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A MODEL FOR THE ACTION OF CEREAL ALPHA AMYLASES ON AMYLOSE*

ELIZABETH A. MACGREGOR
Department of Chemistry, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Canada)

AND ALEX W. MACGREGOR
Grain Research Laboratory, Canadian Grain Commission, 1404-303 Main Street, Winnipeg, Manitoba R3C 3G8 (Canada)

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ABSTRACT

A model is proposed to explain the action of cereal alpha amylases (EC 3.2.1.1) on such linear substrates as amylose. It is suggested that, at the active site of the enzyme, there are nine contiguous subsites, each capable of interacting with a glucose residue. An estimate is made of the energies involved in subsite–glucose interaction, and values obtained for the binding energies are used to predict the distributions of small oligosaccharides to be expected at intermediate stages of amylose hydrolysis catalyzed by cereal alpha amylases.

INTRODUCTION

Investigations of cereal alpha amylases have shown that the enzymes from barley, wheat, rye, and oats have a similar action on amylose[1]. Distinctive distributions of oligosaccharides are obtained at intermediate stages of amylose hydrolysis, but a satisfactory explanation for the observed distributions has been lacking.

In this paper, the enzyme-subsite model, used to explain qualitatively and then quantitatively the action of bacterial alpha amylases (from *B. amyloliquefaciens* and *B. subtilis*)[2–4], has been adapted to offer a quantitative explanation for the action of cereal alpha amylases.

In the model, it is postulated that there are nine contiguous subsites at the active center of a cereal alpha amylase, each subsite being capable of interacting with a glucose residue of a (1→4)-α-D-glucan (Fig. 1). The catalytic center of the enzyme is situated between the sixth and seventh subsites, whilst each subsite has a characteristic free-energy of interaction with a glucose residue. Subsites are assumed to act independently, so that the free-energy of interaction at one site is not affected by binding or lack of binding of a glucose residue at an adjacent site.

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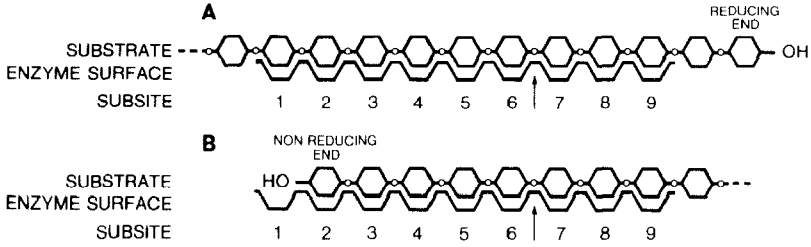

Fig. 1. Enzyme surface showing nine subsites, each capable of binding a glucose residue of a substrate. Amylose molecules are shown at the enzyme active center, in such a way as to give G_5 as product from (*a*) the reducing end of the amylose molecule, or (*b*) the non-reducing end of the amylose molecule. ↑ = catalytic site of the enzyme; ⌐⌙ = subsite of the enzyme; -O-⬡- = glucose residue of the substrate.

Production of any one oligosaccharide during amylose hydrolysis depends on (*i*) the correct positioning of the substrate across the enzyme active site, as shown in Fig. 1 for the formation of maltopentaose; (*ii*) the total free-energy of interaction of substrate with enzyme in the appropriate complex, and (*iii*) the rate of hydrolysis of substrate in the complex.

Relationships can be developed between apparent free-energies of binding at the enzyme subsites and yields of small oligosaccharides (maltohexaose and smaller) obtained at early stages of amylose hydrolysis. From these relationships, estimates of apparent binding-energies can be made once oligosaccharide yields early in amylose hydrolysis have been determined. These apparent binding-energies can then be used to predict oligosaccharide distributions to be expected at later stages in the hydrolysis of amylose by cereal alpha amylases.

THEORY

The binding and hydrolysis of a substrate A_n of chain length n may be represented by:

$$E + A_n \underset{k_{-1,r,n}}{\overset{k_{+1,r,n}}{\rightleftharpoons}} EA_n \xrightarrow{k_{+2,r,n}} E + P_{r,n-m} + P_{r,m},$$

where E is the enzyme, $P_{r,n-m}$ and $P_{r,m}$ are products of chain length $n - m$ and m formed from the non-reducing end and reducing end, respectively, of the substrate. $k_{+1,r,n}$, $k_{-1,r,n}$, and $k_{+2,r,n}$ are the respective rate-constants for binding of substrate to enzyme, dissociation of enzyme–substrate complex, and hydrolysis of the substrate. The subscript r denotes the subsite of the enzyme occupied by the reducing-end residue of the substrate (see Fig. 2a).

Then the rate of production of product, $P_{r,m}$ is given by[5,6]:

$$\frac{d[P_{r,m}]}{dt} = \frac{[E][A_n]\, k_{+2,r,n}/K_{r,n}}{1 + [A_n]/K_{m,n}},$$

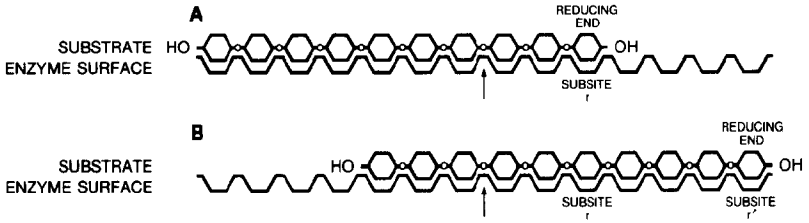

Fig. 2. Formation of m-mer from (a) reducing end, or (b) non-reducing end of a substrate n glucose residues in length. In this example $n = 10$, $m = 3$. ↑ = catalytic site of the enzyme; ⌐_⌐ = subsite of the enzyme; -o⬭= glucose residue of the substrate. This is a generalized figure and the precise number of subsites is not specified.

where $K_{r,n}$ is the Michaelis constant for the reaction yielding $P_{r,m}$ from the reducing end of A_n, i.e., $K_{r,n} = k_{-1,r,n} + k_{+2,r,n}/k_{+1,r,n}$, and $K_{m,n}$ is the macroscopic Michaelis constant for all possible $E\text{–}A_n$ complexes.

An oligosaccharide of chain length m can also be formed from the non-reducing end of substrate A_n. Then the rate of production of $P_{r',m}$ is given by:

$$\frac{d[P_{r',m}]}{dt} = \frac{[E][A_n]\, k_{+2,r',n}/K_{r',n}}{1 + [A_n]/K_{m,n}},$$

where the subscript r' denotes the subsite now occupied by the reducing-end residue of the substrate (see Fig. 2b).

Hence the total rate of formation of P_m is given by the sum of the foregoing rates above, namely:

$$\frac{d[P_m]}{dt} = \frac{[E][A_n]}{1 + [A]_n/K_{m,n}} \left[\frac{k_{+2,r,n}}{K_{r,n}} + \frac{k_{+2,r',n}}{K_{r',n}} \right] \qquad (1).$$

If $k_{+2,r,n} \ll k_{-1,r,n}$ and $k_{+2,r',n} \ll k_{-1,r',n}$, then the Michaelis constants $K_{r,n}$ and $K_{r',n}$ approach the dissociation constants for the EA_n complexes, or $1/K_{r,n} = K'_{r,n}$ and $1/K_{r',n} = K'_{r',n}$ where $K'_{r,n}$ and $K'_{r',n}$ are the association constants for the formation of the appropriate complexes, i.e., $K'_{r,n} = k_{+1,r,n}/k_{-1,r,n}$ and $K'_{r',n} = k_{+1,r',n}/k_{-1,r',n}$.

Thus equation (1) may be expressed as:

$$\frac{d[P_m]}{dt} = \frac{[E][A_n]}{1 + [A]_n/K_{m,n}} [k_{+2,r,n} K'_{r,n} + k_{+2,r',n} K'_{r',n}]$$

By similar arguments, the rate of formation of oligosaccharides of longer chain-length, such as $(m + 1)$, can be written as:

$$\frac{d[P_{m+1}]}{dt} = \frac{[E][A_n]}{1 + [A_n]/K_{m,n}} [k_{+2,r+1,n} K'_{r+1,n} + k_{+2,r'-1,n} K'_{r'-1,n}]$$

Then the ratio of rates of production of oligosaccharides of length m and $(m + 1)$ is given by:

$$\frac{d[P_m]}{dt} \Big/ \frac{d[P_{m+1}]}{dt} = \frac{[k_{+2,r,n} K'_{r,n} + k_{+2,r',n} K'_{r',n}]}{[k_{+2,r+1,n} K'_{r+1,n} + k_{+2,r'-1,n} K'_{r'-1,n}]} \quad (2)$$

Cereal alpha amylases hydrolyze oligosaccharides smaller than maltoheptaose very much more slowly than amylose[7]. Thus in the presence of large substrates, small oligosaccharides may be assumed to be effectively resistant to further attack. Then Eq. (2) gives the ratio of the yields (in terms of numbers of molecules) of the two oligosaccharides during amylolysis of a large substrate.

For any one enzyme–substrate complex, the association constant for the complex can be related to the sum of the binding energies of the occupied subsites by:

$$-RT\ln K'_{r,n} = \sum_{i=r-n+1}^{r} \Delta G_i + 10{,}000 \quad [8] \quad (3)$$

where R is the gas constant, T is absolute temperature, and ΔG_i is the free-energy of binding a glucose residue of the substrate in enzyme subsite i. Hypothetical subsites outside the binding region have zero free-energy of binding.

The 10,000 joules.mole^{-1} is the cratic free-energy contribution to binding[9].

By rearrangement of Eq. (3),

$$K'_{r,n} = \exp[-1/RT(\sum_{i=r-n+1}^{r} \Delta G_i + 10{,}000)],$$

and substitution in (2) for each K' gives:

$$\frac{\text{Yield of } P_m}{\text{Yield of } P_{m+1}} = \frac{d[P_m]}{dt} \Big/ \frac{d[P_{m+1}]}{dt} =$$

$$\frac{k_{+2,r,n} \exp[-1/RT(\sum_{r-n+1}^{r} \Delta G_i + 10{,}000)] + k_{+2,r',n} \exp[-1/RT(\sum_{r'-n+1}^{r'} \Delta G_i + 10{,}000)]}{k_{+2,r+1,n} \exp[-1/RT(\sum_{r-n+2}^{r+1} \Delta G_i + 10{,}000)] + k_{+2,r'-1,n} \exp[-1/RT(\sum_{r'-n}^{r'-1} \Delta G_i + 10{,}000)]}$$

On simplification

$$\frac{\text{Yield of } P_m}{\text{Yield of } P_{m+1}} = \frac{[k_{+2,r,n} \exp(-\sum_{r-n+1}^{r} \Delta G_i/RT) + k_{+2,r',n} \exp(-\sum_{r'-n+1}^{r'} \Delta G_i/RT)]}{[k_{+2,r+1,n} \exp(-\sum_{r-n+2}^{r+1} \Delta G_i/RT) + k_{+2,r'-1,n} \exp(-\sum_{r'-n}^{r'-1} \Delta G_i/RT)]} \quad (4)$$

Two possibilities have been considered for amylases for the dependence of rate constant, k_{+2}, on substrate size and position in an enzyme–substrate complex:

(*i*) k_{+2} varies with the number of subsites occupied by the substrate[10], i.e., it depends on substrate chain-length and position at the active site of the enzyme.

In this case, each k_{+2} may be described by an expression of the type:

$$k_{+2} = k_0 \exp(\sum_i \Delta G_0/RT)^{10} \quad (5),$$

where k_0 is the hypothetical rate-constant for the formation of products from an enzyme–substrate complex if there were no effect due to substrate chain-length, and ΔG_0 is the average contribution of each occupied subsite to acceleration of bond cleavage.

Then Eq. (*4*) can be simplified to:

$$\frac{\text{Yield of } P_m}{\text{Yield of } P_{m+1}} = \frac{[\exp(-\sum_{r-n+1}^{r} \Delta G_i'/RT) + \exp(-\sum_{r'-n+1}^{r'} \Delta G_i'/RT)]}{[\exp(-\sum_{r-n+2}^{r+1} \Delta G_i'/RT) + \exp(-\sum_{r'-n}^{r'-1} \Delta G_i'/RT)]} \quad (6)$$

where $\Delta G_i' = \Delta G_i - \Delta G_0$ and is the apparent free-energy of binding of a glucose residue at subsite *i*, or (*ii*) k_{+2} is constant and independent of substrate chain-length and position. Then Eq. (*4*) simplifies to (*6*) and $\Delta G_i' = \Delta G_i$, the free-energy of binding of a glucose residue at subsite *i* of the enzyme.

If relative yields of oligomers resulting from alpha amylolysis of a large substrate are known, then information can be obtained on free-energies of binding from Eq. (*6*).

If k_{+2} varies with the number of occupied subsites, apparent free-energies of binding can be calculated. Alternatively, free-energies of binding can be obtained if k_{+2} is independent of the number of enzyme subsites occupied by the substrate.

The results presented here do not allow us to distinguish between these two possibilities. We, therefore, refer to the calculated free energies as apparent free energies, assuming condition (*i*) may hold true for cereal alpha amylases, as it appears to do for the alpha amylase of *B. amyloliquefaciens*[6].

A specific example will be used to show how Eq. (6) can be simplified and used to calculate an apparent free-energy of binding.

If $m = 5$ (Fig. 1), Eq. (6) reduces to:

$$\frac{\text{Yield of } G_5^*}{\text{Yield of } G_6} = \frac{[\exp(-\sum_{1}^{9}\Delta G_i'/RT) + \exp(-\sum_{2}^{9}\Delta G_i'/RT)]}{[\exp(-\sum_{1}^{9}\Delta G_i'/RT) + \exp(-\sum_{1}^{9}\Delta G_i'/RT)]}$$

$$= \tfrac{1}{2} + \tfrac{1}{2}\exp(\Delta G_1'/RT) \qquad (7)$$

Thus $\Delta G_1'$ can be found if the relative yields of G_5 and G_6 produced in the early stages of alpha amylolysis of a large substrate are known.

Similar equations can be developed to relate the yields of G_4 and G_3 to $\Delta G_2'$ and $\Delta G_3'$, respectively.

If $m = 2$, however, Eq. (6) becomes:

$$\frac{\text{Yield of } G_2}{\text{Yield of } G_6} = \frac{[\exp(-\sum_{1}^{8}\Delta G_i'/RT) + \exp(-\sum_{5}^{9}\Delta G_i'/RT)]}{[\exp(-\sum_{1}^{9}\Delta G_i'/RT) + \exp(-\sum_{1}^{9}\Delta G_i'/RT)]}$$

$$= \tfrac{1}{2}\exp(\Delta G_9'/RT) + \tfrac{1}{2}\exp(\sum_{1}^{4}\Delta G_i'/RT) \qquad (8)$$

Values for $\Delta G_1'$, $\Delta G_2'$, and $\Delta G_3'$ can be obtained independently. Eq. (8) involves both $\Delta G_9'$ and $\Delta G_4'$ in addition, so that independent values for these energies cannot be obtained. Only a range of values possible for each can be calculated from (8). Similarly, from the yield of G_1, a range of values possible for $\Delta G_8'$ and $\Delta G_5'$ can be estimated.

It should be noted that each productive complex, that is, a complex in which substrate is hydrolyzed, involves substrate binding at subsites 6 and 7 (Fig. 1) of the enzyme. Thus the yield of each product oligosaccharide depends on the binding energies at these subsites. When ratios of yields are considered, these binding energies cancel out from the relationship, and no information on $\Delta G_6'$ and $\Delta G_7'$ can be obtained by the methods used here.

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

RESULTS AND DISCUSSION

Earlier studies[11] of cereal alpha amylases suggested that the action of these enzymes could be explained by postulating eight subsites (at the active site of the enzymes) for interaction with substrate. In work on bacterial alpha amylases, however, a nine-subsite active site was postulated initially[2], but satisfactory quantitative explanations of enzyme action were obtained using a ten-subsite model[3], with the "extra" subsite having an unfavourable (i.e., positive) binding energy. Both the nine-subsite and ten-subsite models for bacterial alpha amylases allow for the formation of maltotriose and maltohexaose as major products from the hydrolysis of amylose, in agreement with experimental observations[2]. Cereal alpha amylases on the other hand yield maltose and maltohexaose as major products of amylose hydrolysis[1]. It seems likely, therefore, that an eight- or a nine-subsite model (with the ninth subsite having a positive binding energy) should be useful in explaining cereal alpha amylase action. Initially, a nine-subsite model was examined.

A typical distribution of oligosaccharides produced during early stages of alpha amylolysis of amylose is shown in Fig. 3. Yields of small oligosaccharides (glucose up to maltohexaose) were determined for six trials (a typical result is shown in Table I) and the results were averaged. Relative numbers of molecules of each oligosaccharide were calculated and from such equations as (*7*) and (*8*), apparent free-energies of binding were obtained (Table II).

It is believed that most of the maltose resulting from amylose hydrolysis is produced from the reducing end of long substrates[12]. This is only possible if $\Delta G_9'$ is greater than

$$\sum_{1}^{4} \Delta G_i'$$

[see Eq. (*8*)], which occurs if $\Delta G_9'$ is greater than 0.8 KJ/mol. Thus, the range given for $\Delta G_9'$ in Table II is limited to 0.8 to 2.5 KJ/mol. The latter figure is the maximum allowable by Eq. (*8*).

It appears, therefore, that $\Delta G_9'$ cannot take the value zero, which would be required for an eight-subsite model (sites 1–8 of Fig. 1). Such an eight-subsite model must be rejected on the grounds that it would lead to the prediction that more maltose should be released from non-reducing than from reducing ends of long substrates. This prediction cannot be reconciled with earlier findings[12].

In addition to subsite 9, the model must contain subsites 1, 2, 3, and 8 to explain satisfactorily the observed oligosaccharide yields from amylose hydrolysis, as may be seen from a comparison of predictions and experimental results in Table III.

Although no information on subsites 6 and 7 is obtained here, we believe it likely that such subsites exist at the enzyme active center, to hold the substrate in the correct position so that the glycosidic bond to be hydrolyzed is in proximity to the catalytic site of the enzyme. Further, if the mechanism of action of alpha

TABLE I

YIELDS OF OLIGOSACCHARIDES OBTAINED FROM EARLY STAGES OF AMYLOSE HYDROLYSIS

Product	*Glucose*	*Maltose*	*Malto-triose*	*Malto-tetraose*	*Malto-pentaose*	*Malto-hexaose*	*Larger products*
	G_1	G_2	G_3	G_4	G_5	G_6	$>G_6$
Weight fraction (%)	0.2	2.5	1.9	2.2	2.4	5.5	85.3

TABLE II

APPARENT ENERGIES OF INTERACTION OF THE ENZYME SUBSITES WITH SUBSTRATE GLUCOSE RESIDUES

Subsite	*1*	*2*	*3*	*4*	*5*	*8*	*9*
Apparent interaction energy (KJoules/mol)	−10.0	+5.3	+1.6	+3.8→−10.0	−12.6→+11.1	−2.8→−12.6	+0.8→+2.5

amylases resembles that of lysozyme[13,14], then distortion of the glucose ring bound at subsite 6 is to be expected[6,15,16] and a positive binding-energy at subsite 6 should be anticipated[4,6]. It may then be postulated that a subsite at position 5 is likely to have a negative binding-energy to hold the substrate in position and overcome the "unfavourable" binding at position 6. The model presented here gives a wide spread for the allowable binding energy at subsite 5, but from the foregoing arguments it may be expected to be negative.

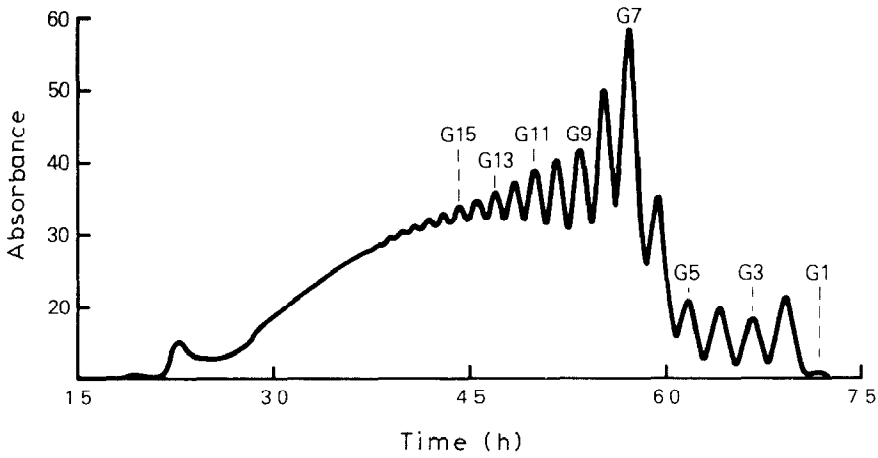

Fig. 3. Oligosaccharides produced during hydrolysis of amylose by malted barley alpha amylase II, elution profile from Biogel P-4. Extent of hydrolysis ≈10% of bonds broken (estimated from total reducing power). G_1, G_2, G_3 etc. denote glucose, maltose, maltotriose etc

A wide spread for the binding energy at subsite 4 is allowed also, including the value zero. This value could be obtained if subsite 4 did not exist. It is possible, therefore, that no interaction may take place between enzyme and substrate at the position of subsite 4. This seems unlikely, however, if interactions between enzyme and substrate take place at the positions corresponding to subsites 1–3 and 5–9; an interaction "gap" of this type appears improbable and has not been found for the other alpha amylases examined thus far[6,17–19].

The values for apparent free-energies shown in Table II were then used to calculate the distribution of small oligosaccharides (G_1 to G_6) to be expected at later stages of hydrolysis of amylose (see Table IV). It was assumed that each long saccharide (longer than G_{11}) produced from amylose would yield smaller products in ratios given by equations of types (*7*) or (*8*). Modifications to these equations must be made to predict the breakdown of G_7 to G_{11}, because such substrates do not span sufficient subsites of the enzymes to allow use of equations (*7*) or (*8*). Calculations of the yields of small oligosaccharides to be expected from the breakdown of large substrates, e.g., consisting of 2000 glucose residues, are long and repetitive and were carried out using programs for an Apple II microcomputer.

Small saccharides such as G_6 are hydrolyzed more slowly than starch[7] or amylose [α-(1→4) bonds in G_6 are hydrolyzed at <0.5% of the rate of hydrolysis of bonds in amylose[20]] and so it was assumed throughout that small saccharides (G_6 and smaller), once formed, were effectively resistant to attack by the enzyme. Quantitative information on the relative rates of hydrolysis of intermediate oligosaccharides (G_8 to G_{12}) and amylose is not available. To facilitate calculations of yields of small oligosaccharides, it was assumed that (*a*) each intermediate oligosaccharide, e.g., G_{11} was hydrolyzed completely before hydrolysis of the next smaller oligosaccharide (G_{10}) began and (*b*) the intermediate oligosaccharides G_7 to G_{11}were hydrolyzed at equal rates. Hence it was not possible to predict, with confidence, distributions of these oligosaccharides (G_7 and larger). The larger oligosaccharides, therefore, are grouped together in the Tables as oligosaccharides larger than G_6. Predicted values are calculated so that the quantity of oligosaccharides larger than G_6 matches that of the experimental determination.

It may be seen (Table IV) that there is, in general, good agreement between the predicted and experimental values. Values obtained for assumption (*a*) are in-

TABLE III

EFFECT OF DELETION OF A SUBSITE FROM THE MODEL

Subsite deleted	*Initial weight ratios to be expected from amylose*	*Experimental results (calculated from Table I)*
1	G_5:G_6 1:1.2	1:2.3
2	G_4:G_5 1:1.25	1:1.09
3	G_3:G_4 1:1.33	1:1.16
8	G_1:G_2 1:2 to 1:4	1:12.5

TABLE IV

COMPARISON OF EXPERIMENTAL OLIGOSACCHARIDE DISTRIBUTIONS (Wt.%) FROM LATER STAGES OF AMYLOSE HYDROLYSIS WITH PREDICTED DISTRIBUTIONS BASED ON A NINE-SUBSITE MODEL

Oligosaccharide	G_1	G_2	G_3	G_4	G_5	G_6	$>G_6$
Experimental	0.4	4.5	3.3	3.7	4.1	10.2	73 8
Predicted[a]	0.4	4.9	3.6	4.2	4.7	11 0	71.2
[b]	0.5	4.9–4.5[c]	3.2–3.5	3.5–3.9	4.3–3.4	9.9–10.5	73.7
Experimental	0 8	10.4	5.6	6.2	6.4	21.7	48.9
Predicted[a]	0.9	10.5	7.6	7.1	6.3	18.4	49.2
[b]	1.0–1.1[c]	9.8–8.8	6.1–6.9	6.6–7.6	8.4–6.0	19.2–20 6	49 0–48.9
Experimental	1.2	13.7	6.3	6.6	6.4	29.4	36.4
Predicted[a]	1.1	13.2	8.1	7.8	7.1	26.5	36.2
[b]	1 3–1.5[c]	12 4–11.0	7.6–8.7	8.0–9 5	10.5–7.0	23.9–25.9	36.4

[a]Each intermediate oligosaccharide, (G_{11}, G_{10}, G_9, G_8), hydrolyzed completely before hydrolysis of the next smaller saccharide begins. [b]Intermediate oligosaccharides (G_7 to G_{11}) hydrolyzed at equal rates [c]The range of values quoted corresponds to results calculated using the range of interaction energies for subsites 4 to 9 shown in Table II.

sensitive to variations in binding energy at subsites 5 to 9 within the ranges quoted in Table II.

In assumption (*a*) we have effectively considered the possibility that G_{10}, for example, is hydrolyzed very much more slowly than G_{11}, that G_9 in turn is attacked much more slowly than G_{10}, etc. This is not likely to prove correct experimentally. Indeed qualitative investigations[21] show that G_8 and larger oligosaccharides are hydrolyzed fairly rapidly, whereas much longer reaction-times are needed for the breakdown of G_7 and smaller substrates. On the other hand, equal rates of hydrolysis of G_7 to G_{11}, as assumed in (*b*), are also unlikely to be found in practice. G_7 appears to be relatively resistant to hydrolysis by cereal alpha amylases[21], and for bacterial alpha amylases an increase in rate of attack with increasing substrate length of intermediate oligosaccharides has been found[6,17]. We believe that a similar situation will be found for cereal alpha amylases.

The "true" situation is likely to be intermediate between that of assumptions (*a*) and (*b*), but as may be seen, both assumptions give results in reasonable agreement with experimental observations.

The range of values obtained by assumption (*b*) arises because the range of interaction energies given in Table II has a marked influence on the distribution of products to be expected from the hydrolysis of G_7.

It has been assumed in the model presented in this report that only one molecule of substrate binds to the active site of the enzyme at one time. It is unlikely that two long substrates ($>G_{10}$) would bind simultaneously at the active site. For shorter substrates the probability of two saccharides binding is increased, but the positive, and hence unfavourable, binding-energies at subsites 2, 3, 9 and probably 6 decrease that possibility.

It has been assumed also that there is no repetitive attack by the cereal alpha

amylases on the substrate. Although it is well accepted that the alpha amylases of mammalian pancreas[22–33] and to a lesser extent the enzymes of saliva[23,26,31,33] and *A. oryzae*[4,23,26,34,35] can hydrolyze substrates by a repetitive-attack mechanism, this is not the case for the alpha amylase of *B. subtilis*[4,24,26,33].

Thus repetitive attack cannot be considered a feature of the action of all alpha amylases. Investigations of a cereal alpha amylase have shown that the enzyme from malted rye shows no repetitive attack in 40% glycerol solution[24]. This does not prove, however, that repetitive attack cannot occur under other conditions.

In an attempt to examine the effect of multiple attack on oligosaccharide production, calculations were carried out of yields of small saccharides to be expected from amylose, if (*a*) hydrolysis were random, but a maltose unit was released from the newly created reducing end of a saccharide formed by the random scission i.e. one maltose is produced by multiple attack for each bond hydrolyzed randomly, or (*b*) hydrolysis was non-random according to a subsite model enzyme, but again one maltose unit is produced by a multiple attack from the newly created reducing end of a saccharide, itself formed by preferential attack. Here the subsite energies of Table II were altered so that no excess of maltose was formed by preferential attack; rather, the extra maltose was generated by multiple attack.

The results of these calculations are shown in Table V. It may be seen that much more maltose would be produced by multiple attack of this kind than is obtained experimentally. Further, yields of other oligosaccharides do not agree well with observed values. It is extremely unlikely, therefore, that cereal alpha amylases act by the following multiple-attack mechanism: within one enzyme–substrate encounter, a bond scission takes place, the newly formed saccharide bound to the right of the catalytic site (at subsites 7 to 9 of Fig. 1) diffuses away from the enzyme, the other newly formed saccharide slides two subsites to the right (to cover at least subsites 6 to 8), and a second bond-scission takes place to release maltose.

A third calculation was undertaken where it was assumed (*c*) the sliding of newly-formed saccharide over the enzyme active site and release of maltose takes place in only 10% of the productive enzyme–substrate encounters. The results are shown in Table V (*c*).

It may be seen that such a calculation predicts oligosaccharide yields closer to the experimental yields than mechanisms where two bonds are hydrolyzed per effective enzyme–substrate encounter. The agreement is not as good, however, as that obtained for schemes of enzyme action without multiple attack (compare Table IV). In particular, an excess of G_3, G_4, and G_5 is predicted, even when G_2 is the postulated product of multiple attack. A further calculation was carried out where it was assumed (*d*) that maltohexaose was formed by multiple attack on each newly formed non-reducing end of a saccharide formed by preferential attack. Results are given in Table V. Formation of G_6 from a non-reducing end was chosen because earlier results[12] suggest ready release of G_6 from non-reducing ends of substrates. Again it can be seen that much more G_6 would be produced by this mechanism

TABLE V

COMPARISON OF EXPERIMENTAL OLIGOSACCHARIDE DISTRIBUTIONS (Wt. %) FROM LATER STAGES OF AMYLOSE HYDROLYSIS WITH PREDICTED DISTRIBUTIONS BASED ON MULTIPLE ATTACK

Oligosaccharide	G_1	G_2	G_3	G_4	G_5	G_6	$>G_6$
Experimental	0.4	4 5	3.3	3.7	4.1	10.2	73.8
Predicted[a]	1.5	17 8	1.8	2.1	2.1	2 5	72.3
[b]	0.2	17.4	0.9	2.4	3.0	3.7	72.4
[c]	0 4	4.4	3.0	3.9	4.4	9.5	74.4
[d]	0.1	0.6	0.5	0 6	1.1	25.1	72.0
Experimental	0.8	10.4	5.6	6.2	6.4	21.7	48.9
Predicted[a]	4.0	28.2	3.4	4.0	4.6	4.3	51.5
[b]	0.5	24.9	2.3	5.9	7.5	9.0	49.9
[c]	0.8	8.0	7.5	8.4	7.5	18.6	49.2
[d]	0.4	1.9	1 7	2.2	3.8	41 5	48.5
Experimental	1.2	13.7	6.3	6.6	6.4	29.4	36.4
Predicted[a]	7.3	37.8	4.7	5.6	5.3	5 0	34 2
[b]	0.8	29.4	3.6	9.4	10.5	11.8	34.1
[c]	1.2	10.4	9.1	9.7	8.9	24.4	36.3
[d]	0.5	2.7	2.5	3.0	5.1	48.4	37.8

[a]Random hydrolysis with one molecule of G_2 produced from the newly formed reducing saccharide/random scission. [b]Hydrolysis based on the subsite model with one G_2 produced per "preferred" scission from the newly formed reducing saccharide. Subsite energies were as in Table II except $\Delta G_4' = 0 \rightarrow -10$ KJ/mol, $\Delta G_9' = 0$. [c]As in *b*, but one G_2 is produced from only 10% of newly formed reducing saccharides. [d]Hydrolysis based on the subsite model with one G_6 produced per "preferred" scission from the newly formed non-reducing end of a saccharide. Subsite energies were as in Table II except $\Delta G_1' = \Delta G_2' = -4.8$ KJ/mol.

than is observed experimentally. Similar calculations, where G_1 or G_3 or G_4 or G_5 is the exclusive product of a multiple-attack mechanism involving two scissions for each enzyme–substrate encounter, would show an overproduction of the oligosaccharide involved. These results suggest that the enzyme cannot be operating by a mechanism where there are two scissions for each effective enzyme–substrate encounter, if such a multiple attack were to release the same oligosaccharide each time. We cannot, however, exclude the possibility of a limited amount of multiple attack (10% or less), particularly if different sizes of oligosaccharide can be released by that multiple attack.

It is possible to account for the action of cereal alpha amylases without invoking multiple attack, although, at present, a limited multiple-attack mechanism cannot be eliminated. It should be noted that if multiple attack is operating it may be possible to explain the enzyme action using an eight-subsite model, as $\Delta G_9'$ was taken as zero to calculate the yields in rows (*a*), (*b*), and (*c*) in Table V and $\Delta G_1'$ was taken as zero to calculate the yields in row (*d*). This would suggest the possibility of no interaction between enzyme and substrate at subsites 9 or 1, i.e., that subsites 9 or 1 do not exist.

The nine-subsite model, however, is capable of giving good approximations for the relative yields of small oligosaccharides to be expected during intermediate

stages of hydrolysis of amylose by the enzymes. It is probable that with further information, such as the relative rates of hydrolysis of oligosaccharides G_7, G_8, and G_9 or information on multiple attack, that refinements in the model can be made and better agreement between predictions and experimental values will be obtained. Such refinements may include increasing the number of subsites at the active center of the model enzyme. We believe that, without multiple attack, nine contiguous subsites is the smallest number which can adequately account for experimentally determined product-distributions during amylose hydrolysis, but this study does not preclude the possibility that substrate–enzyme interaction may involve a larger number of enzyme subsites.

EXPERIMENTAL

Preparation of alpha amylase II. — Malted barley alpha amylase II was prepared as described previously[36], except that the enzyme was passed twice through the affinity-column step to ensure complete removal of beta amylase from the preparation.

Preparation of amylose. — Linear amylose was prepared from potato starch as described previously[37].

Amylose hydrolysis. — Linear amylose (0.1% in 0.01M sodium acetate buffer, pH 5.5, containing mM calcium chloride) was digested with malted barley alpha amylase II (30 IDC units/mg substrate) at 35°. After various periods of time, samples were removed and analysed on a Beckman column (160 × 0.9 cm) packed with Biogel P-4 (400 mesh). The column was maintained at 70°, eluted with degassed, deionized water at the same temperature, and monitored continuously by an automated orcinol–sulfuric acid procedure[38]. Carbohydrate recoveries from the column were at least 95%.

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