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Enzymic Production of Cellotriose from Cellulose

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INTRODUCTION

During the course of preparation of cellobiose by enzyme hydrolysis of cellulose, a second oligosaccharide was detected in rather large proportion in the hydrolyzate. Since it was possible that this oligosaccharide is an intermediate in the enzyme hydrolysis of cellulose, the compound was isolated. It has been characterized as cellotriose.

METHODS

The cellulolytic enzyme solution was obtained by growing *Streptomyces* sp. QM B814 on Walseth (10) cellulose in shake flasks for 2 weeks, and filtering the residual solid material (organism plus undigested cellulose). Walseth cellulose is cotton fiber swollen with cold 85% phosphoric acid, and subsequently dispersed in water. Cellulolytic filtrate (100 ml.) was incubated with 1.4% of the Walseth cellulose (400 ml.) at pH 5.5-6.0 and 50°C. for 6 hr. and for 17 hr., at which time there was between 3.0 and 4.0 mg. of reducing sugar/ml. [estimated as glucose by the dinitrosalicylic acid method (9)].

The hydrolyzates were filtered to remove unhydrolyzed cellulose, boiled for 10 min. to inactivate enzyme, evaporated under reduced pressure to about $\frac{1}{10}$ the original volume, and added to a carbon-Celite column (280 × 40 mm.). The fractions were eluted with water, 7.5% ethanol, and 50% ethanol (11). The cellobiose found in the 7.5% alcohol was crystallized from methanol. The material present in the 50% alcohol eluates was precipitated from methanol in the cold. Both products were washed with acetone and dried *in vacuo* at room temperature.

The enzymic hydrolysis of cellobiose and of cellotriose was followed quantitatively by determination of the reducing value after removal of the glucose with glucose oxidase. The nature of the hydrolysis products was determined by means of paper chromatograms.

RESULTS

Chromatographic examination of the oligosaccharide fraction (50% alcohol eluate) showed the presence of a major component traveling at the rate of cellotriose, and a trace component corresponding to cellobiose. $[\alpha]_D$

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+18.5° (c, 3, water). The powdery material (100 mg.) was taken up in hot water (0.75 ml.), ethanol was added to the point of turbidity, and the solution was stored at 10°C. A crystalline product was obtained which, after two recrystallizations from water-ethanol, had m.p. 205–209°C., undepressed by admixture with α -cellobiose. Weight, 70 mg. [Lit., m.p. 203–214°C. (5); 206–209°C. (13)]. The x-ray diffraction patterns and infrared absorption spectra of the product and α -cellobiose were indistinguishable. $[\alpha]_D^{23} +32.0^\circ$ (5 min.) $\rightarrow +23.9^\circ$ (eqm.) (c, 0.9, water). [Lit., $[\alpha]_D +32.0^\circ \rightarrow 23.2^\circ$ (5); $+35^\circ \rightarrow 21.6^\circ$ (13)].

Anal. Calcd. for $C_{18}H_{32}O_{16} \cdot \frac{1}{2}H_2O:C$, 42.15; H, 6.44. Found: C, 41.80; H, 6.74. [Hess and Dziengel (5) report α -cellobiose to contain $\frac{1}{4}$ – $\frac{1}{2}$ mole water of crystallization.]

Both cellobiose and cellotriose were prepared in good yields. By adding fresh enzyme to the residual cellulose after each hydrolysis (six times), 75% of the Walseth cellulose could be hydrolyzed. In one series of tests, 6.3 g. cellobiose and 1.8 g. of crude cellotriose were produced; in the second series, 10.6 g. cellobiose and 3.8 g. of crude cellotriose were produced. The ratios of cellobiose to crude cellotriose are 3.5 and 2.8, respectively. Only small amounts of glucose were found.

Several experiments were conducted to determine whether the cellotriose arose directly from cellulose, or indirectly by action of a transferase on the cellobiose formed. The action of the *Streptomyces* enzyme was tested on cellobiose and on cellotriose at a concentration of 0.4%. (This was the maximum cellobiose concentration found in the cellulose hydrolyzates.) Under conditions where cellulose was rapidly hydrolyzed in 1 hr., cellobiose and cellotriose were not appreciably affected even after 23 hr. (Table I).

TABLE I

Hydrolysis of Cellulose, Cellobiose and Cellotriose by Enzymes

Substrate: cellulose 0.5%; cellobiose, cellotriose, 0.4%. pH 5.4; 50°C. Cellobiose A is Pfanstiehl, chemically prepared; cellobiose B and cellotriose are enzymically prepared.

Enzyme	Hydrolysis, %						
	Walseth cellu- lose	Cellobiose				Cellotriose	
		A		B		1 hr.	23 hr.
		1 hr.	23 hr.	1 hr.	23 hr.		
<i>Streptomyces</i> sp. QM B 814	32	0	0	0	0	0	3
β -Glucosidase of <i>Aspergillus phoenicis</i> QM 1005	—	55	—	61	—	50	—
Röhm and Haas No. 1	—	88	—	88	—	84	—

These sugars were, however, readily hydrolyzed by two β -glucosidase preparations tested. One of the latter, acting on higher concentrations of cellobiose (2%), produced a transitory reversion product as well as glucose. This material moved more slowly than cellobiose but more rapidly than cellotriose on a chromatogram. No such reversion products were ever formed by the *Streptomyces* enzymes.

The hydrolysis of cellulose by *Streptomyces* cellulase is inhibited by cellobiose. At a cellobiose concentration equal to the cellulose concentration (i.e., 0.5%), 65% inhibition was observed. Glucose gave 34% inhibition under comparable conditions.

DISCUSSION

There have been reports of soluble intermediates resulting from the hydrolysis of cellulosic materials by enzymes of *Myrothecium verrucaria*. Kooiman *et al.* (7) detected intermediates from cellulose dextrin, some of which may be sulfated sugars because (a) they resist enzyme hydrolysis, and (b) sulfate has been detected in them (3). Hash and King (4) reported trace amounts of "tetramer" from hydrolysis of cellulose. (From the properties described, we believe that this may be cellotriose.) Finally, Whitaker (12) measured the relative amounts of cellobiose, cellotriose, and cello-tetraose (10:7:4) produced during the early stages of hydrolysis of a cello-dextrin. He showed that cellotriose and cello-tetraose are indeed hydrolysis products and not products arising by transfer from cellobiose.

We have known for some time that the enzymes of *Streptomyces* sp. QM B814 acting on cellulose give cellobiose as the dominant reducing sugar. It now appears that cellotriose also accumulates as a hydrolysis product. These sugars accumulate because of the absence or near absence of enzymes capable of hydrolyzing them. Unlike some of the dextrin hydrolysis products described by Kooiman (7), the cellobiose and cellotriose are readily hydrolyzed by β -glucosidases. The cellotriose does not arise from cellobiose by transfer. Such reactions require high concentrations of disaccharide and a transferring enzyme, conditions which do not exist in our hydrolyses. In actively growing cultures of *Streptomyces*, cellobiose and cellotriose do not accumulate, probably because they are rapidly absorbed by the living organism, and converted intracellularly to glucose by hydrolytic or phospholytic enzymes (6, 8).

According to the multienzyme theory (1) of cellulose hydrolysis, cellulase is made up of several components of different substrate specificities, some of which may overlap with the β -1,4-oligosaccharases. Each enzyme component acts on a limited range of chain lengths. In order for intermediate products to accumulate in cellulose hydrolysis, the action of enzymes on these intermediates must be minimized. In the results with *M. verrucaria*

(above), the origin of cellulases free of oligosaccharases is different in each instance. Whitaker's (12) cellulase preparation lacks oligosaccharases because of its method of preparation. Kooiman (7) heat-inactivated the oligosaccharase without destroying the cellulase. The *Streptomyces* filtrate apparently never contained an oligosaccharase. Successful accumulation of the water-soluble intermediates depends upon the use of such enzyme preparations as these acting on a highly reactive cellulose (as that developed by Walseth).

The accumulation of cellobiose and cellotriose in our experiments indicates that the rate of enzyme hydrolysis falls off sharply as the degree of polymerization of the substrate decreases from six to two. Cellotetraose would be expected to accumulate if cellulolytic systems were available lacking in the ability to hydrolyze this oligosaccharide. The "polysaccharase" of Grassmann *et al.* (2) seems to possess the desired characteristics, i.e., it hydrolyzes cellodextrin and cellohexaose over 100 times as fast as it hydrolyzes cellotetraose. Unfortunately, Grassmann did not analyze the products of digestion, so that the abundance of cellotetraose in his hydrolyzates is not known.

Polysaccharases may be classified as:

1. Endo-, or random-splitting enzymes, and
2. Exo-, or endwise-splitting enzymes, with
 - a. Removal of one monosaccharide unit at a time, or
 - b. Removal of one disaccharide unit at a time.

The predominance of cellobiose in the hydrolyzates of cellulose eliminates the possibility that the *Streptomyces* enzyme is of the 2a type (above). The relatively large amount of cellotriose produced is indicative of a random type hydrolysis. Most cellulases seem to be of this type.

SUMMARY

Cellotriose is produced in appreciable amounts by action of cellulolytic filtrates of *Streptomyces* sp. on highly reactive cellulose. Cellotriose and cellobiose accumulate because enzymes capable of hydrolyzing them are absent.

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