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Lactosan: 4-*O*-β-D-Galactopyranosyl-1,6-anhydro-β-D-glucopyranose Formation by Heat-Treatment of Lactose *In Vacuo*

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ABSTRACT

Formation of a glycosidic anhydro-disaccharide from heated lactose was studied. Lactosan, 4-O-β-D-galactopyranosyl-1,6-anhydro-β-D-glucopyranose, was isolated from the lactose pyrolysate obtained by heating in vacuo. *It was deduced that the lactosan was a reaction intermediate and contributed to the formation of 1,6-anhydro-β-D-glucopyranose and polysaccharide.*

INTRODUCTION

It is well known that glycosidic anhydro-sugars are formed during the thermal degradation of various carbohydrates *in vacuo* or in an inert atmosphere in a closed system (Peat, 1946), and are important intermediates of polymerization of sugars (Schuerch, 1981). From the pyrolysates of lactose, various anhydro-monosaccharides, namely, 1,6-anhydro-β-D-glucopyranose, 1,6-anhydro-β-D-galactopyranose, 1,6-anhydro-β-D-glucofuranose, 1,6-anhydro-α-D-galactopyranose and 1,4:3,6-dianhydro-D-glucopyranose (Pictet & Cramer, 1920; Hann & Hudson, 1941, 1942; Hohno *et al.*, 1983), have been detected and identified.

An anhydro-disaccharide was detected first by Pictet and Sarasin (1924) from the pyrolyzate of lactose; however the structure was not confirmed. They obtained a nonreducible compound, corresponding to $C_{12}H_{20}O_{10}$, from lactose, by heating at 185°C for 10 to 12 h under reduced pressure and believed it to be lactosan (galactosyl-anhydro-glucose) although they did not

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give supporting evidence for its structure. In our earlier report (Hohno *et al.*, 1983), the anhydro-disaccharide was also detected from the pyrolysates of lactose and was speculated to be a lactosan.

On the other hand, lactosan was synthesized chemically by the alkaline degradation of phenyl-β-lactoside (Montgomery *et al.*, 1943).

In food processing, lactose, as an ingredient in foodstuffs, is frequently subjected to heat. However, little is known about its molecular transformations without cleavage of the carbon skeleton and its polymerization to form polysaccharide. This paper deals with the separation and structural analysis of the titled compound, lactosan, obtained from the pyrolyzate of lactose.

EXPERIMENTAL

Materials

Guaranteed reagent-grade α-lactose hydrate, D-galactose and D-glucose were obtained from Wako Pure Chem. Industries Limited. Crystalline levoglucosan (1,6-anhydro-β-D-glucopyranose) was obtained by dry distillation of starch under reduced pressure (Waad, 1963). Preparation of 1,6-anhydro-β-D-galactopyranose was described in our previous report (Hohno *et al.*, 1983). α-Lactose anhydride was prepared from α-lactose hydrate by heating at 100°C for 5 h in a vacuum drying chamber as usual. β-Galactosidase from *Asp. oryzae* (2000 ONPG unit g^{-1} protein) was purchased from Tokyo Tanabe Company Limited. (1 unit is the quantity of enzyme which will form 1 μmol of *O*-nitrophenol min^{-1} at 30°C from *O*-nitrophenyl-β-D-galactoside.)

Heat-treatment of α-lactose anhydride

α-Lactose anhydride (200 g) was placed into a round-bottomed 1 litre flask and then heated at 180°C for 5 h or 150°C for 10 h in the temperature-controlled air chamber under reduced pressure at 2–5 mm Hg.

Isolation of anhydro-disaccharide

Heated lactose (180 g) was extracted twice with 500 ml of methanol. The combined methanol extracts were evaporated to a syrup and then treated with excess aniline acetate at 80°C for 3 h for elimination of aldoses as described in our previous report (Suyama *et al.*, 1987). After the analine derivatives of aldoses were extracted by ethyl ether, the aqueous fraction was decolorized by treatment with activated charcoal and then the

solution containing anhydro-saccharides was charged on the preparative thin-layer chromatography (TCL) sheet as described below. The zone corresponding to the anhydro-disaccharide on the developed preparative TLC sheet was cut out and extracted by methanol. Thus amorphous but chromatographically pure anhydro-disaccharide was obtained.

Hydrolysis

Chromatographically homogeneous isolated anhydro-disaccharide was kept in boiling 1 N sulfuric acid for 1 h for complete hydrolysis. The reaction mixtures were treated with Amberlite IRA-410 (OH^- form) and then evaporated to a syrup *in vacuo*. Anhydro-disaccharide was hydrolyzed enzymatically by dissolving 2 mg of sugar in 1 ml of 0·05 M McIlvaine buffer (pH 7·0) to which about 20 ONPG units of β-galactosidase and a small drop of toluene were added, incubating at 30°C for 5 h and then heating at 95°C for 5 min to terminate the reaction. Hydrolyzate was dried under a stream of nitrogen to a solid.

Chromatography

Thin-layer chromatography was performed by an ascending technique on silica gel TLC (Merck; precoated on an aluminium sheet Art 5553) in a solvent system of ethylacetate-acetic acid-water (2:1:1, v/v). Sugars were detected by spraying with 5% H_2SO_4-CH_3OH and heating at 150°C for 5 min. Detection of a logging spot of anhydro-disaccharide on preparative TLC was done as usual. Visualization of the separated zones of sugars was carried out by dipping into hexane as described in our previous report (Suyama & Adachi, 1987).

The gas-liquid chromatography (GC) of sugars was carried out as follows. Sugars were converted by trimethylsilyl (TMS) ethers by means of hexamethyldisilazane and trifluoroacetic acid in pyridine (Sweeley *et al.*, 1963). The TMS derivatives of sugars were injected into a Hitachi model 163 gas chromatograph equipped with a hydrogen flame ionization detector and stainless steel column (0·3 × 50 cm) packed with 2% Dexsil 300 GC on Uniport HP (60–80 mesh). Nitrogen was used as a carrier gas at a flow rate 30 ml/min. The column temperatures were 130°C for monosaccharide and programmed from 150° to 350°C at 10°C/min for disaccharide.

Fast-atom bombardment mass spectrometry

Fast-atom bombardment (FAB) mass spectrometry was done on a DX-300 mass spectrometer (JEOL, Tokyo, Japan) using argon atoms with a kinetic energy equivalent to 3 keV. The oligosaccharides were analyzed in a

positive-ion mode. The target was first coated with sodium acetate and the saccharide (about 5 μg), dissolved in 10 μl of water, was added to the glycerol matrix.

^{13}C-NMR

^{13}C-NMR spectra were obtained in D_2O at 100 MHz with 3-(trimethylsilyl)propanesulfonate sodium salt as the internal standard by a JEOL-FX-100 instrument operated in the pulsed Fourier transform mode at 30°C.

RESULT AND DISCUSSION

Thin-layer chromatography of methanol extracts of heated lactose

By evaporation of methanol extracts of 180 g of heated lactose at 180°C for 5 h and 182 g of heated lactose at 150°C for 10 h, 7·3 and 8·0 g of brown-colored, viscous syrup were obtained, respectively. The TLC of both methanol extracts of heated lactose at 180°C for 5 h and 150°C for 10 h after the aniline treatment gave the spot ($Rf = 0{\cdot}39$; lower than that of galactose; 0·45, and higher than that of lactose; 0·26) which was not visualized by spraying with reagent to reduce sugar (aniline-hydrogen phthalic acid reagent and dimedone reagent) but was visualized by spraying with H_2SO_4-methanol. From the *Rf* values by TLC and specificity of spray reagent, it might be concluded that the compound having the 0·39 *Rf* value is an anhydro-disaccharide.

Characteristics of anhydro-disaccharide

The anhydro-disaccharide isolated by preparative TLC gave a single peak on GLC and single spot on TLC. The anhydro-disaccharide was a nonreducing sugar which was negative to Fehling reagent. It yielded equimolar parts of galactose and glucose (1·1:1·0; identified by GLC) by complete acid hydrolysis. Hydrolysis with β-galactosidase yielded equimolar parts of galactose and levoglucosan (1·0:0·95, identified by GLC). The results by GLC suggested that both galactose and levoglucosan were a part of the structure of this anhydro-disaccharide.

In the positive ion FAB mass spectrum, a quasimolecular ion ($M + Na^+$) was observed for anhydro-disaccharide at 347. We concluded that the molecular weight of the anhydro-disaccharide is 324.

^{13}C-NMR was used for further elucidation of the structure of the

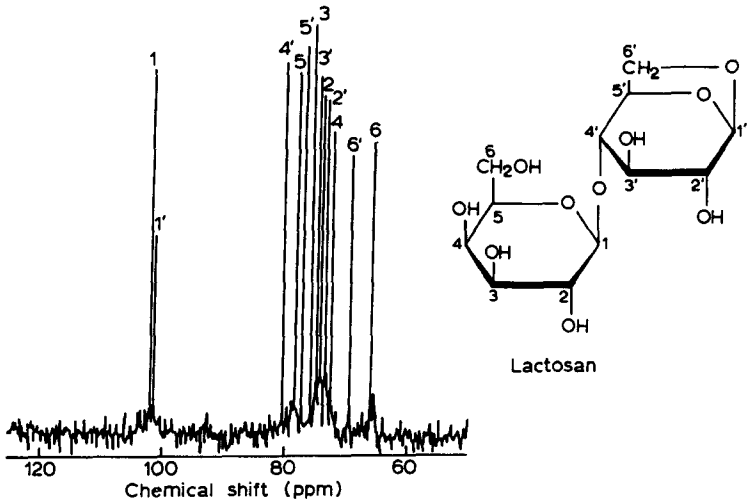

Fig. 1. ^{13}C-NMR of anhydro-disaccharide.

anhydrodisaccharide. Figure 1 shows the ^{13}C-NMR spectrum of the anhydro-disaccharide. The chemical shifts and numbering of each sugar unit are given in Table 1. Resonances were assigned by corresponding reference to authentic lactosan (Matsuda & Tejima, 1980, 1981). Good agreement was obtained between the anhydro-disaccharide and authentic lactosan. The signal at 104·9 ppm could be clearly assigned to the anomeric carbon of the galactose unit, by analogy with the chemical shift of the β-D-form of the galactose unit of lactose (105·8 ppm) and a discrepancy with that of the α-D-form of the galactose unit of melibiose (101·0 ppm). The anomeric configuration of the galactose unit was therefore the β-D-form. The signal at 104·2 ppm could also be clearly assigned to the anomeric carbon of the glucose unit as β-D-form, by analogy with the chemical shift of the β-D-form of glucose units of lactosan (103·6 ppm).

TABLE 1

^{13}C-NMR Data (ppm) for Anhydro-disaccharide and Related Saccharides; Lactosan, Lactose and Levoglucosan

	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Anhydro-disaccharide gal*p*	104·9	73·5	75·4	71·5	78·2	64·0
levoglucosan	104·2	72·7	74·3	80·8	76·9	68·0
Lactosan gal*p*	104·9	73·5	75·4	71·5	78·3	64·1
levoglucosan	104·3	72·3	74·3	81·0	76·9	68·0
Lactose gal*p*	105·8	73·8	75·4	71·4	78·2	63·9
α-glu*p*	94·7	74·3	74·0	81·3	73·0	62·9
β-glu*p*	98·6	76·7	77·2	81·4	77·7	63·0
Levoglucosan	103·6	74·1	73·0	67·1	77·2	66·4

Formation of lactosan

Three possible routes were considered for the formation of lactosan: one is dehydration of the glucosyl residue in lactose; others are liberation of the lactose residue as a lactosan from polysaccharide formed by heating of lactose and condensation of levoglucosan with galactose.

Lactosan might be one of the precursors of 1,6-anhydro-glucose which was detected in the pyrolysate of lactose (Hohno *et al.*, 1983). On the other hand, it is known that the anhydrosugar is easily polymerised by heat-treatment, especially in the presence of an acid catalyst, to form a polysaccharide (Hann & Hudson, 1942). The first investigation of the polymerization of an anhydro-sugar, 1,6-anhydro-glucose, by heat-treatment, was reported by Pictet and Cramer (1920); the resultant polysaccharide structure was found to be multibranched, with random distribution of glycosyl linkages to oxygen atoms on pyranosyl residues and small amounts of furanosyl units (Schuerch, 1981).

It can be concluded that lactosan is also polymerized during the heat-treatment of lactose. In this experiment, an undialyzable polysaccharide was isolated by the dialysis of the pyrolyzates of lactose (9 and 24 g from each 100 g of pyrolyzates of 150°C for 10 h and 180°C for 5 h treatments, respectively; the details, we will report later). No special use or interest of the polysaccharides from lactose thus formed has as yet developed. However, it is concluded that polysaccharides might be formed during the heat-processing of lactose-containing foodstuffs.

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