Carbohydrate Research, 61 (1978) 377–385

REPETITIVE ATTACK BY *Aspergillus oryzae* ALPHA AMYLASE*

JIMMY D. ALLEN AND JOHN A. THOMA
Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701 (U.S.A.)
(Received July 25th, 1977; accepted for publication August 20th 1977)

ABSTRACT

The action of *Aspergillus oryzae* alpha amylase on reducing-end, and uniformly radiolabeled maltotriose through maltodecaose has been studied. The enzyme is found to hydrolyze more than a single glycosidic bond during some enzyme–substrate encounters. The extent of this repetitive attack is quantitated.

INTRODUCTION

The proposal that amylases hydrolyze several glycosidic bonds during the lifetime of a single enzyme–substrate encounter has stirred considerable discussion[1–4]. This repeated attack of substrate, termed multiple or repetitive attack, was originally proposed to account for the action of beta amylase[5]. A number of divergent experimental methods has been devised to test for repetitive attack by other amylases. Each of these procedures shows that porcine-pancreatic alpha amylase exhibits a high degree of repetitive attack, but they appear to give divergent results when applied to other amylases, particularly *Aspergillus oryzae* alpha amylase.

A recent mathematical analysis of the repetitive-attack model has shown that handling and fractionating of substrates that consist of polymer distributions create difficulties in designing experiments to detect repetitive attack[3]. Many of the problems associated with such substrates are alleviated by the use of pure substrates of known chain-length. The amylolysis products of substrates of defined chain-length can be quantitated by chromatographic techniques, and the products of amylolysis are more-readily interpreted in terms of the presence or absence of repetitive attack. In this paper we report the action of *Aspergillus oryzae* alpha amylase on a series of purified, radiolabeled, malto-oligosaccharides and quantitate the extent of repetitive attack.

EXPERIMENTAL

Three-times recrystallized *Aspergillus oryzae* alpha amylase (EC 3.2.1.1) was a generous gift of Y. Nitta. After further purification on a Sephadex A-50 anion-exchange column, the enzyme proved to be a single component by poly(acrylamide)-gel electrophoresis, and ultracentrifugation[6].

*Dedicated to Dexter French, friend and mentor, on the occasion of his 60th birthday.

Uniformly labeled malto-oligosaccharides were prepared from tobacco-leaf [U-^{14}C]starch (ICN) having 1.5 mCi/mg. *Pseudomonas* isoamylase[7] (EC 3.2.1.9) (Hayashibara Lab., Japan) was a generous gift of T. Harada. The starch (500 μCi) was incubated with 0.16 unit of isoamylase for 22 h at 38° and then heated for 10 min at 100°. The oligosaccharides were separated by chromatography, together with standards, on Whatman 3MM paper with a 13:18:19 (v/v) water–ethanol (95%)–nitromethane solvent system[8] and multiple ascents[9] at room temperature. The radio-labeled sugars were detected by autoradiography with Kodak No-screen X-ray film. Using the resulting film as a guide, the sugars were eluted from the paper. The oligosaccharides were further purified by chromatography with a solvent system of 19:36:45 (v/v) water–pyridine–1-butanol for maltotriose through maltohexaose, and 26:39:35 (v/v) for maltoheptaose through maltodecaose. The absence of (1 → 6)-linkages was shown by complete digestion with glucosidase-free beta amylase[10], a generous gift of J.J. Marshall. The concentration of the oligosaccharides was determined by the phenol–sulfuric acid assay[11].

The products of *A. oryzae* amylase hydrolysis of the uniformly labeled sugars were determined as a function of the extent of reaction. The procedure used and the analysis employed were similar to those described previously for hydrolysis of reducing-end labeled malto-oligosaccharides[12]. The starting concentrations of the oligosaccharides in the mixture were* G_3, 19.4; G_4, 8; G_5 and G_6, 19; and G_7–G_{10}, 2.0 μM. At appropriate time-intervals, aliquots were removed from the mixture and the products were separated by paper chromatography. Using an autoradiogram as a guide, the paper was cut up and the products quantitated by liquid-scintillation counting.

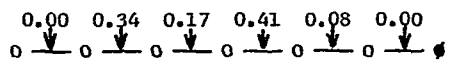
RESULTS

Method of analysis. — If repetitive attack does not occur, or if repetitive attack is toward the nonreducing end of the substrate as with porcine pancreatic amylases[13], frequencies of bond cleavage may be derived from an analysis of the hydrolysis products of a reducing-end labeled substrate[14,15]. Chosing conditions where multi-molecular substrate reactions do not occur[6], the initial mole fractions of the labeled products of a reducing-end labeled oligosaccharide are equal to the relative frequencies of bond cleavage. For example, *A. oryzae* amylase degradation of reducing-end labeled maltoheptaose (G_7) yields as labeled products[12] 8 G_2, 41 G_3, 17 G_4, and 34% of G_5. This gives the bond-cleavage frequencies for maltoheptaose shown in Fig. 1.

Bond-cleavage frequencies may then be used to predict the mole fraction of products when uniformly labeled substrates are degraded in the absence of repetitive attack. As shown in Fig. 1, uniformly labeled maltoheptaose would be expected to yield 21 G_2, 29 G_3, 29 G_4, and 21% of G_5 if *A. oryzae* amylase is not a repetitive-

*G_n denotes a malto-oligosaccharide of n glucopyranosyl residues.

Bond-cleavage frequencies measured from reducing-end labeled G_7



Predicted products of hydroloysis of 100 uniformly labeled G_7 molecules in the absence of repetitive attack

Substrate	Number of molecules	Mole fraction
●—●—●—●—●—●—◆	100(before hydrolysis)	
Products		
◆	0	0
●—◆	42	0.21
●—●—◆	58	0.29
●—●—●—◆	58	0.29
●—●—●—●—◆	42	0.21
●—●—●—●—●—◆	0	0

Fig. 1. Expected results for hydrolysis of uniformly labeled maltoheptaose in the absence of repetitive attack. ○, D-Glucopyranosyl group; ● radiolabeled D-glucopyranosyl group; -●-, reducing radiolabeled D-Glucopyranosyl group; ——, α-D-(1 → 4) bond. The bond-cleavage frequencies calculated from degradation of reducing-end labeled maltoheptaose[12] were used to predict the products of hydrolysis of 100 molecules of uniformly labeled maltoheptaose in the absence of repetitive attack.

attack enzyme. As each substrate G_7 is cleaved only once in the absence of repetitive attack, $[G_2]$ must equal $[G_5]$, and $[G_3]$ must equal $[G_4]$. However, repetitive attack on the substrate will tend to decrease the concentration of the larger oligosaccharides and increase the concentration of the smaller ones. As a result of the redistribution of products due to repetitive attack, the symmetry of $[G_2]$ and $[G_5]$ and of $[G_3]$ and $[G_4]$ will disappear. Consequently, an analysis of the degradation products of reducing-end labeled and uniformly labeled malto-oligosaccharides will give a measure of repetitive attack by alpha amylase.

Application to A. oryzae *amylase.* — Bond-cleavage frequencies by *A. oryzae* amylase[12] were used to predict the hydrolysis products of uniformly labeled G_3–G_{10}, assuming no repetitive attack. The results are tabulated in Table I. Hydrolysis of uniformly labeled G_3–G_{10} by *A. oryzae* amylase was carried out at substrate concentrations where multimolecular reactions have been shown to be insignificant[6]. The data from a typical experiment are shown in Fig. 2. The slopes of the plots, calculated from a weighted least-squares analysis, give the mole fractions of the products.

Subsequent attack on the products of initial bond-cleavage after enzyme–product dissociation, namely, secondary attack, must be carefully distinguished from actual repetitive attack, where reattack occurs before dissociation. Whereas repetitive attack on a substrate malto-oligosaccharide will be constant throughout the course of a hydrolysis, secondary attack on products will become significant only in the later stages of the digestion, after build-up of product is substantial. Consequently, secondary attack will cause a gradual shift in the mole fractions of product. This

TABLE I

COMPARISON OF OBSERVED PRODUCTS OF HYDROLYSIS OF UNIFORMLY-LABELED MALTO-OLIGOSACCHARIDE TO THOSE PREDICTED IN THE ABSENCE OF REPETITIVE ATTACK[a]

Substrate	*Product fraction*								
		G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_8	G_9
G_3										
	No R.A.	0.50	0.50							
	observed	0.51	0.49							
G_4										
	No R.A.	0.005	99	0.005						
	observed	0.01	0.99	0.00						
G_5										
	No R.A.	0.01	0.49	0.49	0.01					
	observed	0.01	0.52	0.47	0.00					
G_6										
	No R.A.	0.00	0.14	0.72	0.14	0.00				
	observed	0.02	0.16	0.69	0.13	0.00				
G_7										
	No R.A.	0.00	0.21	0.29	0.29	0.21	0.00			
	observed	0.00	0.24	0.29	0.28	0.19	0.00			
G_8										
	No R.A.	0.00	0.115	0.33	0.11	0.33	0.115	0.00		
	observed	0.01	0.15	0.35	0.12	0.26	0.11	0.00		
G_9										
	No R.A.	0.00	0.08	0.23	0.19	0.19	0.23	0.08	0.00	
	observed	0.00	0.11	0.25	0.20	0.19	0.18	0.08	0.00	
G_{10}										
	No R.A.	0.00	0.07	0.18	0.14	0.22	0.14	0.18	0.07	0.00
	observed	0.00	0.09	0.20	0.16	0.22	0.15	0.15	0.03	0.00

[a]The product fractions were calculated from bond-cleavage frequencies[12], assuming no repetitive attack (R.A.), as outlined in Fig. 1. The fractions observed were experimentally derived as explained in the text.

change in mole fractions will lead to curvature in the plots used to analyze the product-distribution data. For example, in hydrolysis (Fig. 2) of uniformly labeled G_7, the early points lie in a straight line and curvature becomes evident only after approximately 37% of hydrolysis (abscissa value at 0.55; see caption of Fig. 2). In each case, the initial slopes, in the early stages of the digestion before curvature was evident, were used to compute the product fractions. The mole fractions of products for the degradation of uniformly labeled G_3–G_{10} are reported in Table I. The consistently skewed distribution of products favoring the products of smaller chain-length, a result consistent with repetitive attack, is noteworthy.

Calculation of the extent of repetitive attack. — The results of the degradation of uniformly labeled G_3–G_{10} may be used to estimate the extent of repetitive attack on each substrate. In the absence of repetitive attack, each cleavage of substrate will generate two product-molecules. However, when repetitive attack occurs, more than

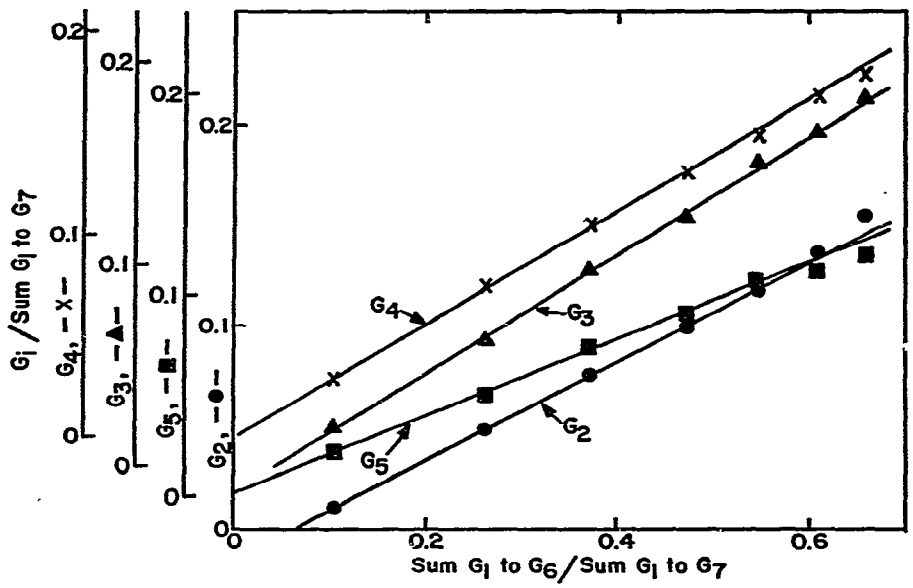


Fig. 2. Plot used to calculated the mole fraction of products from hydrolysis of uniformly labeled maltoheptaose. The radioactivity due to each saccharide in the maltoheptaose hydrolyzate was analyzed as a function of the extent of reaction, as described in the Experimental section. In order to compensate for the different molar specific-activity of the sugars, the radioactivity due to each product was divided by the chain length of that product, to obtain a normalized radioactivity. The normalized radioactivity of each individual product, divided by the total normalized radioactivity due to G_1 through G_7, was then plotted as a function of the sum of the normalized radioactivity of all products (G_1–G_6) divided by the total normalized radioactivity. The slope of the line for each product is the mole fraction for that product. The advantages of this type of plot has been discussed previously[12,16]. The data for G_1 and G_6 are not shown, as production of these sugars was insignificant. The lines are weighted least-squares fits to the first five points. Note that the abscissa values in this plot do not approximate the extent of reaction as with end-labeled substrate data[12,16], as two molecules of product (or more than two when repetitive attack occurs) are formed per molecule of substrate hydrolyzed. The extent of reaction is approximately X/(2 − X), where X is the abscissa value. The maximum degree of hydrolysis for the data used in the least-squares analysis was 37%. In general, in each of the uniformly labeled experiments, the standard errors in the slopes were less than 0.01.

two product molecules will result. The number of product molecules above the two predicted is a measure of repetitive attack.

Maltoheptaose is used here as an example of the calculations used to compute the extent of repetitive attack from mole fractions of uniformly labeled product. For illustrative purposes, consider a mole fraction of product times 100 as molecules of product per 100 total molecules of product. The analysis will thus be based on a calculation of the number of substrate molecules giving rise to these 100 product-molecules.

First, consider a case where maltoheptaose does not undergo repetitive attack, so that uniformly labeled G_7 would have the distribution of products shown in Table II. These values may be converted into molecules of glucose equivalents in each product by multiplication by the product chain-length, as shown in Table II.

TABLE II

METHOD OF CALCULATION OF THE EXTENT OF REPETITIVE ATTACK

Product	*No repetitive attack*[a]		*Repetitive attack*[b]	
	Molecules of product[c]	D-*Glucose equivalents*[d]	*Molecules of product*[c]	D-*Glucose equivalents*[d]
G_1	0	0	0	0
G_2	21	42	24	48
G_3	29	87	29	87
G_4	29	116	28	112
G_5	21	105	19	95
G_6	0	0	0	0
Sum	100	350	100	342

[a]The products are those expected from the hydrolysis of uniformly labeled maltoheptaose in the absence of repetitive attack (Table I). [b]The products are the experimental distribution obtained from degradation of uniformly labeled maltoheptaose (Table I). [c]Molecules of each sugar per 100 molecules of product. [d]Obtained by multiplication of each product by its chain length.

Dividing the sum of the glucose equivalents in the products by the chain length of the substrate reveals the number of substrate molecules hydrolyzed to give 100 product molecules. In this case, 350 glucose residues divided by a chain length of 7 shows that 50 molecules of substrate gave rise to the 100 molecules of product, as would be anticipated in the absence of repetitive attack.

Now consider the actual case where repetitive attack is indicated. The molecules of each product per 100 molecules of total product are shown in Table II. In this case, the sum of the D-glucose residues in 100 molecules of product is 342. Hence, 100 product molecules would arise through the degradation of 342/7 = 48.9 substrate molecules. This is the result expected when a substrate molecule is repetitively attacked, so that more than two molecules of product are formed. In fact, in this case, each substrate degraded gives rise to an average of 2.05 molecules of product, showing that 5% of the G_7 molecules undergo repetitive attack.

In general, the number of substrate molecules (s) giving rise to 100 product molecules may be calculated as

$$s = \frac{\sum_{i=1}^{n-1} (\%\ i\text{-mer})\ (i)}{n} \qquad (1),$$

where n is the substrate chain-length, i is the product chain-length, and $\%$ i-mer is the mole fraction of i-mer times 100. The extents of repetitive attack by *A. oryzae* amylase on G_3 through G_{10}, computed from equation *1*, are reported in Table III.

TABLE III

EXTENT OF REPETITIVE ATTACK BY *A. oryzae* AMYLASE ON MALTO-OLIGOSACCHARIDES

Substrate	G_3	G_4	G_5	G_6	G_7	G_8	G_9	G_{10}
Percent repetitively attacked[a]	1	1	3	5	5	11	8	12

[a]The percent of substrate molecules experiencing more than one bond-cleavage per encounter. Calculated from Eq. *1*.

DISCUSSION

Robyt and French[17] have studied the action of *A. oryzae* amylase on amylase having an average chain-length of 1000 monomer units. The decrease in amylose–iodine color relative to the increase in the reducing value during amylolysis showed that this enzyme exhibits some, but considerably less repetitive attack than does porcine-pancreatic amylase at its optimum pH. To quantitate the degree of repetitive attack, namely, the average number of bond cleavages following initial bond-cleavage, Robyt and French fractionated the digest into polysaccharides and oligosaccharides. Their results revealed that *A. oryzae* amylase has a degree of repetitive attack of 1.7 (ref. 17) or 2.0 (ref. 2), depending on how the results are analyzed. Porcine pancreatic amylase at its optimum pH shows a degree of repetitive attack of 6 (ref. 17) or 3.8 (ref. 2), again depending on how the experimental results are analyzed.

Abdullah *et al.*[18] tested for repetitive attack by alpha amylase using another procedure. The alpha amylolysis of cyclooctaamylose or cyclodecaamylose was carried out in the presence of a large excess of beta amylase. In the absence of repetitive attack, the alpha amylase would cleave the cyclic amyloses to give linear maltooctaose or maltodecaose, which would be rapidly hydrolyzed to maltose by beta amylase. However, if the alpha amylase repeatedly attacked the oligosaccharide, a product having odd number chain-length could result before dissociation and beta amylolysis of the linear oligosaccharide. Detection of D-glucose or maltotriose was taken as diagnostic that repetitive attack had occurred. This experiment showed the presence of repetitive attack by porcine pancreatic amylase, but it was reported that *A. oryzae* amylase showed "little if any" repetitive attack. This test would, of course, lack the sensitivity for detecting a small degree of repetitive attack.

More recently, Suetsugu *et al.*[19] attempted to measure the degree of repetitive attack by *A. oryzae* amylase on cyclohexa-, cycloheptа-, and cycloocta-amylose. The hydrolyzates of these cycloamyloses were examined for the ratio of rings opened to the number of reducing-ends formed. A ratio below unity was presumed to be due to repetitive attack. A degree of repetitive attack of 1.4–1.6 for each of the three cycloamyloses was reported.

In studies such as these, secondary attack on the initial products of cleavage of cycloamyloses must be rigorously excluded. The study of Abdullah *et al.*, which failed to show repetitive attack, excluded secondary attack by including a large excess

of beta amylase so that any dissociated oligosaccharide was rapidly degraded. However, in the study by Suetsugu *et al.*[19], where a high degree of repetitive attack was reported, it is likely that secondary attack contributed significantly to the repetitive attack reported. The linear oligosaccharides produced by initial cleavage of cycloamyloses are better substrates[19,20] than the cycloamyloses and, consequently, would be rapidly reattacked by alpha amylase.

In the study reported here, we have taken care to exclude secondary attack. As malto-oligosaccharides are decreased in chain length they become progressively poorer substrates for alpha amylase[20].** As bond cleavage by *A. oryzae* amylase at the terminal bonds of G_4–G_{10} is very low, the bulk of the products is at least two D-glucose residues shorter than the substrate, decreasing the likelihood of secondary attack. In addition, the product ratios were always obtained from the early stages of hydrolysis before any secondary attack could be detected.

The extents of repetitive attack calculated for G_3 and G_4 are not significantly different from zero (Table III). However, for G_5 through G_{10}, a gradual increase in repetitive attack is observed. The highest incidence of reattack for *A. oryzae* amylase was 12% for G_{10}. From a similar study of the action of porcine pancreatic amylase on maltooctaose, it was found that 27% of the substrate molecules were reattacked[13]. These results are consistent with the reports that porcine-pancreatic amylase exhibits a higher degree of repetitive attack than *A. oryzae* amylase. We conclude that *A. oryzae* alpha amylase has a small but measurable extent of repetitive attack in the degradation of malto-oligosaccharides. We consider that quantitative analysis of the products of digestion of substrates of known chain-length is one of the most sensitive methods for checking for repetitive attack.

REFERENCES

1 W. BANKS, C. T. GREENWOOD, AND K. M. KHAN, *Carbohydr. Res.*, 12 (1970) 79–87.
2 J. A. THOMA, *Biopolymers*, 15 (1976) 729–746.
3 J. A. THOMA, *Carbohydr. Res.*, 48 (1976) 85–103.
4 W. BANKS, C. T. GREENWOOD, AND K. M. KHAN, *Carbohydr. Res.*, 19 (1971) 252–254.
5 J. M. BAILEY AND D. FRENCH, *J. Biol. Chem.*, 226 (1957) 1–14.
6 J. D. ALLEN, Ph.D. Thesis, University of Arkansas, 1975.
7 T. HARADA, A. MISAKI, H. AKAI, K. YOKOBAYASHI, AND K. SUGIMOTO, *Biochim. Biophys. Acta*, 268 (1972) 497–505.
8 J. A. THOMA AND D. FRENCH, *Anal. Chem.*, 29 (1957) 1645–1648.
9 D. FRENCH AND G. M. WILD, *J. Am. Chem. Soc.*, 75 (1953) 2612–2616.
10 J. J. MARSHALL AND W. J. WHELAN, *Anal. Biochem.*, 52 (1973) 642–646.
11 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
12 J. D. ALLEN AND J. A. THOMA, *Biochem. J.*, 159 (1976) 121–132.

**We have previously shown that the alpha amylase of *A. oryzae* exhibits significant transferase activity[6]. Consequently, the Michaelis parameters reported in ref. 20 for the substrates of shorter chain-length are complicated by these multimolecular, substrate reactions to some unassessed degree. However, the general trend of decreasing K_m and increasing V with increasing chain length of the substrate is probably correct, as has been shown for another alpha amylase[21].

13 J. F. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 138 (1970) 662–670.
14 J. A. THOMA, C. BROTHERS, AND J. SPRADLIN, *Biochemistry*, 9 (1970) 1768–1775.
15 J. D. ALLEN AND J. A. THOMA, *Biochem. J.*, 159 (1976) 105–120.
16 J. D. ALLEN, *Carbohydr. Res.*, 39 (1975) 312–315.
17 J. F. ROBYT AND D. FRENCH, *Arch. Biochem. Biophys.*, 122 (1967) 8–15.
18 M. ABDULLAH, D. FRENCH, AND J. F. ROBYT, *Arch. Biochem. Biophys.*, 114 (1966) 595–598.
19 N. SUETSUGU, S. KOYAMA, K. TAKEO, AND T. KUGE, *J. Biochem. (Tokyo)*, 76 (1974) 57–63.
20 Y. NITTA, M. MIZUSHIMA, K. HIROMI, AND S. ONO, *J. Biochem. (Tokyo)*, 69 (1971) 567–576.
21 J. A. THOMA, G. V. K. RAO, C. BROTHERS, J. SPRADLIN, AND L. H. LI, *J. Biol. Chem.*, 246 (1971) 5621–5635.