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Oligosaccharide Structures Formed During Acid Hydrolysis of Lactose

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ABSTRACT

At least 17 disaccharides and four anhydrosugars were formed during hydrolysis of 30 per cent lactose with 2M hydrochloric acid at 100°C for 16 h. The structure elucidation of 11 disaccharides and an anhydrosugar was carried out by methylation analysis, mass spectrometry, enzymatic digestion and ^{13}C-NMR spectrometry. The disaccharides were 4-O-β-D-glucopyranosyl-D-galactose, 3-O-β-D-glucopyranosyl-D-galactose, 2-O-α-D-galactopyranosyl-D-glucose, 6-O-α-D-glucopyranosyl-D-glucose (isomaltose), 6-O-β-D-glucopyranosyl-D-glucose (gentiobiose), α-D-galactopyranosyl-(1↔1)-α-D-glactopyranoside, 6-O-α-D-galactopyranosyl-D-glucose (melibiose), 6-O-α-D-galucopyranosyl-D-galactose, 6-O-β-D-glucopyranosyl-D-galactose, 6-O-α-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose. The anhydrosugar was 1,6-anhydro-β-D-galactopyranose (levogalactosan).

INTRODUCTION

Oligosaccharide formation during hydrolysis of lactose by mineral acid or strongly acidic cation exchange resin was reported by Aronson (1952), De Boer and Robbertsen (1981), Guy and Edmondson (1978) and Huh *et al.* (1990). There are, however, no reports on structural elucidation of

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oligosaccharides formed during acidic hydrolysis of lactose. This paper describes the structures of 11 disaccharides and one anhydrosugar formed during hydrolysis of lactose by hydrochloric acid.

MATERIALS AND METHODS

General

The molar ratios of glucose and galactose were determined as trimethylsilyl derivatives by gas chromatography after acid hydrolysis with 2M trifluoroacetic acid for 6 h at 105°C (Toba *et al.*, 1985).

Reduction of oligosaccharides with sodium borohydride and trifluoroacetylation was performed as described previously (Toba *et al.*, 1985). Alditol trifluoroacetates were analyzed on a Hitachi 163 gas chromatograph equipped with a 10 per cent SE-30 on Chromosorb W AW-DMCS. 80–100 mesh, 1000 mm × 3 mm i.d. stainless steel column. Carrier gas: N_2, 40 ml/min: injection and flame ionization detector: 290°C: column temperature: 90–290°C at a programmed temperature rise of 7·5°C/min.

Reduction of oligosaccharides with sodium borodeuteride, permethylation with the Hakomori method and analysis of the resulting permethylated oligosaccharide alditols by gas chromatography–mass spectrometry were performed as described previously (Toba *et al.*, 1985). Permethylated oligosaccharide alditols were also analyzed on a Hitachi 163 gas chromatograph equipped with a 3 per cent SE-30 on Chromosorb W AW-DMCS. 80–100 mesh, 1000 mm × 3 mm i.d. stainless steel column. Carrier gas: N_2, 40 ml/min; injection and flame ionization detector: 280°C; column temperature: 195–280°C at a programmed temperature rise of 5°C/min. In some cases, permethylated oligosaccharide alditols were also analyzed on a Hitachi 163 gas chromatograph equipped with a 2 per cent SE-30 on Chromosorb W AW-DMCS. 80–100 mesh, 1000 mm × 3 mm i.d. stainless steel column. Carrier gas: N_2, 40 ml/min; injection and flame ionization detector: 250°C; column temperature: 140–240°C at a programmed temperature rise of 5°C/min.

Trimethylsilylated oligosaccharides were prepared as described previously (Toba *et al.*, 1985) and analyzed on a JEOL JGC-20KFP gas chromatograph equipped with a 2 per cent SE-30 on Chromosorb W AW-DMCS. 80–100 mesh. 3000 mm × 3 mm i.d. glass column. Carrier gas: N_2, 40 ml/min; injection and flame ionization detector: 300°C; column temperature: 150–290°C at a programmed temperature rise of 3°C/min.

Gas chromatography of trimethylsilylated anhydrosugars was performed on a Hitachi 163 gas chromatograph equipped with a 1·5% SE-52 on

Chromosorb W AW-DMCS, 80–100 mesh, 2000 mm × 3 mm i.d. stainless steel column. Carrier gas: N_2, 12·5 ml/min; injection and flame ionization detector: 300°C; column temperature: 145°C isothermal.

^{13}C-NMR spectra were recorded as described previously (Toba *et al.*, 1985).

Thin-layer chromatography

Analytical and preparative thin-layer chromatography were performed on TLC plates silica gel 60 (0·25 mm thick, 20 cm × 20 cm, Merck No. 5721) in *n*-butyl alcohol–isopropyl alcohol–water (10:5:4, v/v) by single ascending technique. Spots were detected by spraying 5 per cent sulfuric acid in ethanol and heating.

Paper chromatography

Analytical and preparative paper chromatography were performed on Whatman 3MM chromatography paper (46 cm width × 57 cm long) in *n*-butyl alcohol–pyridine–water (6:4:3, v/v) by seven and four times ascending technique, respectively. Sugars were stained with silver nitrate reagent (Trevelyan *et al.*, 1950).

Enzymatic digestion

α-Galactosidase from green coffee beans (No. 105023), β-galactosidase from *Escherichia coli* (No. 105031), α-glucosidase from yeast (No. 635332) and β-glucosidase from sweet almonds (No. 105422) were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany. Oligosaccharide fractions (1 mg) were incubated at 37°C for 24 h with α-galactosidase (1·8 to 6·0 units), β-galactosidase (4·5 to 6·0 units), α-glucosidase (10 units) or β-glucosidase (0·1 to 0·4 units) in water (1 ml) under one drop of toluene.

Hydrolysis of lactose by hydrochloric acid and isolation of oligosaccharides

Thirty per cent w/w of lactose (55 g) in 2M hydrochloric acid was heated in a boiling water bath for 16 h. After cooling, the acid was neutralized by passage through an Amberlite IRA-45 (OH^-) column (9 cm i.d. × 46 cm long). Neutralized hydrolyzate was then poured onto a mixed layer of charcoal (220 g) and Celite (220 g) on a Buchner funnel. Sugars were eluted with water (25 litres) and 5, 15, 30 and 50 per cent ethanol in water (31, 26, 21 and 17·2 litres, respectively.)

Fractions eluted with 5 per cent ethanol contained disaccharides and were further purified by preparative paper chromatography.

Purification of anhydrosugars

Fractions (100 mg dry weight) eluted with water from charcoal–Celite were dissolved in 75 per cent v/v *n*-propyl alcohol in water (10 ml) and applied onto the column (2 cm i.d. × 30 cm long) packed with Amberlite IRA 400 (bisulfite form) which had been equilibrated with 75 per cent *n*-propyl alcohol (Adachi & Sugawara, 1963). The column was washed with 75 per cent *n*-propyl alcohol (250 ml) and anhydrosugars in the eluent were further purified by preparative thin-layer chromatography.

Structural elucidation of oligosaccharide fractions

Each of the oligosaccharide fractions was purified by preparative paper chromatography until it gave a single spot on paper chromatograms. However, gas chromatographic analysis of it as alditol showed it was still a mixture of two to five oligosaccharides. Structural elucidation of each oligosaccharide was performed without its isolation as follows.

The reduced oligosaccharide fraction was analyzed by gas chromatography to determine the number of sugars in it (as trifluoroacetate and permethylate). Gas chromatography–mass spectrometry of permethylated oligosaccharide alditols was carried out to find the linkage of each peak at the same time. Then the oligosaccharide fraction was treated with α-galactosidase, β-galactosidase, α-glucosidase or β-glucosidase, and the resulting digest was reduced and analyzed by gas chromatography as trifluoroacetates and permethylates. If a particular oligosaccharide(s) in the fraction was hydrolyzed with the glycosidase, the disappearance of corresponding peak(s) and the appearance of constituent monosaccharide-peak(s) must be observed on the gas chromatogram. Non-reducing end residues and their anomeric configurations could be identified from the specificity of glycosidase.

By reference to the methylation analysis, a complete structure was elucidated. ^{13}C-NMR spectrometry was also used for the determination of anomeric configuration of the non-reducing end residue.

RESULTS

Fractionation of hydrolyzate

Table 1 lists results of fractionation of hydrolyzate on charcoal–Celite by a discontinuous gradient of ethanol in water. Fractions eluted by water contained sugars having greater R_{Gal} values than that of glucose. As the 75

TABLE 1
Fractionation of Sugars in Hydrolyzate on Charcoal–Celite Layer

Eluent	*Yield*[a] (*g*)	R_{Gal} *values on paper chromatogram*[b]
Water	33	1·39, 1·34, 1·30, 1·27, 1·17, 1·13, glucose, galactose, 0·73, 0·66, 0·58
5 per cent ethanol	4·3	Glucose, galactose, 0·87, 0·80, 0·73, 0·66, 0·58–0·30[c]
15 per cent ethanol	3·9	0·47–0·07[c]
30 per cent ethanol	1·9	0·21–origin[c]
50 per cent ethanol	0·2	0·05–origin[c]

[a] Dry weight from 55 g initial α-lactose monohydrate.
[b] Mobility relative to galactose in *n*-butyl alcohol–pyridine–water (6:4:3), four ascents.
[c] Inseparable spot.

per cent *n*-propyl alcohol fraction contained only non-reducing sugars by ion-exchange chromatography in 75 per cent *n*-propyl alcohol (Adachi & Sugawara, 1963), anhydrosugars in this fraction were freed from glucose, galactose and oligosaccharides by this procedure. By the next preparative thin-layer chromatography four anhydrosugars were isolated. Their chromatographic characteristics and sugar composition are summarized in Table 2.

Fractions eluted from charcoal–Celite by 5 per cent ethanol in water contained oligosaccharides. Five fractions were then isolated by preparative paper chromatography. Characteristics of these on paper chromatograms

TABLE 2
Characteristics of Anhydrosugars

Anhydrosugar	R_{Gal} *on thin-layer chromatogram*[a]	$R_{TMS\ \alpha\text{-glucose}}$ *on gas chromatogram*[b]	$R_{TMSI,\ 6\text{-anhydro-}\beta\text{-D-galactopyranose}}$ *on gas chromatogram*[c]	*Sugar composition*
AG-1	3·03	0·57	1·16 (1·16)[d]	Galactose
AG-2	2·61	0·67	1·52 (1·49)	Glucose
AG-3	2·16	0·61	1·24 (1·24)	Glucose
AG-4	1·71	0·54	1·00 (1·00)	Galactose

[a] Mobility relative to galactose in *n*-butyl alcohol–isopropyl alcohol–water (10:5:4).
[b] Retention time relative to trimethylsilylated α-D-glucose on 1·5 per cent SE-52, 2 m, 145°C.
[c] Retention time relative to trimethylsilylated 1,6-anhydro-β-D-galactopyranose on 1·5 per cent SE-52, 2 m, 145°C.
[d] Literature value by Sawardeker *et al.* (1965) in parentheses.

and gas chromatograms and the carbohydrate composition of each are given in Table 3.

Although each of the oligosaccharide fractions A–E gave a single spot on paper chromatograms, these split into two to five peaks upon gas chromatography (Table 3). Structural elucidation was performed without further purification.

Structures of oligosaccharide fraction A

Gas chromatographic analysis of trifluoroacetylated reduced oligosaccharide fraction A revealed five peaks (A-1–A-5) (Table 3). Thus, oligosaccharide fraction A contained at least five reducing oligosaccharides.

When oligosaccharide fraction A was treated with glycosidases, only A-1 and A-3 were digested with β-glucosidase to yield glucose and galactose in

TABLE 3

Characteristics of Five Oligosaccharide Fractions Isolated from Lactose Hydrolyzate

Oligosaccharide fraction		*Oligosaccharide*			*Partial mass spectra (m/z) of permethylated reduced oligosaccharide*
Sign	R_{Gal} *on paper chromatogram*[a]	*Sign*	$R_{TFA\ lactitol}$[b]	$R_{perMe\ lactitol}$[c]	
A	0·87	A-1	0·91	1·19[d]	382, 338, 236, 219, 177, 134, 133, 89
		A-2	0·96		
		A-3	1·00		
		A-4	1·10		
		A-5	1·41		
B	0·80	B-1	0·95	0·99	236, 219, 134, 90, 89, 46, 45
		B-2	0·98	0·86	236, 219, 178, 134, 90, 46
		B-3	1·06	1·09	236, 219, 133, 90, 89, 46, 45
C	0·73	C-1	0·91	1·19	236, 219, 177, 133, 89, 59, 46, 45
		C-2	1·03	1·30	236, 219, 178, 134, 90, 46
		C-3	1·11	1·39	236, 219, 178, 134, 90, 46
		C-4	1·17	1·10	235, 219, 207, 147, 145, 127, 111, 101, 71, 45
D	0·66	D-1	0·94	1·30	236, 219, 178, 134, 90, 46
		D-2	1·02	1·27	236, 219, 178, 134, 90, 46
		D-3	1·09	1·41	236, 219, 178, 134, 90, 46
E	0·58	E-1	1·17[e]	1·13[f]	236, 219, 178, 134, 90, 46
		E-2	1·25[e]	1·25[f]	236, 219, 178, 134, 90, 46

[a] Mobility relative to galactose in *n*-butyl alcohol–pyridine–water (6:4:3), four ascents.
[b] Retention time relative to lactitol trifluoroacetate on 10 per cent SE-30, 1 m, 90–290°C (7·5°C/min).
[c] Retention time relative to permethylated lactitol on 3 per cent SE-30, 1 m, 195–280°C (5°C/min).
[d] A-1 to A-5 were not separated from each other.
[e] Retention time relative to trimethylsilylated α-lactose on 2 per cent SE-30, 3 m, 150–290°C (3°C/min).
[f] Retention time relative to permethylated lactitol on 2 per cent SE-30, 1 m, 140–240°C (5°C/min).

the molar ratios of 2·1:1·0. For some reason the others were not digested with any of the glycosidases.

However, permethylated reduced oligosaccharide fraction A gave a single peak by gas chromatography. Mass spectra of this indicated it to be a reducing disaccharide because of the presence of the fragments *m/z* 219 and 236 corresponding to non-reducing and reducing end hexose residues in the parent disaccharide, respectively (Table 3) (Kärkkäinen, 1970). The fragments *m/z* 382 (M^+—89), 338 (M^+—133), 134 and 293, which are characteristic of 1→2, 1→3, 1→4 and 1→5 linkages, respectively, were detected (Table 3).

Therefore, oligosaccharide fraction A is likely to be a mixture of at least five disaccharides which have 1→2, 1→3, 1→4 and 1→5 linkages.

Structures of oligosaccharide fraction B

Permethylated reduced oligosaccharide fraction B gave three peaks by gas chromatography (Table 3). The mass spectrum of each peak showed it to be a reducing disaccharide because of the presence of the fragments *m/z* 219 and 236 (Table 3). Complete acid hydrolysis of oligosaccharide fraction B gave glucose and galactose in the molar ratios of 1·2:1·0. When oligosaccharide fraction B was treated with glycosidases, only B-1 and B-3 were digested with β-glucosidase to yield glucose and galactose in the molar ratios of 1·2:1·0. Oligosaccharide B-2 was not digested with any of the glycosidases. The presence of the fragment *m/z* 134 in the mass spectrum of permethylated reduced oligosaccharide B-1 indicated it to have the 1→4 linkage (Table 3). The fragments *m/z* 134 and 178 in the mass spectrum of B-2 are characteristic of 1→6 linkages. The presence of the fragment *m/z* 133 in the mass spectrum of permethylated reduced oligosaccharide B-3 indicated it to have the 1→3 linkage (Table 3).

On the basis of the above results oligosaccharides B-1 and B-3 were established to be 4-*O*-β-D-glucopyranosyl-D-galactose and 3-*O*-β-D-glucopyranosyl-D-galactose, respectively. Oligosaccharide B-2 is likely to be a 1→6 linked disaccharide which consists of glucose and galactose.

Structures of oligosaccharide fraction C

Permethylated reduced oligosaccharide fraction C gave four peaks by gas chromatography (Table 3). The mass spectrum of each peak showed it to be a reducing disaccharide because of the presence of the fragments *m/z* 219 and 236 (with the exception of C-4) (Table 3).

Acid hydrolysis of oligosaccharide fraction C gave glucose and galactose in the molar ratios of 1·6:1·0. The ^{13}C-NMR signals at 105·5 and 100·7 ppm

TABLE 4
^{13}C-NMR Chemical Shifts[a] of the Anomeric Carbons of the Oligosaccharide Fraction

Oligosaccharide fraction	*Chemical shift*
C	105·5, 100·7, 98·9, 96·0, 95·0
D	105·4, 101·1, 99·3, 98·9, 95·2
E	106·1, 101·4, 99·3, 95·2

[a] ppm downfield from signal for 3-(trimethylsilyl) propionic acid sodium salt.

(Table 4) represented anomeric carbons of β and α anomers of the non-reducing glucose or galactose (Bock *et al.*, 1984). When oligosaccharide fraction C was treated with glycosidases, oligosaccharides C-1 and C-4 were hydrolyzed to galactose and glucose in the molar ratios of 2·1:1·0 with α-galactosidase. Oligosaccharide C-2 was hydrolyzed with α-glucosidase and gave glucose only. Oligosaccharide C-3 was hydrolyzed with β-glucosidase and gave glucose only.

The presence of the fragments *m/z* 177 and 133 in the mass spectrum of permethylated reduced oligosaccharide C-1 indicated it to have a $1 \rightarrow 2$ linkage (Table 3). The fragments *m/z* 46 and 178 in the mass spectra of permethylated reduced oligosaccharides C-2 and C-3 showed them to be $1 \rightarrow 6$ linked disaccharides (Table 3). The absence of fragment *m/z* 236 in permethylated reduced oligosaccharide C-4 indicated it to have no reducing end (Table 3).

On the basis of the above results the structures of oligosaccharide fraction C were established as: C-1,2-*O*-α-D-galactopyranosyl-D-glucose; C-2,6-*O*-α-D-glucopyranosyl-D-glucose (isomaltose); C-3, 6-*O*-β-D-glucopyranosyl-D-glucose (gentiobiose); C-4, α-D-galactopyranosyl-(1 $\longleftrightarrow$ 1)-α-D-galactopyranoside.

Structures of oligosaccharide fraction D

Permethylated reduced oligosaccharide fraction D gave three peaks by gas chromatography (Table 3). The mass spectrum of each peak showed it to be a reducing disaccharide because of the presence of the fragments *m/z* 219 and 236 (Table 3).

Acid hydrolysis of oligosaccharide fraction D gave glucose and galactose in the molar ratios of 0·8:1·0. The signals at 105·4 and 101·1 ppm (Table 4) represented anomeric carbons of β and α anomers of the non-reducing glucose or galactose (Bock *et al.*, 1984). When oligosaccharide fraction D

was treated with glycosidases, oligosaccharide D-1 was hydrolyzed to galactose and glucose in the molar ratios of 1·2:1·0 with α-galactosidase. Oligosaccharide D-2 was hydrolyzed to galactose and glucose in the molar ratios of 1·0:1·1 with α-glucosidase. Oligosaccharide D-3 was hydrolyzed with β-glucosidase and gave galactose and glucose in the molar ratios of 1·0:1·1.

The presence of the fragments *m/z* 178 and 134 in the mass spectra of permethylated reduced oligosaccharides D-1, D-2 and D-3 indicated all of them to have 1 → 6 linkages (Table 3).

On the basis of the above results the structures of oligosaccharide fraction D were established as: D-1, 6-*O*-α-D-galactopyranosyl-D-glucose (melibiose); D-2, 6-*O*-α-D-glucopyranosyl-D-galactose; D-3, 6-*O*-β-D-glucopyranosyl-D-galactose.

Structures of oligosaccharide fraction E

Permethylated reduced oligosaccharide fraction E gave two peaks by gas chromatography (Table 3). The mass spectrum of each peak showed it to be a reducing disaccharide because of the presence of the fragments *m/z* 219 and 236 (Table 3).

Acid hydrolysis of oligosaccharide fraction E gave galactose only. The signals at 106·1 and 101·4 ppm (Table 4) represent anomeric carbons of β and α anomers of the non-reducing glucose or galactose residues (Bock *et al.*, 1984). When oligosaccharide fraction E was treated with glycosidases, oligosaccharide E-1 was hydrolyzed to galactose with α-galactosidase. Oligosaccharide E-2 was hydrolyzed to galactose with β-galactosidase.

The presence of the fragments *m/z* 178 and 134 in the mass spectra of permethylated reduced oligosaccharides E-1 and E-2 indicated both of them to have 1 → 6 linkages (Table 3).

On the basis of the above results the structures of oligosaccharide fraction E were established as: E-1, 6-*O*-α-D-galactopyranosyl-D-galactose; E-2, 6-*O*-β-D-galactopyranosyl-D-galactose.

Structure of anhydrosugars

Table 2 summarizes chromatographic properties and sugar composition of AG-1, AG-2, AG-3 and AG-4; AG-4 gave galactose only on acid hydrolysis. The ^{13}C-NMR spectrum of AG-4 was identical with that of authentic 1,6-anhydro-β-D-galactopyranose (Table 5). Thus, AG-4 was identified to be 1,6-anhydro-β-D-galactopyranose (levogalactosan).

AG-1, AG-2 and AG-3 gave galactose, glucose and glucose on hydrolysis,

TABLE 5
^{13}C-NMR Chemical Shifts[a] of Anhydrosugar AG-4 and Its Assignments

Anhydrosugar	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
AG-4	103·4	74·0	73·0	67·0	77·1	66·3
1,6-anhydro-β-D-galactopyranose	103·6	74·1	73·0	67·1	77·2	66·4

[a] ppm downfield from signal for 3-(trimethylsilyl) propionic acid sodium salt.

respectively. Relative retention times of AG-1, AG-2 and AG-3 to 1,6-anhydro-β-D-galactopyranose agreed with literature values of 1,6-anhydro-α-D-galactofuranose, 1,6-anhydro-β-D-glucofuranose and 1,6-anhydro-β-D-glucopyranose (levoglucosan), respectively (Sawardeker *et al.*, 1965) (Table 2).

DISCUSSION

It is well known ('reversion') that oligosaccharides are formed in solution from monosaccharides by the action of mineral acid (Staněk *et al.*, 1965). Formation of disaccharides having a variety of linkages with α- and β-anomeric configurations has been reported. The same phenomena were observed in maltose, sucrose, lactose and starch during acidic hydrolysis. There are, however, no reports on structural elucidation of oligosaccharides from lactose. As shown in this report complicated oligosaccharide mixtures were formed from lactose by the action of hydrochloric acid. It is very difficult to isolate each oligosaccharide by usually available laboratory techniques. Thus, we have developed a protocol for structural elucidation of disaccharide mixtures without isolation.

In the present paper structural elucidation of 11 oligosaccharides and one anhydrosugar in the hydrolyzate of lactose with hydrochloric acid was carried out. Among these disaccharides isomaltose and gentiobiose were known to be formed from glucose, and 6-*O*-α-D-galactopyranosyl-D-galactose and 6-*O*-β-D-galactopyranosyl-D-galactose were from galactose (Staněk *et al.*, 1965). The others have not yet been reported. A (1 → 6)-linkage is likely to be formed preferentially because eight of the 11 disaccharides have it. Preferential formation of isomaltose and gentiobiose with the (1 → 6)-linkage was also reported in the acid reversion from glucose (Staněk *et al.*, 1965).

In this paper, of four anhydrosugars one is confirmed to be 1,6-anhydro-β-anhydro-β-D-galactopyranose; the others are likely to be 1,6-anhydro-α-D-galactofuranose, 1,6-anhydro-β-D-glucofuranose and 1,6-anhydro-β-D-glucopyranose. There are no reports on formation of

anhydrosugars during acid hydrolysis of lactose. Only 1,6-anhydro-β-D-glucopyranose is reported to be formed in glucose solution with acid (Staněk *et al.*, 1965).

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