar to those obtained earlier [5]. By the criteria of Allen and Thoma [27], no detectable multiple attack is observed here for the hydrolysis of G_9 to G_{12} by either isozyme of barley alpha-amylase.

In a first attempt to estimate subsite energies for the model shown in Fig. 1, it was assumed that each subsite had one characteristic free-energy of interaction with a glucose residue, irrespective of the position of the glucose ring within or at the end of a substrate molecule. Equations such as *3* (see Theory) were used with product distributions obtained on hydrolysis of oligosaccharides G_7 to G_{12} (Tables 3 and 4, and ref 5) to calculate subsite energies as described by Thoma et al. [8]. It was further assumed that oligosaccharides smaller than G_6 were released primarily from reducing ends of longer oligosaccharides, e.g., a dextrin of length n, G_n, would be produced only from the reducing end of any substrate longer than G_{n+5}. Studies of hydrolysis of amylopectin [29] and modified oligosaccharides [5,6] suggest that this assumption is reasonable for cereal alpha-amylases. Product distributions can be used to estimate subsite energies, provided that no appreciable transglycosylation has taken place. While evidence for transglycosylation has been obtained for barley alpha-amylases acting on short maltodextrins up to G_6 [5], the extent of transglycosylation with amylose and a less highly-purified barley alpha-amylase was low [30]. The fact that the products of one-bond scission, e.g., G_y and G_{x-y} from substrate G_x, ($x \neq 6$), are obtained in approximately equal amounts (Tables 3 and 4, and ref 5) indicates that appreciable transglycosylation is unlikely. The largest difference for such a pair is 5%, i.e., 14% G_4 compared to 19% G_7 for the action of alpha-amylase 1 on G_{11} (Table 3). Some of this difference may be due to experimental error. These results, however, would appear to put an upper limit on possible transglycosylation. This first attempt was unsuccessful in obtaining a set of subsite energies which could adequately account for the yields of G_7 resulting from the action of either isozyme or for the yields of G_3 obtained during hydrolysis by alpha-amylase 1.

The effect of different binding energies associated with one subsite was then considered, where the free-energy of interaction could depend on whether an internal glucose, a reducing-end, or a nonreducing end residue was bound at a particular subsite. If this were the case, then for a cereal alpha-amylase with a 10-subsite active site, 30 free-energies of interaction would be required to describe adequately the action of the enzyme. Insufficient data are available to allow estimation of so many binding energies, as several experimental results lead to the

same information about subsite energies, e.g., the relative yields of glucose and maltose from hydrolysis of G_{10}, G_{11}, and G_{12} would all give values for $(\Delta G_7 - \Delta G_{re,7} + \Delta G_{re,8})$ from Eq. 4. It was decided, therefore, to investigate the minimum number of free-energies of interaction necessary to account for the yields of products which could not be explained on the basis of one binding energy per subsite. It was found that reasonable agreement between experimental results and values predicted from a model could be obtained if the outer two subsites, 0 and 9 (Fig. 1), were considered to have different energies of interaction for end, compared to internal, glucose residues.

If, for example, $\Delta G_0 \neq \Delta G_{nr,0}$, then from the action of barley alpha-amylase on G_{12}, the ratio

$$\frac{\text{Yield of } G_4}{\text{Yield of } G_5} = \exp\left[\frac{1}{RT}(\Delta G_{nr,0} - \Delta G_0)\right]$$

by arguments similar to those shown in the Theory section. Other product distributions, e.g., ratio of G_3 to G_4 released from G_{11} also give information on $(\Delta G_{nr,0} - \Delta G_0)$. When these were taken into account, $(\Delta G_{nr,0} - \Delta G_0)$ was found to be -1000 ± 300 J mol^{-1} for alpha-amylase 1 and -800 ± 300 J mol^{-1} for alpha-amylase 2. This suggests that indeed there is differential binding of a nonreducing end compared to an internal glucose residue at subsite 0. A negative free-energy of interaction indicates a favourable interaction between subsite and a glucose residue, and the negative value of $\Delta G_{nr,0} - \Delta G_0$ suggests more favourable binding of a nonreducing end glucose ring than an internal glucose unit at subsite 0.

From G_{12} hydrolysis,

$$\frac{\text{Yield of } G_3}{\text{Yield of } G_4} = \exp\left[\frac{1}{RT}(\Delta G_9 - \Delta G_{re,9})\right]$$

Again, hydrolysis of other substrates also gives information on $(\Delta G_9 - \Delta G_{re,9})$; after calculation and averaging, it was found that $(\Delta G_9 - \Delta G_{re,9}) = 400 \pm 200$ J mol^{-1} for alpha-amylase 1 and 0 ± 200 J mol^{-1} for alpha-amylase 2. These values suggest preferential binding of a reducing-end glucose residue at subsite 9 by alpha-amylase 1, but no significant difference for alpha-amylase 2 between binding a reducing-end or internal glucose ring.

Since independent values cannot be obtained for all $\Delta G_{nr,i}$, ΔG_i, and $\Delta G_{re,i}$, we proceed on the basis $\Delta G_{nr,i} = \Delta G_i = \Delta G_{re,i}$ for subsites other than 0 or 9. This allowed the evaluation of interaction energies for subsites 0, 1, 2, 8, and 9 (Table 5). Methods for calculating subsite energies based solely on distributions of products formed do not yield information on subsites 6 and 7 [8,9]. Hydrolysis products of G_8 and longer substrates are not sensitive to interaction energies for inner subsites 3, 4, and 5 (see Tables 1–4). In order to investigate these subsites further, previous results [5] for hydrolysis of G_5, G_7, and modified oligosaccharides, *p*-nitrophenyl maltotetraoside, maltohexaoside, and maltoheptaoside, were examined. For breakdown of small oligosaccharides such as G_5 and G_7, it cannot

Table 5
Energies of interaction of enzyme subsites with substrate residues

Substrate residue	Subsite									
	0	1	2	3	4	5	6	7	8	9
Interaction energy (J mol^{-1}) for alpha-amylase 1										
Glucose (internal)	−1600 [a]	−11000 ± 700	−1800	−500	900	−3500 ± 700	nd [b]	nd	−6000	1200
Glucose (chain end)	−2600 [c]									800 [d]
p-Nitrophenyl group	nd	nd	nd	nd	nd	nd	nd	(ΔG_7 + 2100)	−6600	−1900
Interaction energy (J mol^{-1}) for alpha-amylase 2										
Glucose (internal)	−1800	−10500 ± 700	−1500	−1900	2300	−9500 ± 700	nd	nd	−6900	800
Glucose (chain end)	−2400 [c]									
p-Nitrophenyl group	nd	nd	nd	nd	nd	nd	nd	(ΔG_7 + 1600)	−5000	300

[a] Unless otherwise stated, uncertainty in the values given is ±300 J mol^{-1}. [b] nd, Not determined. [c] Values for interaction with a nonreducing end glucose-residue. [d] Value for interaction with a reducing end glucose-residue.

be assumed that G_3, for example, is released only from the reducing end of the substrate, and so total production of a product from both ends of the substrate had to be considered. Although transglycosylation has been observed during action of barley alpha-amylase on G_5 [5] and $pNPG_4$ * [6], it has been reported that a major change of product distribution with substrate concentration does not occur in the latter case [6] and G_2 and G_3 remain major products in the former case [5], and so results from these substrates were taken into consideration. Results for hydrolysis of G_6 and $pNPG_5$ were not considered, because of possible complications of transglycosylation and effects of substrate concentration on products formed [5,6]. Little concentration effect on $pNPG_6$ or $pNPG_7$ hydrolyses has been observed, however [6]. It was, therefore, considered reasonable to use results for breakdown of $pNPG_6$ and $pNPG_7$. In order to evaluate subsite energies from work with modified substrates, Ajandouz et al. [6] made the assumption that a *p*-nitrophenyl group does not bind significantly at a barley alpha-amylase subsite. In contrast, a comparison of the hydrolysis of short maltodextrins (G_5 to G_8) with that of *p*-nitrophenyl oligosaccharides ($pNPG_4$ to $pNPG_7$) suggests, rather, that a *p*-nitrophenyl group does mimic a glucose residue in interaction with subsites, e.g., enzyme action on $pNPG_7$ resembles that on G_8 more than that on G_7 (ref 5). In the current study, therefore, on the evaluation of subsite energies from work on *p*-nitrophenyl maltodextrins, three additional subsite interaction energies, $\Delta G_{p,7}$,

* Abbreviations pNPG, $pNPG_2$, ...etc., are used for *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl α-maltoside,... etc.

$\Delta G_{p,8}$, and $\Delta G_{p,9}$, were considered for the binding of a *p*-nitrophenyl group to subsites 7, 8, or 9, respectively. Since the method for calculating subsite energies does not allow evaluation of a value for ΔG_7, only the difference $(\Delta G_{p,7} - \Delta G_7)$ could be calculated for each isozyme from product yields of hydrolysis of modified oligosaccharides. Results are shown in Table 5. There is some qualitative agreement between the energy profile obtained here for the active sites and previous studies [6,7], in that subsites 1, 5, and 8 are believed to have large favourable energies of interaction with glucose residues. Quantitatively, there is disagreement between the earlier model based on amylose hydrolysis [7] and the present results only at subsites 2 and 3. This disagreement may have arisen through lack of consideration of subsite 0 and lack of information on the breakdown of oligosaccharides (G_8 to G_{12}) of intermediate length in the earlier study. The greater disagreement between the results in Table 5 and the energies determined by Ajandouz et al. [6] may result in large part from the assumption by these authors that a *p*-nitrophenyl group does not bind at an enzyme subsite and their use only of short *p*-nitrophenyl maltodextrins to study enzyme action, because these substrates are hydrolysed by the barley alpha-amylase isozymes with difficulty [5]. In our study, also, information on subsites 3, 4, and 5 could be obtained only from work on short substrates which do not span the whole active site. Further, the results given for substrates 3, 4, and 5 in Table 5 represent "best" values computed from experimental results. In some cases (results not shown), no one value for the energy at a subsite (subsite 3, for example) was compatible with all the experimental data. This may indicate that the assumption that subsites act independently is not always valid, at least for short substrates. Indeed Ajandouz et al. [6] have provided evidence that subsite energies may depend on the length of the substrate and hence on the number of subsites occupied. They found, for example, higher subsite affinities when dealing with $pNPG_2$ and $pNPG_3$ than for longer substrates. The values given here for binding energies of subsites 3 to 5 should, therefore, be viewed with caution, and are probably more reliable for indicating differences between alpha-amylases 1 and 2 than for providing absolute values.

The results in Table 5 indicate major differences between alpha-amylases 1 and 2 at subsites 3 to 5 and 8 and 9. While the hydrophobic *p*-nitrophenyl group interacts at subsites 8 and 9 in alpha-amylase 1 more favourably than a glucose residue, the opposite is true at subsite 7. In alpha-amylase 2, the *p*-nitrophenyl group interacts with subsites 7 and 8 much less favourably than a glucose residue, and the binding of the *p*-nitrophenyl group is slightly more favourable than that of glucose at subsite 9. It is therefore likely that at least one amino acid residue involved in subsites 8 and 9 is more hydrophobic in alpha-amylase 1 than alpha-amylase 2.

Once subsite energies have been calculated, they can be used to predict yields of products from a substrate, provided no transglycosylation or multiple attack is taking place. There is no evidence that multiple attack on oligosaccharides up to G_{12} occurs with barley alpha-amylases. Further, failure to observe formation of oligosaccharides larger than substrates in digests of G_9 to G_{12} together with the finding of approximately equal yields of G_y and G_{x-y} in these digests ($x = 9$ to 12)

indicate a low level of transglycosylation. In Tables 3 and 4, yields of oligosaccharides to be expected from G_9 to G_{12} hydrolysis on the basis of the subsite energies of Table 5 are shown. Since the subsite energies were calculated from the experimental yields, good agreement between experimental data and yields predicted from the energies of Table 5 is to be expected. Yields of oligosaccharides were also predicted using interaction energies of Table 5 for subsites 0, 1, 8, and 9 and values of zero for energies of subsites 2–5. It can be seen in Tables 3 and 4 that these yields are almost identical to the predicted yields obtained for non-zero values of subsite 2–5 energies. The interaction energies of subsites 2–5, therefore, appear to have little effect on enzyme action on malto-oligosaccharides such as G_9 to G_{12}. Yields are also independent of binding energies for subsites 6 and 7 provided these remain constant [8,9]. Thus, the hydrolysis of substrates long enough to span across subsites 1 and 8 is determined to a large extent by the outer subsites, 0, 1, 8, and 9 only. The large negative free-energies of interaction at subsites 1 and 8 imply favourable binding at these two subsites. Substrates which can span the two subsites, i.e., G_8 or longer, are hydrolysed relatively quickly by barley alpha-amylases and the hydrolysis rate drops off dramatically for shorter oligosaccharides [5,6]. Because of the favourable binding energy at subsite 1, long substrates tend not to bind to the enzymes with a nonreducing end glucose residue at subsites 2 to 6; hence G_5 and smaller oligosaccharides are rarely produced from the nonreducing ends of such substrates. Only when the substrate is too short to span from subsite 1 to 7 are products smaller than G_6 released from the substrate nonreducing end, and in this case the hydrolysis pattern is influenced by the binding energies of subsites 2 to 6. The positive free-energy at subsite 9 implies an unfavourable glucose–subsite interaction. This in turn causes the barley alpha-amylases to show a slight "preference" for a reducing-end glucose residue at subsite 8 and gives preferential release of maltose from a substrate reducing end. It should be noted that the subsite energies of Ajandouz et al. [6], calculated from barley alpha-amylase action on small modified substrates, consistently give underestimates of the yield of maltose and over-estimates of the yield of maltohexaose from longer oligosaccharides (see Tables 3 and 4).

Yields of oligosaccharides to be expected from hydrolysis of very long substrates such as amylose can be calculated using subsite energies, on the assumption that small oligosaccharides (G_7 or smaller) are not hydrolysed at an appreciable rate while longer oligosaccharides are present in the digest. The results in Fig. 3 show little evidence of any breakdown of dextrins smaller than G_7 and only when 55–60% (by weight) of the material in the digest is smaller than G_7 can noticeable G_7 hydrolysis be observed. Also, previously determined values for molecular activity, k_0, and Michaelis constant K_m [5] suggest that hydrolysis of small oligosaccharides is slow. Yields of oligosaccharides of intermediate length (G_8–G_{12}) cannot be predicted unless values for k_0 and K_m are known for these substrates, but such information is available [5] only for G_8. It is interesting to note [5] that, for G_8, K_m is smaller and k_0/K_m is larger for alpha-amylase 1 than for alpha-amylase 2. Thus, in amylose digests when small amounts of G_8 are formed, the G_8 is likely to be degraded faster by alpha-amylase 1 than 2. This probably explains

the observed lower yields of G_8 during amylose hydrolysis by alpha-amylase 1 when total products smaller than G_7 constitute 40% or less of the digests (Tables 1 and 2).

In the predictions of yields from amylose, calculations were carried out in order to estimate the amounts of G_1 to G_7 to be expected when the yields of G_8 to G_{12} and total of products larger than G_{12} matched those found experimentally. Predicted yields are shown in Tables 1 and 2, using the subsite energies of Table 5. It can be seen that there is reasonably good agreement between predicted and experimental values, although the amount of glucose predicted is consistently lower than that found experimentally. This may be a result of the simplifying assumption that G_7 is not degraded appreciably while larger substrates exist in a digest, since G_7 hydrolysis appears to be a major source of glucose production during alpha-amylolysis by cereal enzymes [5]. At the stage in amylose hydrolysis where 60% (by weight) of the products are smaller than G_7, it is likely that G_7 breakdown is occurring (Fig. 3), but this has not been taken into account in the predictions, and for both alpha-amylase 1 and 2 the amount of G_7 determined experimentally is indeed less than that predicted, while the amount of glucose predicted is appreciably less than found (Tables 1 and 2).

Again, the predicted yields of oligosaccharides smaller than G_8 are not sensitive to the values for free-energies of interaction at subsites 2 to 5, as shown by the second set of predictions in Tables 1 and 2. The pattern of hydrolysis of amylose is dictated, therefore, by interaction of glucose residues with subsites 0, 1, 8, and 9. In vivo, it is unlikely that alpha-amylase in barley is important for the hydrolysis of short oligosaccharides — the beta-amylase present in barley will degrade these substrates more quickly than alpha-amylase. Thus, the pattern of breakdown of starch by alpha-amylase in the germinating seed is probably determined primarily by the binding energies of the alpha-amylase "outer" subsites, 0, 1, 8, and 9, and also by the ability of each subsite to accommodate glucose residues carrying the α-(1 → 6)-linked branches of amylopectin.

It has been assumed that the interaction energy of a glucose residue at a subsite is independent of binding at other subsites. We show here that, for substrates

Table 6
Kinetic parameters for intermediate length substrates

Substrate		G_7		G_8		$pNPG_6$	$pNPG_7$
Kinetic parameter		$K_{m,n}$ (mM)	$k_0/K_{m,n}$ ($M^{-1}s^{-1}$)	$K_{m,n}$ (mM)	$k_0/K_{Km,n}$ ($M^{-1}s^{-1}$)	$k_0/K_{m,n}$ ($M^{-1}s^{-1}$)	$k_0/K_{m,n}$ ($M^{-1}s^{-1}$)
Alpha-amylase 1	Experimental [5]	1.2	2.2×10^4	0.65	4.0×10^5	2.0×10^4	1×10^6
	Predicted with $(\Delta G_6+\Delta G_7)$ $=-6000$ J mol^{-1}	2.4	3.2×10^4	0.68	3.4×10^5	2.1×10^4	0.4×10^6
Alpha-amylase 2	Experimental [5]	2.9	1.3×10^4	2.0	2.3×10^5	2.5×10^4	2.6×10^5
	Predicted with $(\Delta G_6+\Delta G_7)$ $=+1000$ J mol^{-1}	3.0	2.2×10^4	1.0	2.5×10^5	1.3×10^4	1.3×10^5

capable of spanning the active site from subsites 1 to 7, the hydrolysis pattern is not influenced by the binding energies for subsites 2 to 5. Thus, even if binding energies at these subsites are not independent of binding at other subsites, this would have no effect on the yields of products to be expected from hydrolysis of G_8 or larger substrates, but would undoubtedly affect degradation of short oligosaccharides.

The possible results of transglycosylation cannot be quantitated at present because the mechanism is not understood for barley alpha-amylases. It has been shown, however, that transglycosylation and/or condensation reactions are brought about by alpha-amylases only with poor, short substrates at high concentrations [6,31,32]. Thus, reactions of this type are likely to be of little importance during the hydrolyses studied here.

There is no evidence for multiple attack by barley alpha-amylases on oligosaccharides up to G_{12}. It was shown previously that the possibility of a limited amount of multiple attack on amylose (10% or less) could not be excluded [7], and changes made to the subsite model in this paper do not alter that conclusion.

The Michaelis constant, $K_{m,n}$, and molecular activity, k_0, of an enzyme for a particular substrate can be predicted from Eqs. *5* and *6* if values for free-energies of interaction of all subsites are known. Alternatively, if $K_{m,n}$ and k_0 are known, these can be used to investigate subsite energies. Here an attempt was made to find the value of $(\Delta G_6 + \Delta G_7)$ giving the best agreement between K_m and k_0/K_m values predicted, using subsite energies of Table 5 and Eqs. *5* and *7*, and those found experimentally [5]. Both $K_{m,n}$ and k_0 depend on all possible complexes of enzyme with substrate, while $k_0/K_{m,n}$ depends on productive complexes only, where the substrate spans both subsites 6 and 7. In order to find $(\Delta G_6 + \Delta G_7)$ and evaluate $K_{m,n}$ and k_0 for *p*-nitrophenyl maltodextrins, binding energies of the nitrophenyl group at subsites 0–5 would have to be known, and at present there is no easy method to determine their values. For $k_0/K_{m,n}$, however, only complexes with the nitrophenyl group at subsites 7, 8, or 9, or protruding beyond 9, need be considered and the binding energies of Table 5 can be used. A comparison of experimental values of $K_{m,n}$ and $k_0/K_{m,n}$ with values predicted from energies given in Table 5 and the "best" value for $(\Delta G_6 + \Delta G_7)$ is shown in Table 6 for G_7, G_8, $pNPG_6$, and $pNPG_7$. Independent values for ΔG_6 and ΔG_7 were not readily obtained because the $k_0/K_{m,n}$ values depended only on their sum. In order to predict $k_0/K_{m,n}$ from Eq. *7* values of $k_2 = 270$ and 340 s^{-1} were used for alpha-amylases 1 and 2, respectively. These were the values found for molar rates of hydrolysis of G_9–G_{12} at a substrate concentration that is likely to be appreciably greater than the $K_{m,n}$ and hence should correspond [14] to k_2. It can be seen that the predicted values are within a factor of 2.5 of the experimental results. Since the $(\Delta G_6 + \Delta G_7)$ values are found essentially from $(\Delta G_6 + \Delta G_7)$ = total binding energy for a substrate:

$$\sum_{0}^{5} \Delta G_i - \sum_{8}^{9} \Delta G_i$$

any errors in interaction energies for subsites 0 to 5, 8, and 9 will contribute to

errors in $(\Delta G_6 + \Delta G_7)$. Secondly, if the assumption is incorrect that the interaction energies for subsites 7 and 8 do not depend on whether the bound glucose residue is internal or a reducing-end group, then further error can occur in $(\Delta G_6 + \Delta G_7)$. It is, therefore, more useful to consider the values found, -6000 J mol^{-1} for alpha-amylase 1 and $+1000$ J mol^{-1} for alpha-amylase 2, as being indicative of differences between the isozymes, than to place reliance on the absolute values. It is expected that strong similarities will be found for all alpha-amylases at the subsite equivalent to subsite 6 (Fig. 1) of the model discussed here, since two histidine residues postulated to be important at this subsite in *A. oryzae* alpha-amylase [33] are conserved in almost all alpha-amylases, including the cereal alpha-amylases [34–39], and have been shown by mutagenesis to be important for barley alpha-amylase activity [40]. It is, therefore, concluded that the values of $(\Delta G_6 + \Delta G_7)$ are likely to reflect differences at subsite 7 between the two barley isozymes. Alpha-amylase 1 is then expected to have a much more favourable interaction energy for binding a glucose residue than alpha-amylase 2, and this in turn helps to explain the higher yields of glucose liberated by alpha-amylase 1 during hydrolysis of oligosaccharides or amylose.

5. Conclusions

The action of barley alpha-amylases on α-(1 → 4)-linked glucans can be understood in terms of an active site consisting of 10 contiguous subsites capable of interacting with glucose residues. The catalytic site is situated between the seventh and eighth subsites. Free-energies of interaction of subsites with glucose can be calculated and used to predict hydrolysis patterns of oligosaccharides and amylose, but such calculations show that the breakdown of substrates longer than G_8 is influenced mainly by binding energies for the outer subsites, i.e., the first, second, ninth, and tenth subsites. Differences in action of barley alpha-amylase isozymes 1 and 2 can be described in terms of differences in interaction energy at some subsites. Values obtained for individual subsite energies in this and previous work [5,6] depend to some extent on assumptions made when developing a model, but there is general agreement that the second, sixth, and ninth subsites have high affinity for glucose residues, i.e., large negative values for the free-energy of interaction.

6. Experimental

Materials.—Alpha-amylases 1 and 2 were purified from barley malt as described previously [41]. Enzyme activity, in iodine dextrin colour (IDC) units, was assayed using a modification of the Briggs method [42] with beta-limit dextrin prepared from waxy maize starch as the substrate. Malto-oligosaccharides G_1–G_7 were obtained from Sigma Chemical Co., and G_8–G_{12} were purified from debranched glycogen by cellulose column chromatography [43].

Amylose hydrolysis.—Linear amylose was prepared from potato starch as described previously [44]. Amylose (ca. 6.5 mg/mL) was suspended in acetate buffer (0.2 M; mM $CaCl_2$, pH 5.5), heated at 100°C for 2–3 min, and filtered through a 0.5 μm membrane. Enzyme digests contained 300 IDC units of alpha-amylase/mL of amylose solution and they were incubated at 35°C. Samples were removed at 5, 10, 20, 30, 40, and 50 min intervals, boiled for 2 min to inactivate the enzyme, and analysed for total carbohydrate and amylose hydrolysis products. Total carbohydrate was determined by amyloglucosidase digestion followed by measurement of the resulting glucose with hexokinase reagent (Gluco-quant kit, Boehringer-Mannheim).

Product analysis.—Amylose hydrolysis products were analysed by anion-exchange HPLC using a Waters 625 pump and 715 WISP sample injector (Waters Associates, Milford, MA), and a Dionex CarboPac PA1 column (4 × 250 mm) with a PA1 guard column and pulsed amperometric detector (Dionex Canada Ltd., Etobicoke, ON). Digest samples (5–25 μL) were injected directly on to the column and eluted with the following gradient:

Time (min)	A (%)	B (%)	Sodium acetate (mM)
0	25	75	125
30	75	25	375
35	100	0	500
40	25	75	125

A = 150 mM NaOH in 500 mM sodium acetate; B = 150 mM NaOH.

A linear gradient of 125–375 mM sodium acetate in 150 mM NaOH was used for the initial 30 min. Samples were injected at 50-min intervals and the flow rate was 0.75 mL/min. The detector response was calibrated with a mixture of G_1 to G_{12} prepared from stock solutions of the individual dextrins.

Maltodextrin hydrolysis.—Maltodextrin digests were prepared and analysed as described previously [14]. All hydrolyses were carried out at 35°C. For each isoenzyme, at least three samples were taken at different times from duplicate hydrolyses and results were averaged.

Analysis of results.—Calculations of interaction energies, yields of products expected from oligosaccharide hydrolysis, and predicted k_0/K_m values were carried out using Mathcad version 3.1 on an IBM-compatible personal computer. Predicted hydrolysis patterns of amylose were calculated using QBasic programs written for the same computer. The programs were written specifically for amylose hydrolysis by cereal alpha-amylases where maltoheptaose and smaller oligosaccharides are degraded much more slowly than larger oligosaccharides. These programs, therefore, are not generally applicable to other alpha-amylases.

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