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Note

The action of amylase on 6-amino-6-deoxyamyloses

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As part of a continuing study of the action of amylases on amylose molecules in which a fraction of the D-glucosyl residues have been chemically modified, a series of 6-amino-6-deoxyamyloses was prepared by the method of Whistler and Medcalf[1], and used as substrates for amylases.

The replacement of a fraction of the primary hydroxyl groups by primary amino groups makes these products extremely interesting. Amino glycosides are less readily hydrolyzed by acid than normal glycosides, presumably because the amino group is protonated in the acid medium[2]. Whistler and Medcalf[1] found that their 6-amino-6-deoxyamyloses required more time for complete hydrolysis by acid than amylose itself, an observation confirmed in this work.

At the normal pH optima of the enzymes, these aminated amylose molecules would also be protonated. Crystalline, porcine pancreatic amylase does, however, retain something less then ten percent of its activity at pH 10.5. This observation, made by Robyt and French[3], was verified in the current investigation. In addition, it was found that the crystalline, liquefying amylase of *Bacillus subtilis* also retains activity at pH 10.5, a pH value at which only a small fraction of the amino groups would be protonated. Any marked difference in the activity of these two enzymes on these substrates at this high pH value might be of value in elucidating the mechanism of the enzyme action.

Four samples, of decreasing value of degree of substitution (d.s.), were used as substrates for (*a*) crystalline, porcine pancreatic amylase, (*b*) crystalline, liquefying amylase of *Bacillus subtilis*, and (*c*) a mold-enzyme mixture that also contained glucamylase. The reducing sugars in the 24-h limit hydrolyzates, calculated as D-glucose (% of the theoretical), are shown in Table I.

The relatively highly substituted product, d.s. 0.61, was also investigated at pH 10.5. The yields of D-glucose were just slightly below those obtained at the pH optima of the enzymes. Protonation of the amino group did not seem to be an important factor in the action of these two amylases on this substrate.

The hydrolyzates were examined by paper (solvents *A* and *B*) and thin-layer (solvents *B* and *C*) chromatography. The monomer 6-amino-6-deoxy-D-gluco-

TABLE I

ACTION OF AMYLASES ON 6-AMINO-6-DEOXYAMYLOSES IN 24 HOURS

Amylase	*Enzyme (mg) per mg of substrate*	*Degree of substitution*			
		0.85	0.61	0.33	0.22
		Reducing sugars calculated as yield of D-*glucose (% of the theoretical)*			
Porcine pancreatic	9.4×10^{-4}	0	5	14	40
Same at pH 10.5	190×10^{-4}		trace[a]		
Mold-enzyme mixture	0.8	0	trace[a]	19	66
Bacillus subtilis	0.2	0	7	18	26
Same at pH 10.5	4.0		6		

[a]Trace means <5%.

pyranose was not detected in any of the hydrolyzates, even when relatively large amounts were applied to the paper or plate. The absence of a modified monomer in the hydrolyzates is consistent with the results obtained with 6-*O*-methylamylose and with 6-deoxy-6-iodoamylose[4,5].

No products at all were detected in the reaction mixtures from the substrate of d.s. 0.85. Glucose and maltose were detected in the hydrolyzates produced by the action of the two crystalline *alpha*-amylases on the aminated amylose of d.s. 0.61.

The hydrolyzate produced by the action of the *Bacillus subtilis* enzyme on the substrate of d.s. 0.33 was chromatographed by use of solvent *A*. Glucose (R_{Glc} 1.00), maltose (R_{Glc} 0.58), maltotriose (R_{Glc} 0.37), a trace of maltotetraose (R_{Glc} 0.22), and a ninhydrin-positive fraction (R_{Glc} 0.18 to origin) were detected.

With solvent *A*, the fractions glucose through maltotetraose all had mobilities greater than the ninhydin-positive fraction. A larger sample (150 mg) of the sample of d.s. 0.33 was subjected to limit hydrolysis with the crystalline, *Bacillus subtilis* amylase. The entire hydrolyzate was chromatographed, and the fractions corresponding to glucose through maltotriose were excised and extracted, and the extracts concentrated, and analyzed by the phenol–sulfuric acid method[6]. The results, calculated relative to D-glucose as unity, are shown in Table II. They are compared with the results obtained by Robyt and French[7] for the limit hydrolysis of amylose catalyzed by the same enzyme.

TABLE II

COMPARISON OF YIELDS OF OLIGOSACCHARIDES OF LOW MOLECULAR WEIGHT FROM AMYLOSE (ROBYT AND FRENCH[7]) AND FROM 6-AMINO-6-DEOXYAMYLOSE, D.S. 0.33

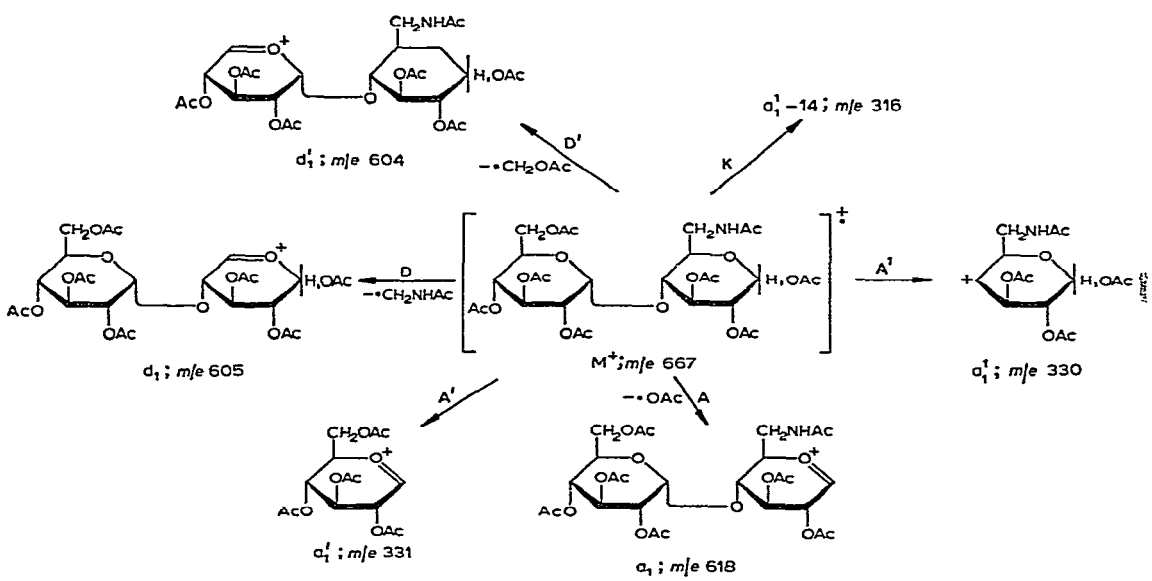
Product	*Amylose*	*6-Amino-6-deoxyamylose*
Glucose	1.00	1.00
Maltose	2.32	0.89
Maltotriose	4.15	0.33

Robyt and French[7] obtained four times as much maltotriose as D-glucose; the results with the substrate of d.s. 0.33 showed only one third as much maltotriose as D-glucose. The results were valuable, because they helped to prove that there was not too much clustering of the aminated D-glucose residues. Had there been long segments of non-aminated D-glucose residues, the results would have resembled those obtained by Robyt and French[7].

The ninhydrin-positive fractions near the origin were also excised and extracted, and the extracts concentrated, and chromatographed by use of solvent *B*. The fractions having R_{Glc} 0.68, 0.52, and 0.40 were removed and extracted, and the extracts concentrated. The peracetates of the first two were prepared, and examined by electron-impact, mass spectrometry (e.i.–m.s.); there was insufficient of the third fraction to permit the preparation of the peracetate.

The peracetate (**1a**) of the fraction of R_{Glc} 0.68 was subjected to e.i.–m.s. A molecular ion was not observed, but its value at *m/e* 677 was inferred from fragment ions at *m/e* 619 and 618 (ref. 8), which correspond to the loss of $\cdot NHCOCH_3$ and $\cdot OCOCH_3$, respectively, from the molecular ion. Peaks at *m/e* 605 ($M^{\ddagger} - \cdot CH_2NHCOCH_3$) and *m/e* 604 ($M^{\ddagger} - \cdot CH_2OCOCH_3$) not only supported the assignment of the molecular ion, but also indicated that the amino group is situated on one of the primary carbon atoms of the disaccharide.

The relative intensities of the ions a_1' and a_1^1, formed by pathways A′ and A^1 (see Scheme 1), were used in making a tentative assignment of the position of the amino group. Fragment a_1' should be formed in greater abundance, because it has greater stability than a_1^1 (ref. 8). The greater stability of the a_1' fragment has been



Scheme 1. Fragmentation pathways for compound **1a**.

demonstrated by an examination of the mass spectra of the peracetates of six maltoses which had been modified at one of the primary carbon atoms (ref. 9). The value of ion a'_1 would be *m/e* 330 were the acetylated amino group on the terminal, non-reducing D-glucosyl group of the original disaccharide. An *m/e* of 331 would be found if the acetylated amino group was on the D-glucose residue of the disaccharide. The relative intensities of the peaks at *m/e* 331 and 330 were 3.8 and 0.7% (*m/e* 43 = 100%), respectively, suggesting that the fraction of R_{Glc} 0.68 was 6^1-amino-6^1-deoxymaltose.

Placement of the amino group on the D-glucose residue was also suggested by ions 14 mass units less than a_1 (see Scheme 1). These ions at *m/e* $a_1^1 - 14$ are observed for disaccharides[9], and, in most cases, their relative abundances are greater than for a_1^1. It has been observed that these ions are fragments of the D-glucose residue and contain C-6. The value of the ion $a_1^1 - 14$ would be 316 if the D-glucose residue carried the amino group, and 317 if the amino group was on the D-glucosyl group. The relative intensity of the peak at *m/e* 316 was more than twice the intensity of that at *m/e* 317, again indicating that this fraction was 6^1-amino-6^1-deoxymaltose.

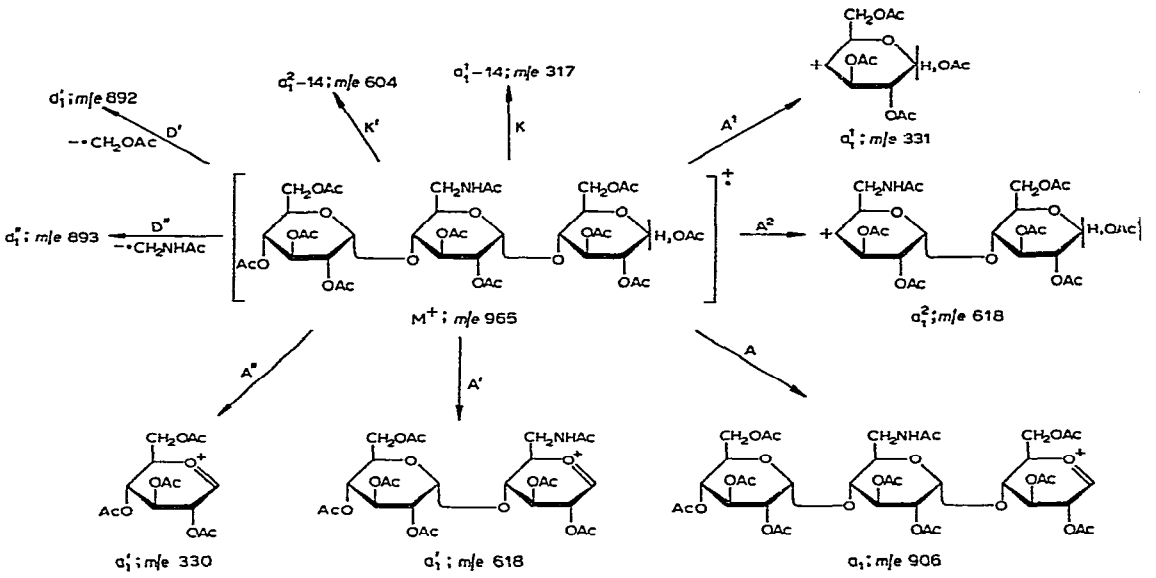
The mass spectrum of the acetate (**2a**) of the fraction of R_{Glc} 0.55 also showed no molecular ion, but ions at *m/e* 906 ($M^+ - \cdot OCOCH_3$) and *m/e* 907 ($M^+ - \cdot NHCOCH_3$) indicated that the molecule was the peracetate of a trisaccharide of *m/e* 965. Fragment ions at *m/e* 892 ($M^+ - \cdot CH_2OCOCH_3$) and 893 ($M^+ - \cdot CH_2NHCOCH_3$) supported the placement of the molecular ion at *m/e* 965. The existence of the ion at *m/e* 893 also suggested that the amino group had been on a primary carbon atom. All of the evidence suggested that the original fraction of R_{Glc} 0.55 was a trisaccharide containing one primary amino group.

Had the amino group been on the terminal D-glucosyl group of the trisaccharide, cleavage along pathway A″ (see Scheme 2) would have produced a relatively intense peak at *m/e* 330. The fact that the intensity of this peak was less than one sixth that of a peak at *m/e* 331 suggested that the amino group had not been on the terminal D-glucosyl group.

Cleavage along pathway A′ of Scheme 2 would have produced an ion of *m/e* 618 if the central D-glucosyl residue had contained the acetamido group, or *m/e* 619 if the central D-glucosyl residue had contained only an acetoxyl group. The relative intensities of the ions *m/e* 618 and 619 were 0.15 and 0.09%, suggesting that the central D-glucosyl residue had contained the amino group.

Placement of the amino group on the central D-glucosyl residue was also supported by the results of extrapolating the fragmentation pathway by which the $a_1 - 14$ ions are formed. Fragmentation along pathway K of Scheme 2 would have produced an ion at *m/e* 317 if the central D-glucosyl residue had contained the acetamido group, or at *m/e* 316 if the central D-glucosyl residue had contained only the usual acetoxyl group. The relative intensities of *m/e* 317 and 316 were 0.32 and 0.007%, again suggesting that the amino group had been on the central D-glucosyl residue, and that the fraction having R_{Glc} 0.55 was 6^2-amino-6^2-deoxymaltotriose.

The presence of 6^2-amino-6^2-deoxymaltotriose in the enzyme hydrolyzate had been expected, based on previous results with 6-deoxy-6-iodoamylose[5]. The presence



Scheme 2. Fragmentation pathways for compound **2a**.

of 6^1-amino-6^1-deoxymaltose had not been expected[10], but the mass-spectral data suggested that this compound was, indeed, a product of the enzymic hydrolysis. There have not been enough investigations of the action of the crystalline, liquefying amylase of *Bacillus subtilis* on modified amyloses to eliminate this possibility on the basis of previous experiments.

The replacement of the primary hydroxyl groups of the D-glucosyl residues in amylose evidently causes some change in the nature of the enzyme–substrate fit, so that the modified D-glucose monomer has never been detected in any of the hydrolyzates. This substitution does not seem to hinder the activity of the enzyme towards the rest of the molecule so long as the degree of substitution is small.

EXPERIMENTAL

General. — Corn amylose (A. E. Staley Co.), crystalline, *Bacillus subtilis* amylase (Enzyme Development Corp.), crystalline, porcine pancreatic amylase (Worthington Biochemical Corp.), and the mold-enzyme mixture termed glucamylase (Clinton Corn Processing Co.) were commercial samples. Samples of 6-amino-6-deoxyamylose were prepared by the method of Whistler and Medcalf[1].

Methods. — Descending paper-chromatograms were developed with 6:4:3 (v/v) 1-butanol–pyridine–water (solvent *A*), and 3:1:1 (v/v) butanone–glacial acetic acid–water (solvent *B*) on Whatman 3MM paper, with detection by the alkaline silver nitrate method of Trevelyan *et al.*[11]. Aminated fractions were detected by dipping the strips of paper in 0.2% ninhydrin in acetone, and allowing the color to develop

overnight in a dark cabinet. Thin-layer chromatograms were obtained on prepared plates of Silica Gel G (Analtech) by using solvent *B*, and 9:7:4 (v/v) ethanol–toluene–diethyl ether (solvent *C*), with detection by spraying with 5% sulfuric acid in ethanol, and heating at 105°. The reducing-sugar determinations shown in Table I were conducted by the iodometric method of McLeod and Robison[12]. Mass spectra were recorded with an RMUGL Hitachi instrument having a direct-insertion probe. The source temperature was 180°, and the ionizing potential was 70 eV.

Enzyme hydrolyses. — These were conducted at 25° at a substrate concentration of 10.0 mg/ml in those experiments reported in Table I. The solutions were 0.10M with respect to acetate at pH 5.7 (*Bacillus subtilis* enzyme), phosphate and sodium chloride at pH 7.0 (porcine, pancreatic enzyme), and acetate at pH 4.5 (mold-enzyme mixture). The buffer solution used in the experiments at pH 10.5 was 0.05M with respect to carbonate and hydrogen carbonate. Preparative experiments with the product of d.s. 0.33, and the crystalline *Bacillus subtilis* amylase were conducted in a 4.0% solution, with 4 mg of substrate per mg of enzyme, at pH 5.7, 0.01M with respect to acetate. Initial development of the papers was performed with solvent *A*, and secondary development of isolated, aminated fractions was performed with solvent *B*. Analyses of extracted fractions for glucose, maltose, and maltotriose were conducted by the phenol–sulfuric acid method[6]. Peracetates of the isolated, aminated fractions were prepared by means of acetic anhydride–pyridine in the usual way.

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