Isoraffinose (6^G- β -galactosylsucrose) Synthesized by the Intermolecular Transgalactosylation Reaction of *Escherichia coli* β -Galactosidase

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Received February 13, 1986

The enzymatic transgalactosylation reaction of *O*-nitrophenyl- β -D-galactoside and lactose in the presence of sucrose as an acceptor was studied. The main product of *E. coli* β -galactosidase was a hitherto undescribed trisaccharide, *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\leftrightarrow$ 2)-*O*- β -D-fructofuranoside, to which we assigned the trivial name isoraffinose. Sucrose was an excellent acceptor to accumulate isoraffinose in good yield. Allolactose and trace of unidentified oligosaccharides were also formed by this reaction.

β -Galactosidase [β -D-galactoside galactohydrolase, EC 3.2.1.23] is present in a wide variety of sources including plants, animals, and microorganisms.¹⁾ Of these β -galactosidase, the enzyme from *Escherichia coli* is readily isolated in large amounts, and many of its physical and chemical characteristics are known.^{2~5)} β -Galactosidase catalyzed two reactions toward glycosides or oligosaccharides.^{6,7)} One is hydrolysis to their individual constituents. The other is transgalactosylation,^{6~9)} transferring the galactose moiety of the substrate to an acceptor molecule. Either intramolecular or intermolecular transgalactosylation reactions can occur. When the natural substrate, lactose is acted upon by the *E. coli* β -galactosidase, Huber *et al.*⁷⁾ showed that approximately 50% of the molecules are converted by intramolecular transgalactosylation to allolactose (*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucose: independent of the initial lactose concentration), while about 50% is either hydrolytic products or intermolecular transgalactosylation products (depending on the initial lactose concentration). In the presence of sugar or alcohol as an acceptor for the intermolecular transgalactosylation reaction of lactose by *E. coli* β -galac-

tosidase, Huber *et al.*¹⁰⁾ suggested that the ability for the intermolecular transgalactosylation reaction is highly dependent on the structure of the hydroxyl acceptor.

In the presence of sucrose as an acceptor with lactose as a substrate, with growing cultures of *Leuconostoc mesenteroides*, Aso and Yamauchi¹¹⁾ have reported the production of lactosucrose, *i.e.*, 4^G- β -galactosylsucrose. Suzuki and Hehre¹²⁾ have reported the production of lactulosucrose, *i.e.*, 4^F- β -galactosylsucrose. However, there have been no reports the production of trisaccharide by the intermolecular transgalactosylation reaction of the enzyme with sucrose as an acceptor and lactose or *O*-nitrophenyl- β -galactoside (ONPG) as substrates.

We can now report that the product formed by the intermolecular transgalactosylation reaction of *E. coli* β -galactosidase is, in fact, neither lactosucrose nor lactulosucrose but a hitherto undescribed trisaccharide, 6^G- β -galactosylsucrose, to which we assigned the trivial name isoraffinose.

MATERIALS AND METHODS

Materials. Raffinose, lactose, melibiose, sucrose, D-

glucose, D-fructose, D-galactose, and *O*-nitrophenyl- β -D-galactoside (ONPG) were purchased from Wako Pure Chemical Industries, Ltd. Allolactose was enzymatically synthesized as shown in our previous report.¹³⁾ A trimethylsilylation reagent (TMSI-H) containing hexamethyldisilazane and trimethylchlorosilane in pyridine was from a commercial source (Gasukuro Kogyo Inc., Ltd.). All other chemicals were of analytical grade, and purchased either from Wako Pure Chemical Industries, Ltd., or from Tokyo Kasei Ltd.

Enzyme source. Both β -galactosidase from *E. coli* (560 ONPG unit/mg protein) and β -fructosidase from yeast (280 unit/mg protein), lyophilized, were from Boehringer Mannheim Ltd.

Transgalactosylation reaction. The following two experiments were done; (I) eight ml of a reaction mixture of 0.1 M McIlvaine buffer, pH 7.0, containing 1.6 g of sucrose, 0.8 g of ONPG, and 300 ONPG units of *E. coli* β -galactosidase was incubated at 30°C for 5 hr, or (II) 2 g of sucrose, 2 g of lactose, and 160 units of *E. coli* β -galactosidase, in 8 ml of 0.1 M McIlvaine buffer (pH 7.0) were incubated at 30°C for 5 hr or 24 hr. After incubation, the reaction mixtures were heated in boiling water to end the reaction. Both reaction mixtures were examined at intervals for 24 hr on TLC.

Hydrolysis. Chromatographically homogeneous isolated trisaccharide was kept in boiling 1 N sulfuric acid for 1 hr for complete hydrolysis, and 0.5 N trifluoroacetic acid at 60°C for 2 hr for partial hydrolysis. Both reaction mixtures were treated with Amberlite IRA-410 (OH⁻ form), and then evaporated to a syrup *in vacuo*. Trisaccharides were hydrolyzed enzymatically by dissolving 2 mg of trisaccharide in 1 ml of 0.05 M McIlvaine buffer (pH 7.0) to which 5 units of β -fructosidase or 10 ONPG units of β -galactosidase and a small drop of toluene were added, incubating at 30°C for 5 hr, and then heating at 95°C for 5 min. Both hydrolyzates were dried under a stream of nitrogen to a solid.

Chromatography. Thin-layer chromatography (TLC) was done by a multiple ascending technique (two times) on silica gel TLC (Merck; precoated on an aluminum sheet Art 5553) in a solvent system of ethylacetate-acetic acid-water (2:1:1, by vol.). Sugars were detected by spraying with 5% H₂SO₄-CH₃OH and heating at 150°C for 5 min.

The crude mixture of sugars was fractionated on a gel filtration column (2.5 × 82 cm) packed with Toyopearl HW-40 Fine (Toyo Soda, MFG., Co., Ltd.). The column was eluted with deaerated distilled water at a flow rate of 15 ml/hr. The sugar in each fraction was measured by the phenol-sulfuric acid method.

Sugars were converted to trimethylsilyl (TMS) ethers by TMSI-H reagent, and then used in gas-liquid chromatography (GLC) on a Hitachi model 163 gas chromatograph

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 temperature was programmed from 130°C to 350°C at 10°C/min.

Fast-atom bombardment (f.a.b.) mass spectrometry. F.a.b. 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 μ g), dissolved in 10 μ l of water, was added to the glycerol matrix.

¹³C-NMR. ¹³C-NMR spectra were obtained in D₂O at 25.00 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.

RESULTS AND DISCUSSION

Isolation of transgalactosylation reaction products

To remove excess ONPG in the ONPG-sucrose system, the reaction mixture was treated with 5 ml of ethyl ether twice. The aqueous fraction thus obtained was chromatographed on a gel filtration column. The elution pattern is shown in Fig. 1. The first peak of effluent containing a nonreducing trisaccharide, which was abbreviated as GS, and a small amount of unidentified tetrasaccharide, was combined and concentrated to syrup using rotary evaporator. A large amount of sugar was also eluted as the second peak Fig. 1. The sugar in the second peak consisted of sucrose and a negligible amount of trisaccharide. The syrup from the first peak was further purified by TLC on an aluminium sheet. The zone corresponding to the spot of GS was cut out of the chromatogram. GS was extracted with hot water from the strips and then the aqueous extract was evaporated to syrup. The reaction mixture of the lactose-sucrose system was put on TLC. The zone corresponding to the spot of GS was also cut out of the chromatogram. GS was extracted and evaporated to syrup.

FIG. 1. Elution Profile of Transgalactosylation Reaction Products of the ONPG-Sucrose System from a Toyopearl HW-40 Fine.

FIG. 2. GLC of TMS-derivatives of β -Galactosidase (A) and β -Fructosidase (B) Hydrolyzates of GS.

Characteristics of GS

Both GSs isolated from the reaction mixtures of ONPG-sucrose and lactose-sucrose systems by preparative TLC gave single peaks on GLC and single spots on TLC. GS was a nonreducing sugar which was negative to Fehling reagent. $[\alpha]_D^{20}$: $+41^\circ$ ($c=5.0$, H_2O). Calculated for $C_{18}H_{32}O_{16}$ (as trisaccharide): C, 42.9; H, 6.4. Found: C, 42.3; H, 6.7 (%). It yielded equimolar parts of galactose, glucose, and fructose (1:0.9:0.7:identified by TLC and GLC) by complete acid hydrolysis. Hydrolysis with acid under mild conditions (partial hydrolysis), or yeast β -fructosidase

yielded fructose and allolactose. Hydrolysis with β -galactosidase yielded galactose and sucrose. Figure 2 shows the GLC of TMS-derivatives of β -fructosidase and β -galactosidase hydrolyzates. The retention times for the TMS-derivatives of sugars in the hydrolyzate agreed well with those of galactose, fructose, sucrose, and authentic allolactose. The results on GLC suggested that both sucrose and allolactose were a part of the structure of this trisaccharide, GS.

F.a.b. mass spectrometry (positive ion) was done on the GS. Figure 3 shows the region of the quasimolecular ions $M+Na^+$ at 400~600

a.m.u. We concluded that the molecular weight of GS is 504, as a trisaccharide.

^{13}C -NMR was used for further elucidation of the structure of GS. Figure 4 shows the ^{13}C -NMR spectrum of GS. The chemical shifts and numbering of each sugar unit are given in Table I. Resonances of GS were assigned by corresponding reference sugars of sucrose, allolactose, melibiose, and raffinose. The signal at 106.3 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 galactose unit of allolactose (106.1 ppm) and discrepancy with that of α -D-form of galactose unit of melibiose and raffinose (101.0 and 101.2 ppm, respectively). The anomeric configuration of the galactose unit of GS was therefore β -D-form. The signal at 94.8 ppm could be also clearly assigned to the anomeric carbon of the glucose unit as the α -D-form, by analogy with the chemical shift of the α -D-form of glucose units of raffinose and sucrose (94.7 and 94.9 ppm, respectively).

Signals of 64.2 and 106.4 ppm were assigned to the C-1 and C-2 of the fructofuranose unit of GS, by analogy with the chemical shifts of raffinose and sucrose (Table I).

On the basis of all the experimental results, it seemed most reasonable to conclude that GS is 6^G- β -galactosylsucrose, that is, *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-glucopyranosyl-(1 $\leftrightarrow$ 2)- β -D-fructofuranoside. The new trisaccharide was named isoraffinose.

FIG. 3. The Quasimolecular-ion Region of GS ($M + \text{Na}^+ = 527$) in F.a.b.-Mass Spectrum.

The composition of transgalactosylation products

Figures 5 and 6 show GLC of TMS-deriv-

FIG. 4. ^{13}C -NMR Spectrum of GS.

TABLE I. ^{13}C -NMR DATA FOR ISORAFFINOSE AND RELATED SACCHARIDES, RAFFINOSE, SUCROSE, ALLOLACTOSE, AND MELIBIOSE

		C-1	C-2	C-3	C-4	C-5	C-6
GS (Isoraffinose)	galp	106.3	73.6	75.5	71.5	77.9	63.9
	glup	94.8	73.8	75.3	72.2	74.4	71.5
	fruf	64.2	106.4	79.3	76.8	84.2	65.4
Raffinose	galp	101.2	71.2	72.2	72.0	73.7	63.9
	glup	94.8	73.7	75.4	72.2	74.1	68.7
	fruf	64.2	106.5	79.2	76.8	84.1	65.2
Sucrose	glup	94.9	73.8	75.4	72.0	75.1	63.0
	fruf	64.2	106.5	79.3	76.8	84.1	65.2
Allolactose	galp	106.1	73.6	75.5	71.5	78.0	63.8
	α -glup	94.9	74.2	75.5	72.3	73.3	71.5
	β -glup	98.8	76.9	78.5	72.3	77.7	71.5
Melibiose	galp	101.0	72.0	73.7	71.3	72.3	63.9
	α -glup	95.0	74.2	75.8	72.3	72.9	68.7
	β -glup	98.9	77.0	78.7	72.4	77.2	68.8

FIG. 5. GLC of TMS-derivatives of Transgalactosylation Reaction Products of the ONPG-Sucrose System.

FIG. 6. GLC of TMS-derivatives of Transgalactosylation Reaction Products of the Lactose-Sucrose System.

atives of sugars in the reaction mixtures of the ONPG-sucrose system and the lactose-sucrose system incubated for 5 hr, respectively. As can be seen from Fig. 5, in the ONPG-sucrose system, the amount of free galactose was very small compared with that of isoraffinose (peak area of isoraffinose was about 8 times larger than that of galactose). And in the lactose-sucrose system, glucose and traces of galactose and fructose were found as monosaccharides, and both allolactose and isoraffinose accumulated during the transgalactosylation.

Peaks of traces of unidentified oligosaccharides were also found as shown on GLC.

The composition of sugars of the reactants obtained by incubation of the lactose-sucrose system for 5 and 24 hr under the conditions described in the EXPERIMENTAL section is summarized in Table II. At the incubation

TABLE II. SUGARS COMPOSITION (%) OF THE ENZYMIC TRANSGALACTOSIDATION REACTION PRODUCTS

	5 hr*	24 hr*
Galactose	—	Trace
Glucose	8.5	22.8
Sucrose	45.9	39.4
Lactose	35.8	8.1
Allolactose	4.4	8.9
Isoraffinose	4.9	17.0
Others	0.3	2.5

* Incubation time

Experimental details are given in the text.

period of 24 hr, lactose was transferred to galactose (trace), and glucose (22.8%), allolactose (8.9%), isoraffinose (17.0%), and others (2.5%; included unidentified trisaccharide and tetrasaccharide).

For the transgalactosylation reaction of lactose using β -galactosidase of *E. coli*, Huber *et al.*⁷⁾ showed that the main reaction products at low initial concentrations of lactose were the hydrolyzates, galactose and glucose (53%), and the intramolecular transgalactosylation reaction product, allolactose (43%), and the accumulation of trisaccharides, so called galactosyl-lactose, was very low.

The mechanism of action and reaction kinetics with natural and synthetic substrates of *E. coli* β -galactosidase have been studied quite extensively.^{3,7,10,15,16)} From these articles, β -galactosidase is known to have two subsites for binding, one for galactose and the other for glucose, the two components of lactose. When the enzyme has caused the breakage of the glycosidic bond and the aglycone moiety has diffused away, the enzyme is in the galactosyl form, because of the "galactose" site has quite good affinity for galactose. Then acceptors bind and interact at the glucose site of the galactosyl form of the enzyme to form the transgalactosylation reaction product.

For the intramolecular transfer reaction of lactose, Huber *et al.*¹⁰⁾ suggested that the glucose site of the galactosyl form of β -galactosidase becomes highly specific for binding

glucose after the glycosidic bond is broken, and it is best to bind glucose more tightly at the 6-hydroxyl group, not the 4- or other hydroxyl group, because it is in close contact with the active galactose. For the intermolecular transfer reaction, Huber and Gaunt¹⁴⁾ suggested that definite specificities for binding and for productive formation of transgalactosylation reaction products were found at the glucose site of β -galactosidase.

Considering our results and those findings, sucrose would be a excellent acceptor in terms of binding, and sucrose would be highly reactive compared to other sugars such as lactose or galactose. With free glucose as an acceptor, Huber *et al.*⁷⁾ showed that there was significantly more allolactose produced than when no glucose was present. This indicates that not only glucose but also sucrose can substitute easily for bound glucose (from lactose which is being acted on by β -galactosidase).

Accumulation of isoraffinose and allolactose rather than hydrolysis of lactose by the transgalactosylation reaction of *E. coli* β -galactosidase in lactose-sucrose system might coincide with the fact that *E. coli* has difficulty in consuming these di- and trisaccharides.

On the other hand, the beneficial role of bifidobacteria in the human intestinal flora have been elucidated. It is known that some sugars promote the growth of bifidobacteria. In these sugars, Tamura¹⁷⁾ showed 4^G- β -galactosylsucrose was one of the excellent sugar sources for bifidobacteria. 6^G- β -Galactosylsucrose, isoraffinose, may also have this effect.

Acknowledgment. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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