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Note

Influence of pH on the hydrolysis of *p*-nitrophenyl maltodextrins by alpha-amylase 2 from malted barley[1]

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Abstract

The action of barley malt alpha-amylase 2 (E.C. 3.2.1.1) on *p*-nitrophenyl derivatives of maltodextrins was studied at pH 4.8, 6.0 and 7.8. Distributions of products from any one substrate changed little with pH, but a difference in behaviour was observed at pH 4.8 between longer and shorter substrates. With long substrates, such as *p*-nitrophenyl alpha-malto-octaoside, the enzyme showed high activity at pH 4.8 and 6.0, while for shorter substrates, enzyme activity was much less at pH 4.8 than at 6.0. © 1998 Elsevier Science Ltd. All rights reserved.

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Alpha-amylases (EC 3.2.1.1) are endo-acting enzymes that hydrolyse the α-(1→4) bonds of starch and related polysaccharides. General properties, such as pH–activity profiles, and action patterns of alpha-amylases on oligo- and poly–saccharides differ according to the source of the enzyme [1]. For mammalian alpha-amylases [2–5] and, to a lesser extent for a fungal enzyme [6], the variation of activity with pH has been found to depend on the size of the substrate. Thus, differences in the pH for optimum activity for large and small substrates have been observed, as well as changes with pH in the distribution of products formed on hydrolysis of one substrate. In this study, the effect of pH on the hydrolysis of *p*-nitrophenyl derivatives of maltodextrins by alpha-amylase 2, the high pI isozyme of barley malt alpha-amylase, was determined to characterise more fully the action of this enzyme. *p*-Nitrophenyl maltoheptaoside, modified at the non-reducing end with a 4, 6-*O*-benzylidene group (B*p*NPG$_7$) is widely used as a substrate for the assay of cereal alpha-amylase [7]. Complications in the assay might arise if the nature of the major products formed on α-amylolysis changed markedly with pH. We, therefore, included this substrate in the study.

Abbreviations: *p*NP; *p*NPG$_1$; *p*NPG$_2$...etc.; are used for *p*-nitrophenol; *p*-nitrophenyl α-D-glucopyranoside; *p*-nitrophenyl α-maltoside, etc.

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p-Nitrophenyl glycosides of α-malto-oligosaccharides and B*p*NPG$_7$ were hydrolysed by barley malt α-amylase 2 in Tris–acetate buffer at pH 4.8, 6.0 and 7.8. The *p*-nitrophenol or *p*-nitrophenyl glycosides released at less than 20% hydrolysis of the substrate were quantified using HPLC. Extents of hydrolysis were kept low in an attempt to minimise secondary hydrolysis of primary products and transglycosylation. There was little hydrolysis in the zero time subsamples indicating that the alpha-amylase was rapidly inactivated in the boiling-water bath. Nevertheless, digest products in the zero time hydrolysates were subtracted from the amount measured in the 5–20 min digests.

Product distributions, determined as averages of two to six determinations, are shown in Fig. 1. It can be seen that variations in pH bring about little change in these distributions, the largest effect being observed for the hydrolysis of *p*NPG$_6$ at pH 6.0, where *p*-nitrophenol production is decreased and the yield of *p*-nitrophenyl α-maltoside is increased by ~ 5% of total products, compared to the situation at pH 4.8 or 7.8. Hydrolysis of *p*NPG$_5$ at a higher concentration (5 mg/mL compared to 4 mg/mL for all substrates of Fig. 1) gave results that agree within experimental error with those shown in Fig. 1. Product yields from *p*NPG$_5$, *p*NPG$_6$, *p*NPG$_7$, and B*p*NPG$_7$ are comparable to those reported earlier for hydrolysis at pH 5.5 in acetate buffer at a substrate concentration of 5 mg/mL [8].

In earlier studies the action of barley malt alpha-amylases on oligo- and poly-saccharides was explained in terms of an enzyme active site that consists of ten contiguous subsites, where each subsite is capable of interacting with one glucose residue of a (1→4)-α-D-glucan substrate, as illustrated in Fig. 2 [8–10]. Differences in interaction energy between a glucose residue and amino acid sidechains at each subsite were thought to account for characteristic product distributions obtained on hydrolysis of various substrates. Subsite interaction energies were calculated for barley malt alpha-amylase 2 and used to predict satisfactorily the action of the enzyme at pH 5.5 on amylose and oligosaccharides containing 9–12 glucose residues [10]. Here we find that, for any one substrate, there are only minor changes in product distributions with pH, suggesting that pH has little effect on the subsite energies for a single substrate. Calculated energies from the literature, along with proposed interaction energies for a *p*-nitrophenyl group [10], were used, therefore, to calculate expected product

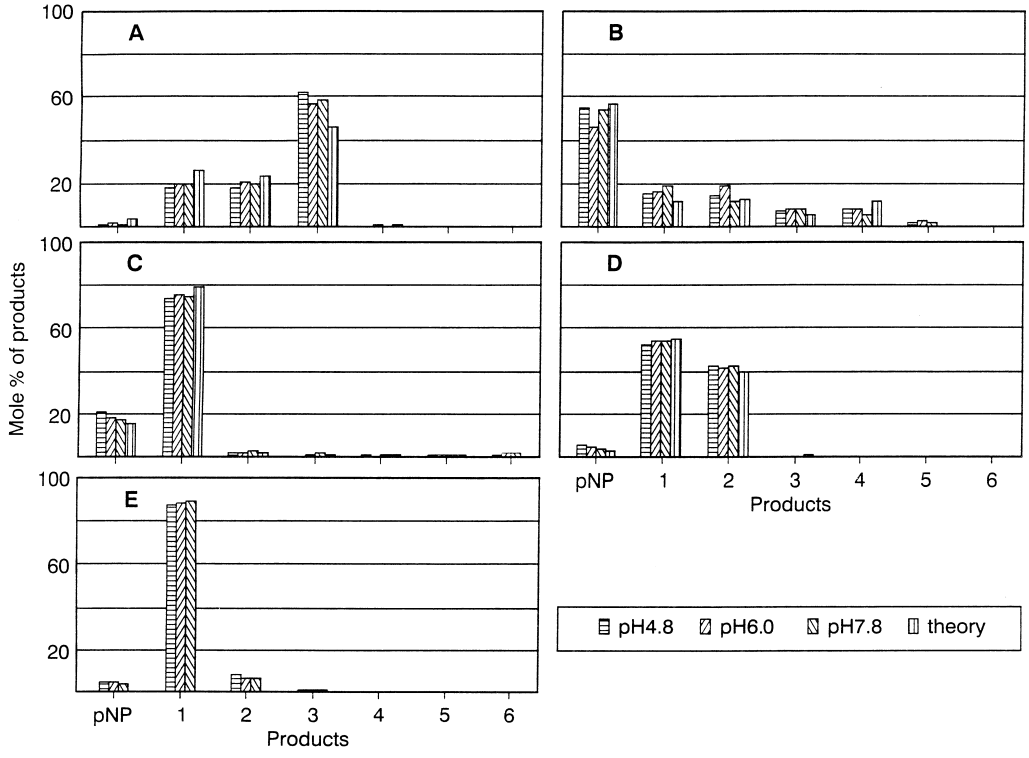

Fig. 1. Distribution of products obtained on hydrolysis of (A) *p*NPG$_5$, (B) *p*NPG$_6$, (C) *p*NPG$_7$, (D) *p*NPG$_8$, and (E) B*p*NPG$_7$ by barley malt alpha-amylase 2. 1, 2, ...= *p*NPG$_1$, *p*NPG$_2$, etc., for yields greater than 15 mol%, the uncertainty is ±2 mol%. Uncertainty in smaller yields is ±1 mol%.

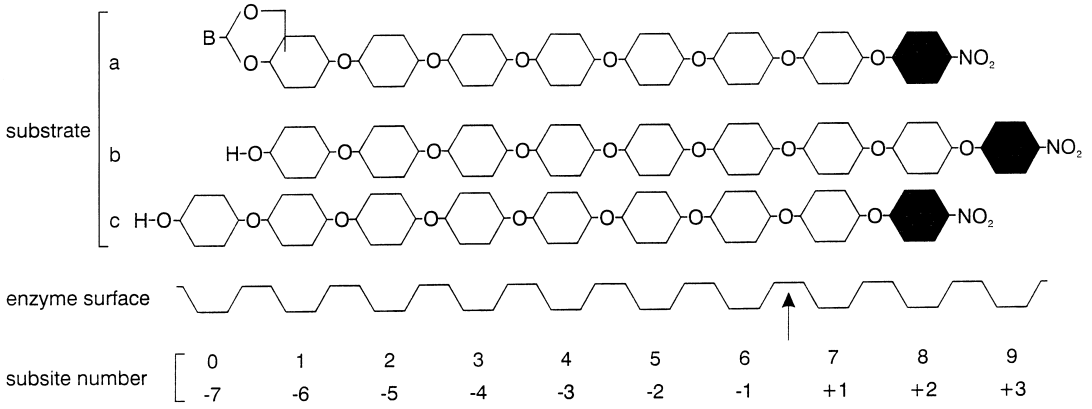

Fig. 2. Active centre of barley malt alpha-amylase 2 showing 10 subsites each of which is capable of interacting with a glucose residue of the substrate. In previous papers [8,10] subsites were numbered 0–9, starting with subsites binding the non-reducing end of the substrate. The nomenclature −7 to +3, assuming bond cleavage to take place between subsites −1 and +1 [11], was adopted here, and is given in addition to the previous system of subsite numbering. (a) preferred mode of binding B*p*NPG$_7$; (b) and (c) preferred modes of binding *p*NPG$_8$: ↑, catalytic site of the enzyme: ⌒, an enzyme subsite; O⬡, a glucose residue of the substrate; O⬢-NO$_2$, *p*-nitrophenyl group of the substrate; B, benzylidene group of B*p*NPG$_7$.

yields from the *p*-nitrophenyl maltodextrin derivatives studied here. Predicted yields are shown in Fig. 1, and it can be seen that the predicted yields are close to the experimental ones. The greatest discrepancy between predicted and experimental results occurs for *p*NPG$_5$. Previously published subsite energies were calculated on the assumption that the interaction energy for a subsite is independent of substrate binding at other subsites [8–10]. This may not hold true, however, when substrates are too short to span key subsites −6 and +2 (Fig. 2, using −7 to +3 numbering for subsites [11]), so that interaction energies may depend on substrate length [10]. Variation in subsite energies, caused by substrate length, may explain the discrepancies found here for *p*NPG$_5$. Changes in substrate–enzyme interactions at subsites −4 to −2 could bring about differences shown in Fig. 1 between calculated and experimental values (calculations not shown).

The small change in product distributions with pH, found for *p*NPG$_6$, may be connected to the fact that this substrate, also, is too short to span the critical region of the active site. Again, subsites may not act independently of one another, but at this stage it is difficult to offer an explanation of the observed results in terms of enzyme structure and possible changes in ionization of amino acid side chains at the enzyme active centre. For the larger substrates, longer than G$_8$ or *p*NPG$_7$, it has been shown that the hydrolysis pattern is determined mainly by the interaction energies at subsites −7, −6, +2 and +3 [10]. The lack of major effect of pH on product distributions then indicates that functional groups of the alpha-amylase, probably amino acid side chains, that are most important at subsites −7, −6, +2, and +3 do not change ionization state in the pH range 4.8–7.8. Comparison of amino acid sequence and structure of barley malt alpha-amylase 2 with Taka amylase indicates that subsites +2 and +3 may be formed by residues lysine 182, tryptophan 206 and threonine 207 [12–14]. Thus, little effect of pH, in the range 4.8–7.8, would be expected. At present, it is not possible to identify the residues constituting subsites −7 and −6. Histidine is, however, the only common amino acid with a side chain capable of ionizing in the pH range used (pK = 6.0 for the side chain); it therefore seems unlikely that histidine residues are involved.

Rates of hydrolysis of the substrates were estimated at the three pH values used and are shown in Table 1. In order to make direct comparisons of

Table 1
Initial rates of hydrolysis of *p*-nitrophenyl malto-oligosaccharides at pH 4.8, 6.0 and 7.8 by barley malt alpha-amylase 2

Substrate	Rates of hydrolysis (moles of bond hydrolysed/mol of enzyme/s) pH 4.8	pH 6.0	pH 7.8
*p*NPG$_5$	2.0 ± 0.5	7.0 ± 0.5	0.65 ± 0.05
*p*NPG$_6$	4.0 ± 0.5	20 ± 3	3.0 ± 0.5
*p*NPG$_7$	15 ± 7	140 ± 30	31 ± 4
*p*NPG$_8$	340 ± 30	340 ± 10	55 ± 5
B*p*NPG$_7$	430 ± 20	370 ± 40	72 ± 3

results for different substrates, all rates were calculated from samples taken after 20 min of hydrolysis. It can be seen that, for the longer substrates, pNPG$_8$ and BpNPG$_7$, the rate at pH 4.8 is equal to or greater than that at pH 6.0, while the rate at pH 4.8 is substantially less than that at pH 6.0 for the shorter substrates. The *p*-nitrophenyl group is believed to be able to mimic a glucose residue in terms of enzyme–substrate binding [10]. Thus, a difference is obvious at pH 4.8 between the action of the enzyme on a substrate that can span subsites -7 to $+2$ or -6 to $+3$, i.e., pNPG$_8$, and the smaller substrates which cannot do so. Since BpNPG$_7$ behaves like pNPG$_8$ and not pNPG$_7$ at pH 4.8, it seems likely that the blocking benzylidene group can also mimic a glucose residue to some extent in binding to the alpha-amylase. It therefore appears that substrate-binding spanning subsites -7 to $+2$ or -6 to $+3$ is necessary for high activity at pH 4.8, and that the enzyme acts much less efficiently at that pH if these subsites are not occupied simultaneously. We interpret this to mean that a conformational change may take place at the enzyme active site when substrate is bound across subsites -7 to $+2$ or -6 to $+3$ and that this change increases the hydrolytic activity at pH 4.8. A similar suggestion has been made for pig pancreatic alpha-amylase, i.e., that binding of the substrate at subsites near the extremities of the active site influences the catalytic activity of the enzyme [5].

The rate of hydrolysis of BpNPG$_7$ at pH 6.0 is comparable to that determined earlier at pH 5.5 in acetate buffer [8], while the rate for pNPG$_8$ at pH 6.0 is similar to that obtained for oligosaccharides G$_9$ to G$_{12}$ at pH 5.5 [8,15]. This helps to confirm that maximum catalytic efficiency is exhibited by the enzyme when one or no subsite of the active centre is unoccupied. Rates for pNPG$_5$, pNPG$_6$ and pNPG$_7$ at pH 6.0, however, are lower than obtained earlier at pH 5.5 [8]. This difference may be caused by the lower concentration of substrate, the pH difference, or possibly the nature of the buffer used here, coupled with the shorter length of these substrates.

The results obtained here on BpNPG$_7$ indicate that it is a useful substrate in the pH range 4.8–6.0, but that sensitivity of the assay would be markedly decreased at higher pH values. In principle, then, assay of cereal alpha-amylase activity using BpNPG$_7$ as substrate is practical in the pH range 4.8–6.0, with no complications likely to arise because of variation in major products formed with changes in pH. The substrate, however, is often used at pH 5.2 in kit form, along with excess glucoamylase and α-glucosidase for alpha-amylase determinations [7]. This study indicates that variation of assay sensitivity with pH in the range 4.8–6.0 is likely to depend more on the relative activities of the glucoamylase and α-glucosidase than on the alpha-amylase itself.

The benzylidene blocking group appears capable of mimicking a glucose residue sufficiently to cause BpNPG$_7$ to be hydrolysed by the alpha-amylase at a high rate at pH 4.8, like pNPG$_8$ but unlike pNPG$_7$. It is obvious, however, that the barley enzyme can distinguish between the groupings, as shown by the differences in product distributions from the substrates BpNPG$_7$ and pNPG$_8$ (Fig. 1). The results suggest that, while there appears to be little hindrance to binding the benzylidene group at subsite -7, a glucose residue binds more easily than the benzylidene grouping at subsite -6, thus giving more pNPG$_2$ from pNPG$_8$ than from BpNPG$_7$ (see Fig. 2).

In summary, we found little difference in distributions of products formed with changes in pH but observed variations in relative rates of hydrolysis that appeared to depend on substrate length. We interpret this to mean that pH changes in the range 4.8–7.8 do not affect binding of glucose residues at subsites -7, -6, $+2$, and $+3$, the key subsites for enzyme interaction with long substrates. If two or more of these subsites are unoccupied, the enzyme is catalytically less efficient, and this effect is more marked at pH 4.8 than at pH 6. Further studies of barley alpha-amylase–substrate interactions will be necessary before this effect can be understood more fully.

1. Experimental

Materials.—*p*-Nitrophenol was obtained from Sigma, pNPG$_1$ to pNPG$_7$ from Boehringer-Mannheim, and pNPG$_8$ from Calbiochem. Blocked *p*-nitrophenyl maltoheptaoside. (BpNPG$_7$) was purchased from Genzyme Corporation. All other reagents were of reagent grade or better.

Alpha-amylase 2 was purified from barley malt as described earlier [16]. Concentration of the enzyme stock solution was determined by assaying the activity in iodine dextrin colour units (IDC) using a modification of the Briggs method [17],

with beta-limit dextrin prepared from waxy maize starch as substrate. Enzyme concentration was then calculated using the previously determined specific activity of barley malt alpha-amylase 2, i.e., 2.8×10^{13} IDC units per mole of enzyme [16].

Rates of hydrolysis of p*NP-malto-oligosaccharides and product distributions*.—Digests containing 4 mg of substrate per mL of Tris–acetate buffer (25 mM, mM $CaCl_2$, pH 4.8, 6.0 or 7.8) were incubated at 35 °C. Known amounts, approximately 38, 22, 4, 1 and 1 pmol of enzyme per mL of digest, were added to pNPG$_5$, pNPG$_6$, pNPG$_7$, pNPG$_8$, and BpNPG$_7$ assays, respectively. Aliquots (0.5 mL) were removed at zero time and at intervals of 5 min (all substrates at pH 6.0), 10 min (pNPG$_7$, pNPG$_8$ and BpNPG$_7$ at pH 4.8 or 7.8) or 20 min (pNPG$_5$ and pNPG$_6$ at pH 4.8 or 7.8), and boiled for 12 min to inactivate and precipitate the enzyme. Hydrolysates were diluted 1:4 with acetonitrile and analysed directly by HPLC. Samples were injected automatically onto a Cosmosil 5NH_2–MS analytical column (4.6×250 mm) from Nacalai Tesque Inc., (Japan) and eluted with 80% acetonitrile in water for 5 min and then with a 80–65% gradient of acetonitrile in water at 30 °C and 1 mL/min. The eluate was monitored at 305 nm (for pNPG$_1$ to pNPG$_8$) and 420 nm (for pNP) with a Waters 490 multiwavelength detector. Product distributions and rates of hydrolysis were determined by quantifying all pNP-containing products against standards.

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