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The action of germinated barley alpha-amylases on linear maltodextrins*,†

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ABSTRACT

The actions of barley alpha-amylase isozymes 1 and 2 (EC 3.2.1.1) on malto-oligosaccharides and their *p*-nitrophenyl glycosides were similar, but not identical. For each isozyme, transglycosylation occurred with small substrates that were hydrolysed with difficulty, whereas the rates of hydrolysis increased with increase in the size of the substrate for both the malto-oligosaccharides and the *p*-nitrophenyl glycosides. A *p*-nitrophenyl group was found to mimic a glucose residue to a large extent. The differences in action of the isozymes are believed to be caused by differences at more than one subsite of the active site. A lysine–arginine substitution is postulated to account for some of the observed variations.

INTRODUCTION

Large amounts of alpha-amylase are synthesised during the germination of barley and two major groups of isozymes, alpha-amylases 1 and 2, have been identified[1]. The isoelectric points are in the pI ranges 4.5–5.1 and 5.9–6.6 for alpha-amylases 1 and 2, respectively[2], whereas alpha-amylase 1 has greater stability[3] at pH 3.6 and greater affinity for calcium ions[4] than alpha-amylase 2. In addition, an endogenous inhibitor of barley alpha-amylase reacts with alpha-amylase 2 and appears to have no effect on alpha-amylase 1 (ref. 5). These differences can be explained by differences in amino acids and their sequences in the enzymes. Indeed, sequences deduced from genes or full-length c-DNA clones show significant differences between the two major groups of isozymes[6–10]. Although the overall structure and tertiary folding of the polypeptide chains have not yet been determined, they are expected to be similar for alpha-amylases 1 and 2 (refs. 11 and 12).

Less is known, however, of differences in the action of the two groups of isozymes. Each hydrolyses intact starch granules, but alpha-amylase 1 appears to be more efficient

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than alpha-amylase 2 when equal amounts of activity are used[13,14]. Also, the profiles of malto-oligosaccharides that result from the hydrolysis of starch granules differ significantly for the two groups of isozymes[13]. We now report on the hydrolysis of malto-oligosaccharides and their *p*-nitrophenyl glycosides by alpha-amylases 1 and 2.

The action of alpha-amylase 2 on amylose has been explained in terms of subsite theory[15], and an attempt has been made to use this theory to explain qualitatively the differences observed in the actions of alpha-amylases 1 and 2.

RESULTS

Product distributions. — The distributions of products formed on hydrolysis of malto-oligosaccharides by alpha-amylases 1 or 2 are shown in Table I. The yields given are an average of at least six determinations and each hydrolysis was allowed to proceed until no more than 20% of the substrate was hydrolysed.

TABLE I

Yields of products from hydrolysis of malto-oligosaccharides by barley alpha-amylases 1 and 2

Substrate	*Isozyme*	*Products (mol. % of total products)*[a]							
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8
G_6	1	9	41	14	27	9	-	tr.[b]	-
	2	3	50	9	35	3	-	tr.	-
G_7	1	43	5	2	2	5	43	-	-
	2	35	11	4	4	11	35	-	-
G_8	1	12	38	1	1	1	34	14	-
	2	9	43	1	1	2	36	9	-
G_9	1	4	34	13	1	1	13	30	4
	2	3	39	9	1	1	9	35	3

[a] Experimental error is ± 5% of values given. [b] Trace = tr.

Similar amounts were obtained of the two fragments produced by the scission of one bond in malto-oligosaccharides, except in the case of G_6* (Table I). For example, the same molar quantities, within experimental error, of G_3 and G_6 were produced from G_9, but a significant excess of G_2 over G_4 was produced from G_6. Each isozyme gave G_2 and G_3 almost exclusively from G_5 (data not shown) and mainly G_2 from G_4. Some transglycosylation also occurred, and small amounts of G_6 and G_7 appeared in the digest of G_5; G_6 was formed in the digest of G_4 (Fig. 1), and G_7 was also produced in the digest of G_6.

p-Nitrophenyl glycosides of α-malto-oligosaccharides and a derivative (B*p*NPG_7) of *p*-nitrophenyl α-maltoheptaoside, blocked at the non-reducing end with a 4,6-*O*-

benzylidene group, were hydrolysed by alpha-amylases 1 and 2. Only the *p*-nitrophenol* released and the *p*-nitrophenyl glycosides formed were quantified, and the results are given in Table II as averages of at least six determinations for $<20\%$ hydrolysis of the substrate. Transglycosylation was indicated by the appearance of $p\text{NPG}_6$ in the digest of $p\text{NPG}_4$.

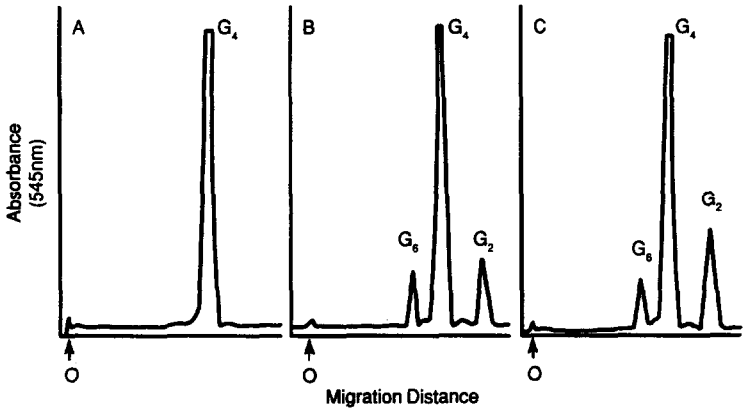

Fig. 1. H.p.t.l.c. of the products of hydrolysis of maltotetraose by barley alpha-amylases 1 and 2: A, before hydrolysis; B, digest with alpha-amylase 1; C, digest with alpha-amylase 2; G_2, G_4, G_6 denote maltose, maltotetraose, maltohexaose, respectively; O, origin.

TABLE II

Yields of *p*-nitrophenyl glycosides from hydrolysis of *p*-nitrophenyl malto-oligosaccharides by barley alpha-amylases 1 and 2

Substrate	*Isozyme*	*Products (mol.% of nitrophenyl products)*[a]						
		p*NP*	p*NPG*	p*NPG*$_2$	p*NPG*$_3$	p*NPG*$_4$	p*NPG*$_5$	p*NPG*$_6$
*p*NPG$_4$	1	4	23	61	13	-	-	tr.[b]
	2	5	19	76	1	-	-	tr.
*p*NPG$_5$	1	-	40	44	13	3	-	-
	2	-	25	23	50	2	-	-
*p*NPG$_6$	1	49	21	13	6	5	5	-
	2	55	14	14	6	6	4	-
*p*NPG$_7$	1	7	91	2	-	-	-	-
	2	18	80	2	-	-	-	-
B*p*NPG$_7$	1	-	95	5	-	-	-	-
	2	5	89	5	-	-	-	-

[a] Experimental error is ± 5% of values given. [b] Trace = tr.

* Abbreviations G_1, G_2, G_3, ... *etc.*, are used for glucose, maltose, maltotriose, ... *etc.*, and *p*NP, *p*NPG, *p*NPG$_2$, ... *etc.*, are used for *p*-nitrophenol, *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl α-maltoside, *etc.*

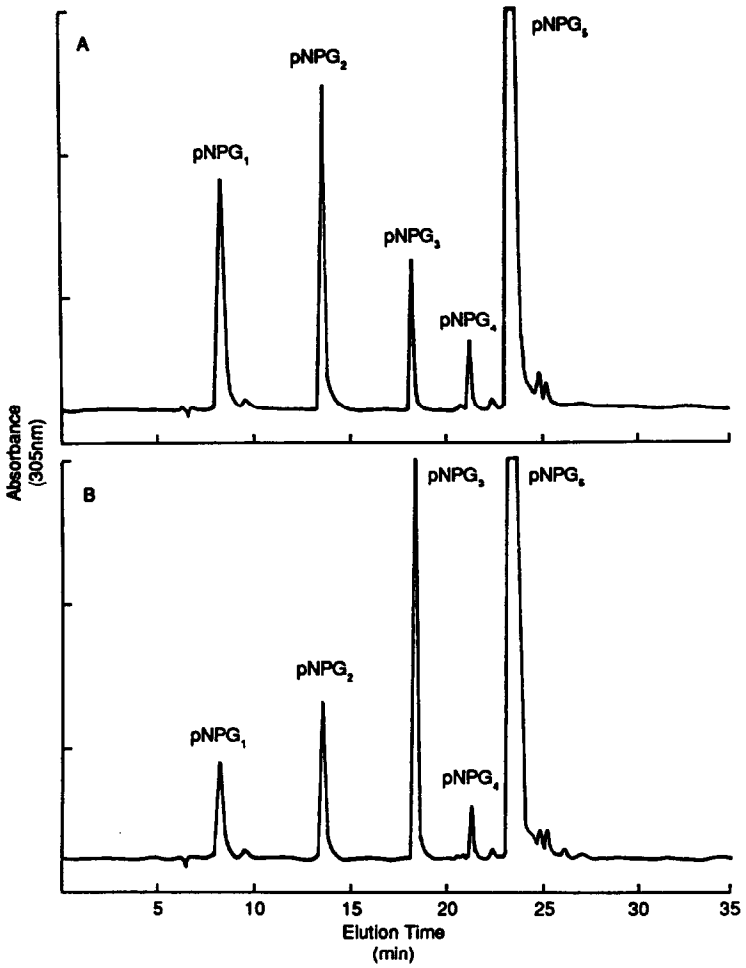

Fig. 2. H.p.l.c. of the products of hydrolysis of *p*-nitrophenyl α-maltopentaoside by barley alpha-amylases 1 and 2: A, digest with alpha-amylase 1; B, digest with alpha-amylase 2; pNPG$_1$, pNPG$_2$, pNPG$_3$, *etc.* denote *p*-nitrophenyl α-glucosides of D-glucopyranose, maltose, maltotriose, *etc.*

The results show that there are differences in the actions of alpha-amylases 1 and 2. This difference is small for the hydrolysis of G_9 and most marked for the hydrolysis of pNPG$_5$ (Fig. 2).

Rates of hydrolysis. — Michaelis–Menten saturation curves for the rates of reaction versus the concentrations of the substrate were obtained for the hydrolysis of G_6, G_7, G_8, pNPG$_6$, pNPG$_7$, and BpNPG$_7$ by each isozyme. Those for the hydrolysis of G_8 are shown in Fig. 3A. The results were analysed using Eadie–Hofstee plots to determine K_m and V_{max}, and hence the catalytic constant k_{cat} (Fig. 3B, Table III); k_{cat} is expressed as moles of bond hydrolysed per s per mole of enzyme added.

No attempt was made to study the effect of the concentration of the substrate with

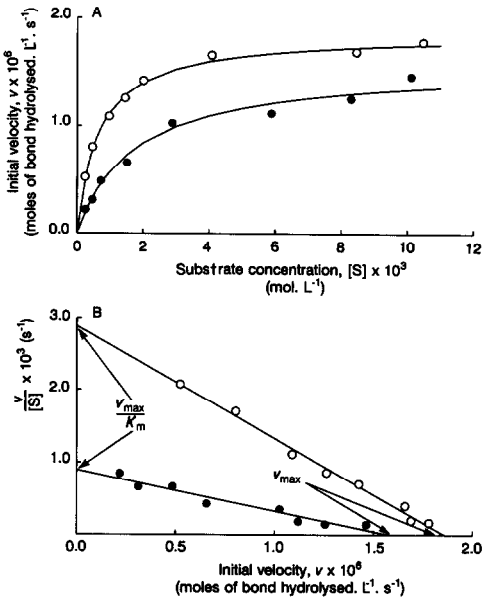

Fig. 3. Reaction kinetics for the hydrolysis of G_8 by alpha-amylases 1 and 2: A, variation of initial velocity of reaction with substrate concentration; B, Eadie–Hofstee plot used to determine K_m and V_{max}; o, apha-amylase 1; •, alpha-amylase 2; solid lines represent plots calculated from the values of K_m and k_{cat} in Table III.

TABLE III

Kinetic parameters for oligosaccharide hydrolysis by barley alpha-amylases 1 and 2

Substrate	*Isozyme*	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
G_6	1	10 ±2	8 ±3	0.8×10^3
	2	13 ±2	25 ±8	2.0×10^3
G_7	1	1.2 ±0.2	27 ±3	2.2×10^4
	2	2.9 ±0.5	40 ±8	1.3×10^4
G_8	1	0.65 ±0.07	260 ±30	4.0×10^5
	2	2.0 ±0.2	450 ±80	2.3×10^5
pNPG_6	1	5 ±0.5	100 ±10	2.0×10^4
	2	4 ±0.4	100 ±10	2.5×10^4
pNPG_7	1	0.35 ±0.04	350 ±40	1.0×10^6
	2	1.4 ±0.1	370 ±40	2.6×10^5
B*p*NPG_7	1	0.35 ±0.04	530 ±50	1.5×10^6
	2	0.8 ±0.1	420 ±40	5.3×10^5

TABLE IV

Initial rates of hydrolysis of malto-oligosaccharides (5 mg/mL) by barley alpha-amylases 1 and 2

Substrate	*Isozyme*	*Rate* (s^{-1})	*Substrate*	*Isozyme*	*Rate* (s^{-1})
G_6	1	3 ±2	*p*NPG_4	1	12 ±3
	2	8 ±3		2	12 ±3
G_7	1	21 ±3	*p*NPG_5	1	7 ±2
	2	27 ±4		2	12 ±2
G_8	1	200 ±20	*p*NPG_6	1	60 ±6
	2	260 ±30		2	48 ±5
G_9	1	280 ±40	*p*NPG_7	1	270 ±30
	2	360 ±40		2	280 ±30
			B*p*NPG_7	1	420 ±40
				2	330 ±30

smaller substrates due to the complexity caused by transglycosylation which is pronounced at higher concentrations.

For comparative purposes, however, the rate of hydrolysis of several oligosaccharides was determined at one concentration of the substrate, giving approximately the same concentration of α-(1→4) linkages in each digest, for both isozymes. The results are given in Table IV, expressed as moles of bond hydrolysed per s per mole of enzyme used. These results show that, on a molar basis, alpha-amylases 1 and 2 hydrolyse substrates at comparable rates, but the rates increase with increasing numbers of glucose residues in the substrate.

DISCUSSION

Alpha-amylases 1 and 2 differ in their actions on malto-oligosaccharides and their *p*-nitrophenyl α-glycosides. The distributions of products resemble each other more than they do those produced by alpha-amylases from pig pancreas[16], *Aspergillus oryzae*[17,18], or *Bacillus amyloliquefaciens*[19]. In particular, the alpha-amylases 1 and 2 produce more G_1 from G_7, G_8, and G_9; more G_2 from G_6, G_8, and G_9; and less G_3 from G_6, G_8, and G_9 than other alpha-amylases[16–19].

Several alpha-amylases, from mammalian[20–25], bacterial[21,22,26,27], and fungal sources[17,18,21,28–32], catalyse transglycosylation reactions, and such activity has been reported[33,34] for the major isozyme of germinated barley, alpha-amylase 2. The appearance of G_6 in a digest of G_4 (Fig. 1), and higher oligosaccharides in digests of G_5, G_6, and *p*NPG_4, confirms that alpha-amylases 1 and 2 each effects transglycosylation. The decreasing k_{cat}/K_m values in Table III and the decreasing rates of hydrolysis shown in Table IV indicate that alpha-amylases 1 and 2 each hydrolyses smaller substrates with great difficulty. For many alpha-amylases[17,18,20,24,30], transglycosylation becomes more evident

as the size of the substrate and the rate of hydrolysis decrease, and this trend occurs with alpha-amylases 1 and 2. Because of the increase in transglycosylation with G_4, G_5, G_6 and pNPG$_4$, the values given in Tables III and IV for the smaller substrates are measures of apparent hydrolysis only, *i.e.*, appearance of products smaller than the original substrate.

The results in Table I show little evidence of multiple attack by the alpha-amylases 1 and 2 on G_7, G_8, or G_9, since a small, or no, excess of, say, G_2 over G_5 (from G_7), G_6 (from G_8), or G_7 (from G_9) was found in the digests. It is possible that the significant excess of G_2 in G_6 digests was formed during the hydrolysis of G_6 by a multiple attack mechanism, but it is also likely that the reaction is complicated by transglycosylation. Although multiple attack is well documented for mammalian alpha-amylases[35–44] and the enzyme from *A. oryzae*[36,41,45–47], there is little evidence that it is important in the action of bacterial alpha-amylases[38,41,47] and is not, therefore, a universal feature of hydrolysis by alpha-amylases. An earlier study of the action of alpha-amylase 2 on amylose was inconclusive on this point. The results could be explained with or without multiple attack[15], although no evidence of multiple attack was found for an alpha-amylase from malted rye[38].

The active sites of alpha-amylases are generally considered to be composed of subsites, with each subsite capable of interacting with a glucose residue of a (1→4)-α-D-glucan substrate[15,16,18,19,47] (Fig. 4). The differences in the actions of alpha-amylases from various sources are then explained in terms of differences in the numbers of subsites at each active site and variations in energies of interaction of individual subsites and glucose residues of the substrate. The similarities in product distributions and reaction

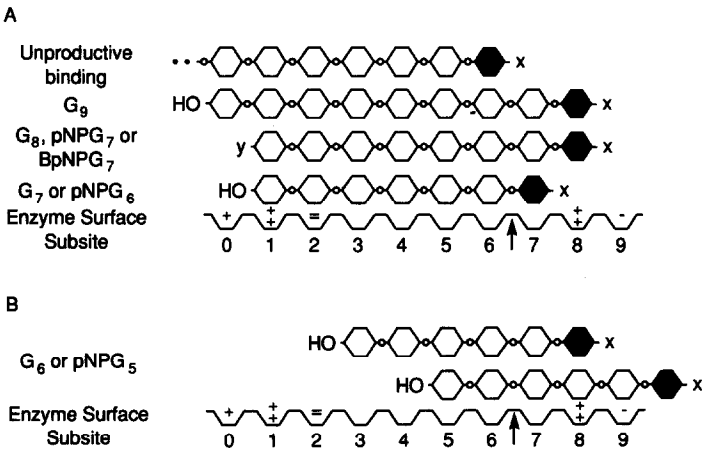

Fig. 4. Active centre of the enzyme showing 10 subsites (0–9), each capable of interacting with a glucose residue of the substrate: A, productive and unproductive modes of binding; B, two possible modes of binding G_6 or pNPG$_5$, of which the lower mode would be more favourable for alpha-amylase 2 and the upper for alpha-amylase 1; ↑, catalytic site of the enzyme; ⌒, an enzyme subsite; ⬡, a G_1 residue of the substrate; y, HO-4 or a 4,6-*O*-benzylidene group; ●, a G_1 or phenyl group; X, HO-1 on G_1 or NO_2 of a p-nitrophenyl group; +, favourable glucose–enzyme interactions; -, unfavourable interactions.

kinetics of alpha-amylases 1 and 2 (Tables I–IV) suggest that the arrangements of subsites at the active sites should be broadly similar but that the differences observed may be due to differences in the amino acids at individual subsites.

In general, for alpha-amylases 1 and 2, K_m decreases and k_{cat} increases as n in the substrate G_n increases (Table III), a trend which has been observed for other alpha-amylases[19,48,49]. This trend is believed to be a consequence both of better binding of larger substrates that can span a complete active site, and of a larger proportion of possible binding modes of a long substrate leading to hydrolysis[48,49] [if substrate binds to enzyme so that glucose residues do not occupy both subsites 6 and 7, no hydrolysis can take place (Fig. 4A)]. The values obtained for alpha-amylases 1 and 2 have orders of magnitude the same as those for other alpha-amylases[16,19,43,44,48–50]. McCleary and Sheehan[51] reported a K_m value of 0.6mM for wheat alpha-amylase (probably alpha-amylase 2) acting on B*p*NPG$_7$ at 40° and pH 5.2, and Sirou *et al.*[52] found a value of 0.73mM for barley malt alpha-amylase (mainly alpha-amylase 2) acting on the same substrate at 30° and pH 5.2, Søgaard and Svensson[53], on the other hand, obtained K_m values of 0.7 or 3.1mM and values of k_{cat} of 690 or 720 s^{-1} for alpha-amylases 1 or 2, respectively, with *p*NPG$_7$ at 37° and pH 6.2. It should be noted that these results were obtained under experimental conditions that were different from those used here.

For most substrates, K_m is higher for alpha-amylase 2 than for alpha-amylase 1. Thus, binding to the former isozyme is less favourable and must reflect differences at one or more subsites. The general similarity in the values of k_{cat} for the two isozymes suggests that the mechanism of catalysis and the amino acid residues involved are similar. Three carboxyl groups have been implicated in the mechanism of catalysis by alpha-amylases[54–56] and alignments of amino acid sequences[8,11] show that these are conserved in each isozyme. The carboxyl groups are associated with aspartic acid 180 and 291 and glutamic acid 205 of alpha-amylase 1 and the corresponding amino acids 181, 291, and 206 of alpha-amylase 2.

Kinetic parameters (K_m, k_{cat}, and hydrolysis rates, Tables III and IV) suggest that the *p*-nitrophenyl moiety of the malto-oligosaccharide α-glycosides can mimic a glucose residue, *e.g.*, K_m and k_{cat} for G_8 and *p*NPG$_7$ are similar. Consequently, it can be deduced from the results in Table II that most G_1, G_2, and G_3 produced during hydrolysis of such substrates as G_7, *p*NPG$_6$, or larger, come from scission of linkages near the reducing end of the substrate (Fig. 4A), in agreement with earlier work on barley alpha-amylase[33,57].

The subsite model proposed[15] for alpha-amylase 2 suggests that "preferred" products from longer malto-oligosaccharides should be G_2 and G_6 because of favourable energies of interaction at subsites 1 and 8 (Fig. 4A). This interpretation is indeed true for the hydrolysis of G_8 (Table I) and can also be considered to hold true for the hydrolysis of *p*NPG$_7$ and B*p*NPG$_7$ (Table II) if it is assumed that a *p*-nitrophenyl grouping mimics a glucose residue at the active site of the enzyme. Hydrolysis of G_9 yields more $G_2 + G_7$ than $G_3 + G_6$ (Table I). This result can occur if the energy of interaction at subsite 9 is unfavourable, as was proposed[15], or if an extra subsite, subsite 0 (Fig. 4A), has a small favourable energy of interaction. Such a subsite was not considered hitherto, but the apparent high yield of G_7 from amylose obtained earlier (see Fig. 3 of ref. 15) suggests this possibility.

The substrate G_7 cannot bind to the enzyme and involve subsites 1 and 8 simultaneously. Binding of the non-reducing end of G_7 at subsite 1 leaves the reducing moiety at subsite 7 (Fig. 4A), whereas binding of the non-reducing end at subsite 2 (believed[15] to have an unfavourable energy of interaction) allows the reducing moiety to occupy subsite 8. The results in Tables I and II suggest that the former binding mode is preferred. The decreased efficiency of enzymic action on substrates which cannot bind at subsites 1 and 8 simultaneously is reflected in the much reduced k_{cat}/K_m values for G_7 and pNPG$_6$ compared to those for G_8 and pNPG$_7$ (Table III).

Differences between the two isozymes, in the yields of G_1 and G_2 from malto-oligosaccharides (Table I) and of *p*-nitrophenol or *p*-nitrophenyl α-D-glucopyranoside from their *p*-nitrophenyl glycosides (Table II), probably reflect differences between alpha-amylases 1 and 2 at subsites 7 and 8. Possible identities of amino acid residues at particular subsites have been suggested for the alpha-amylase of *A. oryzae*[54], and lysine (residue 209 of *A. oryzae* alpha-amylase[54]) was suggested to be important at subsites equivalent to 7 and 8 of alpha-amylases 1 and 2. Amino acid sequence alignment of barley and *A. oryzae* alpha-amylases[11] indicates that an equivalent lysine is present in alpha-amylase 2 (residue 184), but is replaced by arginine in alpha-amylase 1 (residue 183)[6–10].

The fact that barley alpha-amylases appear to form more G_1 than other alpha-amylases[16–19], at least from G_7, G_8, and G_9, may reflect differences at subsite 7. Matsuura *et al.*[54] have suggested that histidine (residue 210 of *A. oryzae* alpha-amylase) makes up part of the subsite equivalent to subsite 7 of the barley enzymes. Again, the amino acid sequences show[11] that the histidine, present in most alpha-amylases, is replaced by glycine (residue 184 of alpha-amylase 1 and 185 of alpha-amylase 2). Site-directed mutagenesis of the equivalent histidine to aspartic acid in *Bacillus stearothermophilus* alpha-amylase gave an active enzyme which produced more G_1 from soluble starch than the wild-type alpha-amylase[58].

The preferred mode of binding of G_6 to alpha-amylases may differ. Each isozyme yields G_2 and G_4 as the major products, which could arise from binding the substrate reducing-end residue at subsite 8 or the non-reducing-end residue at subsite 5 (Fig. 4B). The results for the hydrolysis of pNPG$_5$ (Fig. 2 and Table II) suggest the former binding would be more likely for alpha-amylase 1 and the latter for alpha-amylase 2. For pNPG$_6$, pNPG$_7$, and BpNPG$_7$, there are relatively small differences between the two isozymes in the production of *p*-nitrophenol, *p*-nitrophenyl α-D-glucopyranoside, and *p*-nitrophenyl α-maltotrioside. This finding suggests that the major differences found for the hydrolysis of pNPG$_5$ may arise from differences in binding the non-reducing end of the substrate, *i.e.*, at subsites 3 or 5. There is little homology in the amino acid sequences of barley alpha-amylases and *A. oryzae* alpha-amylase[11] in the regions believed to constitute subsites 3 or 5 (ref. 54) and it is not yet possible to ascribe such subsite variations to particular amino acids in the barley isozymes. This difference is not likely to be important for the hydrolysis of large substrates, such as amylose, which are likely to bind to the whole active site. Glucose–enzyme interactions at subsites 1, 8, and possibly 0 and 9 are then likely to determine how hydrolysis proceeds (Fig. 4). The

difference may be more important for the hydrolysis of amylopectin where short branches or short inter-branch linear chains may not completely span the active site. The differences at subsite 3 or 5 may influence the rate of hydrolysis of amylopectin, but it is more difficult to predict whether they affect the nature of the branched dextrins which result. Earlier studies[59] of the smallest branched dextrins produced by barley alpha-amylase 2 suggest that the glucose residue carrying the branch at position 6 cannot bind at subsites 5 or 6, and may have some difficulty in binding to subsite 7. There is probably little hindrance to binding of the branch-carrying glucose residue at other subsites, and the branch is likely to protrude from the active site.

The 4,6-*O*-benzylidene group of B*p*NPG$_7$ does not make this oligosaccharide less susceptible to hydrolysis. Rather, it is hydrolysed slightly faster than *p*NPG$_7$ (Tables III and IV) to give less *p*NP and more *p*NPG$_1$ than from *p*NPG$_7$ (Table II). This result suggests an unfavourable interaction of the non-reducing 4,6-*O*-benzylideneglucosyl residue at subsite 0 or a more favourable binding at subsite 1. The latter possibility seems more likely in view of the increased rate of hydrolysis and k_{cat}/K_m of each isozyme for B*p*NPG$_7$ compared to *p*NPG$_7$ (Tables III and IV). The presence of a hydrophobic or aromatic amino acid side chain in the vicinity of subsite 1 of the enzyme could explain these observations.

The results presented here suggest that alpha-amylases 1 and 2 can be described by the nine-subsite model[15], although one additional subsite (subsite 0, Fig. 4) may be present. Differences at individual subsites can account for differences observed in the distributions of products and kinetic parameters, as a slightly less favourable energy of binding at one or two subsites of alpha-amylase 2 could account readily for differences in K_m between alpha-amylases 1 and 2 (refs. 48 and 49). Where high sequence homology exists between barley alpha-amylases and alpha-amylases with known three-dimensional structures[11,54,55], it is possible to suggest differences in amino acids at the active site, which may be responsible for the observed variations in action of alpha-amylases 1 and 2. It will not be possible to locate all of the amino acid substitutions responsible for differences in activity until more information is available on differences in action of alpha-amylases 1 and 2 on larger substrates, such as amylose and amylopectin, and the tertiary structure of a barley alpha-amylase is known.

EXPERIMENTAL

Materials. — Malto-oligosaccharides (G_4–G_7) were obtained from Sigma Chemical Co., and G_8 and G_9 were purified from debranched glycogen by column chromatography on cellulose[60]. *p*-Nitrophenol and *p*-NP-maltodextrins were obtained from Boehringer-Mannheim, and B*p*NPG$_7$ from Genzyme Corp. (Boston, MA). All other reagents used were of Reagent grade or better.

Alpha-amylases 1 and 2 were purified from barley malt, essentially as described[61,62]. Enzyme activity, in iodine dextrin colour (IDC) units, was assayed using a modification of the Briggs method[63] with beta-limit dextrin prepared from waxy maize starch as substrate. IDC units were converted to specific activities using factors of 1.5×10^{13} IDC units/mole of alpha-amylase 1 and 2.8×10^{13} IDC units/mole of alpha-amylase 2. Determination of these conversion factors will be discussed elsewhere.

Hydrolysis of G_4 and G_5. — Digests containing G_4 or G_5 (5 mg/mL) and alpha-amylase 1 or 2 (1500 IDC U/mL) in acetate buffer (0.1M, mM $CaCl_2$, pH 5.5) were incubated at 35°. Samples were removed at intervals, boiled rapidly to inactivate the alpha-amylase, and analysed by high-performance t.l.c. (h.p.t.l.c.) using a modified version[64] of the method described by Wursch and Roulet[65]. Stained plates were scanned on a Schimadzu scanner and the signal was integrated with a Hewlett–Packard integrator.

Rates of hydrolysis of G_6–G_9 and product distributions. — Digests containing 5 mg of malto-oligosaccharide per mL of acetate buffer (0.1M, mM $CaCl_2$, pH 5.5) were incubated at 35°. Known amounts of approximately 400, 200, 75, and 30 IDC U of alpha-amylase 1 or alpha-amylase 2 per mL of digest were added to G_6, G_7, G_8, and G_9 assays, respectively. Aliquots were removed at intervals of 10 min (G_6 and G_7) or 5 min (G_8 and G_9), placed in 2-mL capped polypropylene micro test-tubes (Bio-Rad Laboratories), and boiled for 4 min to inactivate the enzyme. Samples were filtered using a 1-mL Becton Dickson tuberculin syringe and Nalgene 4-mm syringe filter (0.2-μm pore, nylon membrane) before h.p.l.c. analysis.

Digests were analysed by anion-exchange h.p.l.c. using a hybrid Waters/Dionex system consisting of a Waters 625 pump and 715 WISP (Waters Associates, Milford, MA), a Dionex CarboPac PA1 column (4 × 250 mm, no guard column), and a Dionex pulsed amperometric detector (p.a.d.) with a gold working electrode (Dionex Canada Ltd., Etobicoke, Ont.). Chromatography was controlled with a Waters 840 Data and Chromatography Control Station (Waters Expert Chromatography software running on a Digital Professional 380 computer). Filtered samples (25 μL) were injected onto the column and G_1–G_9 digest products were resolved by using a linear gradient of 50→200mM sodium acetate in 150mM sodium hydroxide over 25 min. The flow rate was 1 mL/min.

Hydrolysis rates were calculated as moles of bond hydrolysed per mole of enzyme per unit time from the total amounts of the following products: G_6, $G_1 + G_2 + \frac{1}{2}G_3$; G_7, $G_1 + G_2 + G_3$; G_8, $G_1 + G_2 + G_3 + \frac{1}{2}G_4$; G_9, $G_1 + G_2 + G_3 + G_4$.

The detector response was calibrated using a standard mixture of G_1–G_9 prepared from solutions of the individual malto-oligosaccharides. Concentrations of the malto-oligosaccharide solutions were determined by amyloglucosidase digestion followed by measurement of the resulting glucose with hexokinase reagent (Glucoquant kit, Boehringer-Mannheim). Amyloglucosidase digests were monitored by h.p.l.c. to ensure that glucose was the sole product. Concentrations of the individual malto-oligosaccharide solutions were adjusted to account for the small but variable amounts (as determined by h.p.l.c.) of contaminating malto-oligosaccharides present.

Rates of hydrolysis of p-NP-malto-oligosaccharides and product distributions. — Digests were similar to those described for the malto-oligosaccharides, except that the following concentrations of isozyme were used per mL of digest: 400 IDC U of alpha-amylase 1 and 500 U of alpha-amylase 2 for *p*NPG_4, 200 U of alpha-amylase 1 and 250 U of alpha-amylase 2 for *p*NPG_5, 100 U of alpha-amylase 1 and 150 U of alpha-amylase 2 for *p*NPG_6, and 15 U of alpha-amylase 1 and 25 U of alpha-amylase 2

for B*p*NPG$_7$. Samples were removed at intervals (6 min), boiled rapidly, and analysed directly by h.p.l.c. Samples were injected automatically onto a Cosmosil amino-bonded column (25 × 4.6 cm) from Nakarai Chemicals Ltd. (Japan), and eluted with an 80→60% gradient of acetonitrile in water at 30° and 0.75 mL/min. The eluate was monitored at 305 nm with a Waters 490 multi-wavelength detector. Product distributions and rates of hydrolysis were determined by quantifying all *p*NP-containing products.

Determination of kinetic constants. — (*a*) *Malto-oligosaccharide substrates.* — All digests were prepared in sodium acetate buffer (0.1M, mM $CaCl_2$, pH 5.5). Maltohexaose (2–14 μmol/mL) was digested with 500 IDC U/mL of alpha-amylase 1 or 2 for 10 and 30 min at 35° and boiled to inactivate the enzyme. Maltoheptaose (0.5–14 μmol/mL) was digested with 325 IDC U/mL of alpha-amylase 1 or 2 for 10 and 20 min. Maltooctaose (0.25–10 μmol/mL) was digested with 110 IDC U/mL of alpha-amylase 1 or 2 for 5 min. Samples were filtered and analysed by h.p.l.c. as described above.

Kinetic constands V_{max} and K_m were derived from Eadie–Hofstee plots, using the PROC REG procedure of the SAS statistical software (SAS Institute Inc., Cary, NC).

(*b*) p*NP-Malto-oligosaccharide substrates.* — Digests were prepared in acetate buffer (0.2M, mM $CaCl_2$, pH 5.5) and contained the following: 0.28–11.1 mg/mL of pNPG$_6$ and 150 IDC U/mL of alpha-amylase 1 or 2, 0.32–12.7 mg/mL of *p*NPG$_7$ or 0.34–13.6 mg/mL of B*p*NPG$_7$ and 25 IDC U/mL of alpha-amylase 1 or 2. Digests were incubated at 35° for 5 min, then boiled, and appropriate aliquots were analysed directly by h.p.l.c. All *p*NP-containing products were quantified.

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