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Note

Enzymic hydrolysis of ketodextran

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Chemically modified substrates offer a means of studying the specificity of hydrolytic enzymes. For instance, enzymic-degradation studies of partially methylated amylose indicated that substitution at position 6 of D-glucopyranosyl residues prevents hydrolysis of the glucosidic bond of the substituted residue, but allows hydrolysis if the substituent is in the aglycon[1]. Other chemical modifications are possible; indeed, Melton and Slessor[2] have studied the relative effect of seven different substituents on the initial rate of hydrolysis of cyclohexa-amylose derivatives by *Aspergillus oryzae* amylase. Ketodextran[3] and ketopustulan[4] stimulate the production of dextranase and (1→6)-β-D-glucanase by moulds, presumably because of their resistance to hydrolysis. Oxidation offers a relatively easy method of chemical modification of substrates, and we now report studies of the enzymic hydrolysis of ketodextran.

Oxidation renders dextran relatively resistant to enzymic hydrolysis (Fig. 1). The hydrolytic rate of ketodextran I is less than 10% of the rate for dextran, yet reduction and subsequent sugar analysis[5] (Table I) indicates that only 12% of the residues have been oxidized (assuming all the 3-keto substituted residues yield D-allose, and the 2-keto substituted residues yield equal quantities of D-mannose and D-glucose on reduction). Increased oxidation of dextran, as indicated by the hydrolytic rate of ketodextran II, does not significantly affect the initial rate of hydrolysis but does decrease the extent of hydrolysis.

Sugar analysis (Table I) of the reduced ($NaBD_4$) hydrolytic products from ketodextran II (after 18-h incubation with enzyme) indicates that the monosaccharide and disaccharide fractions are enriched in D-glucose and depleted in D-allose, whereas, in the tetrasaccharide and higher oligosaccharide fractions, the reverse occurs. The content of D-mannose and D-galactose, although low, is similar in all fractions except for the monosaccharide fraction. Methylation analysis[6] demonstrates that 83% of the 3-keto substituted residues occur at the non-reducing end of the trisaccharide fraction (Table II); 17% occur in the middle, while only D-glucose occurs at the reducing end. Analysis of the tetrasaccharide fraction shows that 32% of the

3-keto substituted residues occur at the non-reducing end, whilst the remaining 68% occur in the middle and probably one residue from the non-reducing end. The mass spectra of 2,3,4,6-tetra-*O*- and 2,3,4-tri-*O*-methyl-D-glucose did not indicate significant quantities of deuterium in the 2-position, which would indicate the presence of D-glucose derived from C-2 substituted residues or the corresponding D-mannose

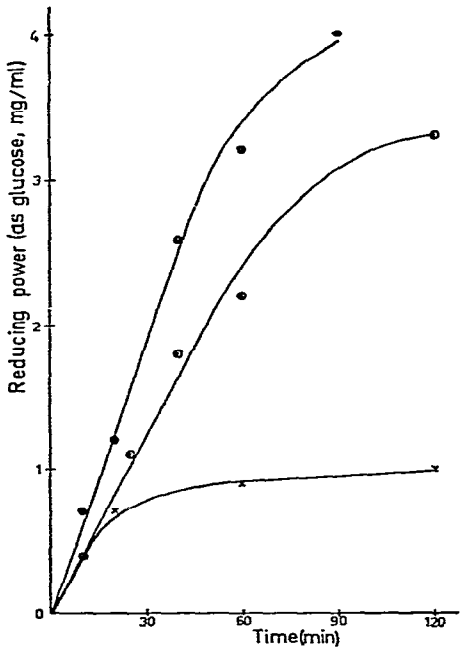

Fig. 1. Production of reducing groups during treatment of dextran (●—●), ketodextran I (⊙—⊙), and ketodextran II (×—×) with dextranase. Ketodextrans I and II were incubated with ten times as much enzyme as dextran. Controls receiving boiled enzyme did not change reducing power during incubation nor, after reduction, was the sugar composition of the incubated material altered.

TABLE I

SUGAR COMPOSITION OF SAMPLES OF REDUCED KETODEXTRAN I AND II, AND OF THE REDUCED PRODUCTS OF ENZYMIC HYDROLYSIS OF KETODEXTRAN II

Sample	*Weight (mg)*	*Percent*			
		D-*Glucose*	D-*Allose*	D-*Mannose*	D-*Galactose*
Ketodextran I	—	89	10	1	—
Ketodextran II	—	80	17	2	1
Monosaccharide	9	100	—	—	—
Disaccharides	30	95	2	3	1
Trisaccharides	53	80	17	2	1
Tetrasaccharides	41	75	22	2	1
Higher oligosaccharides	102	77	21	1	2

derivatives which have similar retention times. The mass spectrum of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl-D-glucitol was consistent with the location of deuterium at the 1-position. Authentic samples of the methylated allose derivatives were not available. The methylation patterns of these sugars were evident from the mass spectra of the derived alditol acetates. In addition, the mass spectra showed deuteration at C-3, indicating that they were allose derivatives. Further, the quantities of the methylated allose derivatives detected corresponded to the percentages of allose found in the sugar analyses of the trisaccharide and tetrasaccharide fractions.

TABLE II

METHYLATION ANALYSIS OF THE REDUCED TRISACCHARIDES AND TETRASACCHARIDES PRODUCED DURING ENZYMIC HYDROLYSIS OF KETODEXTRAN II

Sugars	T[a]	*Percent*	
		Trisaccharides	*Tetrasaccharides*
1,2,3,4,5-Penta-*O*-methyl-D-glucitol	0.46	35	24
2,3,4,6-Tetra-*O*-methyl-D-allose	0.69	15	8
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	19	16
2,3,4-Tri-*O*-methyl-D-allose	1.64	3	17
2,3,4-Tri-*O*-methyl-D-glucose	2.32	28	35

[a]Retention times of the corresponding alditol acetate on ECNSS-M relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

The results indicate that the presence of a keto group in the 3-position prevents hydrolysis of the glucosidic bond of the substituted residue and strongly inhibits hydrolysis of the bond one residue removed towards the reducing end, but allows hydrolysis if the substituent is in the aglycon. Although the results show that oxidation at the 3-position affects hydrolysis of the two adjacent glycosidic bonds, they do not explain the rapid decline in hydrolysis rate caused by even a low degree of oxidation. Studies of bacterial dextranase have indicated that the enzyme requires six binding subsites[7]. If oxidation decreases binding, which seems to be a reasonable assumption, then the effect of oxidation on the hydrolysis rate would be more apparent.

EXPERIMENTAL

General methods. — G.l.c. was conducted with a Perkin–Elmer model 900 instrument, using the following column: ECNSS-M, 3% on Gas-chrom Q, at 195° for alditol peracetates, and at 160° for partially methylated alditol acetates. For g.l.c.–m.s., a Perkin–Elmer 270 gas chromatograph–mass spectrometer was used.

Substrates and hydrolysis. — Ketodextran was prepared from dextran-20, as previously described[8]. Oxidation times of 30 and 60 min were used to produce

ketodextrans I and II, respectively. To measure hydrolytic rates, dextran-20 or the ketodextran derivatives (200 mg) were dissolved in 0.01M phosphate buffer (pH 6.0, 10 ml) and dextranase (0.8 unit for dextran-20, and 8.3 units for ketodextrans I and II, in 1 ml of water) was added. The liberation of reducing groups at 30° was measured by the copper reduction method[9]. Following enzymic hydrolysis, the products were reduced with $NaBD_4$, and a portion was chromatographed on a column (1.5 × 50 cm) of Sephadex G-15 with distilled water at a flow rate of 1.4 ml/h. Fractions (0.7 ml) were collected and optical rotations measured. Fractions corresponding to a d.p. of 1, 2, 3, 4, and higher were isolated, and the purity of the preparations of low molecular weight was verified by paper chromatography. Fractions which were not homogeneous were chromatographed again using the same Sephadex column. The sugar composition of each fraction, as well as of reduced ketodextran I and II, was determined by g.l.c. of the alditol acetate derivatives[5] after hydrolysis. The tri- and tetra-saccharide fractions were methylated[10], and the methylated sugars produced on hydrolysis were analyzed by g.l.c.–m.s. of their alditol acetate derivatives[6].

Enzyme. — The methods used for dextranase production and activity measurements have been previously described[3]. To purify the dextranase used in this study, the extracellular enzyme produced by cultivating *Penicillium funiculosum* on ketodextran (23 units.ml^{-1}) was dialyzed, concentrated by lyophilization (2,090 units, 7.1 units/mg), and then chromatographed by using a column (5 × 100 cm) of Bio-Gel P-150 (100–200 mesh) developed with 0.01M phosphate buffer (pH 6.0) at a flow rate of 20 ml/h. Fractions (20 ml) were collected and the enzyme activity was measured. The most active fractions of the single peak of activity were combined, dialyzed, and lyophilized (1,740 units, 20 units/mg). A portion (50 mg) of this preparation was further purified by isoelectric focusing as follows. The density gradient was formed with sucrose, and the ampholyte (pH 3–10, LKB Producktor AB) was used at a concentration of 1% in a total volume of 440 ml. The dextranase preparation was dissolved in the light solution. The voltage was adjusted to keep the power input at ~6 Watts. After 26 h at 5°, focusing was terminated and the column was emptied in 10 ml fractions. After the pH of each fraction had been measured, it was adjusted to 6.0 and enzyme activity measured. The main peak of activity, which focused at pH 4.45, was recovered by dialysis and lyophilization (680 units, 90 units/mg). Polyacrylamide gel-electrophoresis[11] of this preparation indicated the presence of a single protein. For convenience in handling, the dextranase preparation was dissolved in water, and crystalline egg-albumin was added to give, on lyophilization, a product having a specific activity of 8.3 units/mg.

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