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Note

Trehalose as a common industrial fermentation byproduct

Steven R. Hull [a], James S.S. Gray [b], Theodore A.W. Koerner [a,b], Rex Montgomery [b,*]

[a] *Department of Pathology, University of Iowa, Iowa City, IA 52242, USA*

[b] *Department of Biochemistry, 4-611 Bowen Science Building, University of Iowa, Iowa City, IA 52242, USA*

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During the analysis of several industrial fermentation byproduct streams significant amounts of α,α-trehalose and α,β-isomaltose were found. These disaccharides were purified from one of the fermentation-byproduct streams and identified by chromatography and 600-MHz ^{1}H NMR. Examination of fermentation streams before and after the addition of active organisms established that α,α-trehalose is a product of the fermentation and α,β-isomaltose is a component of the starting nutrients.

Analysis of a number of agribusiness industrial-byproduct streams has had the objective of identifying compounds of potential value for isolation, or determing those that may be toxic to microorganisms in the anaerobic methanogenesis of organic compounds in the streams. The frequent occurrence of two disaccharides was noted in those streams from fermentation processes and prompted this study.

1. Results and discussion

Two unknown carbohydrates, **1** and **2**, after isolation from a fermentation-byproduct stream by sequential chromatography over charcoal–Celite and Bio-gel P2, were found to be 90–95% pure by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (Fig. 1) and gas–liquid chromatography of the trimethylsilyl

* Corresponding author.

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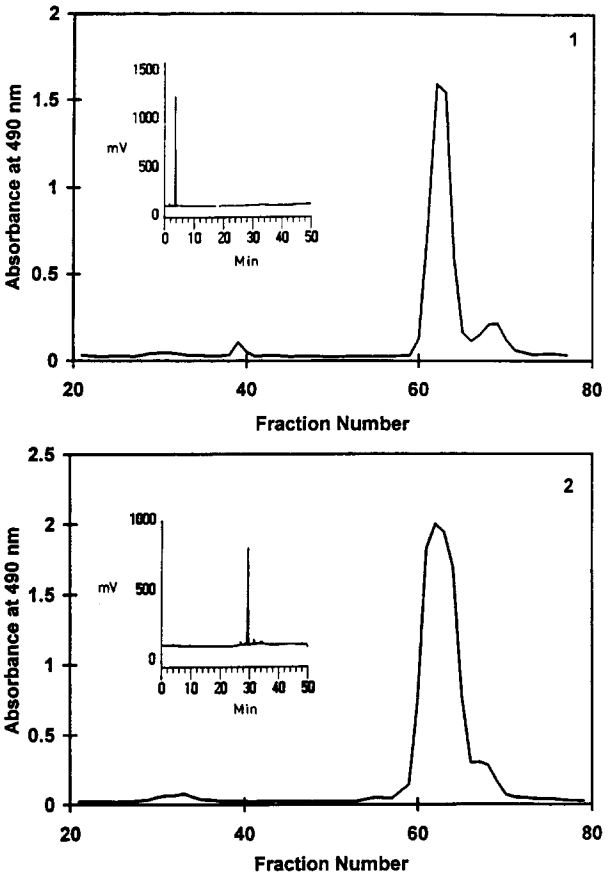

Fig. 1. The two unknown saccharides, **1** (upper panel) and **2** (lower panel), after their partial purification over carbon were further purified to homogeneity by passage through a column (1×90 cm) of Bio-gel P2 (>400 mesh) eluted with water. The purity of the peak fractions was ascertained by HPAEC–PAD analysis (inset).

ethers (Fig. 2). Each compound was composed only of glucose, **1** being nonreducing and **2** a reducing sugar.

^{1}H NMR spectroscopy at 600 MHz identified **1** as α,α-trehalose and **2** as α,β-isomaltose, the methylene and methine proton chemical shifts (Table 1) and vicinal coupling constants (Table 2) being established by COSY-45 connectivity experiments. The NMR spectrum for α,α-trehalose was clearly indicative of two identical α-D-glucosyl residues in a molecule with a plane of symmetry. The data are in agreement with that published earlier [1] at 300 MHz and at 270 MHz when corrected for the acetone standard assignment [2].

The NMR spectrum for the anomeric mixture of isomaltose (36% α; 64% β) was similar to that reported earlier [3] for methyl β-isomaltoside at 500 MHz and 300 MHz for the disaccharide [1]. Precise data were obtained for the chemical shifts of all the protons in isomaltose, thus clarifying some earlier data.

Of thirty streams analyzed from fermentation processes, α,α-trehalose was identified in sixteen. Nine of these streams (0.1–1.5 g/L trehalose) were from corn wet-milling steeping in which *Lactobacillus* spp. were predominant, four (0.3–8.0 g/L trehalose) were from a

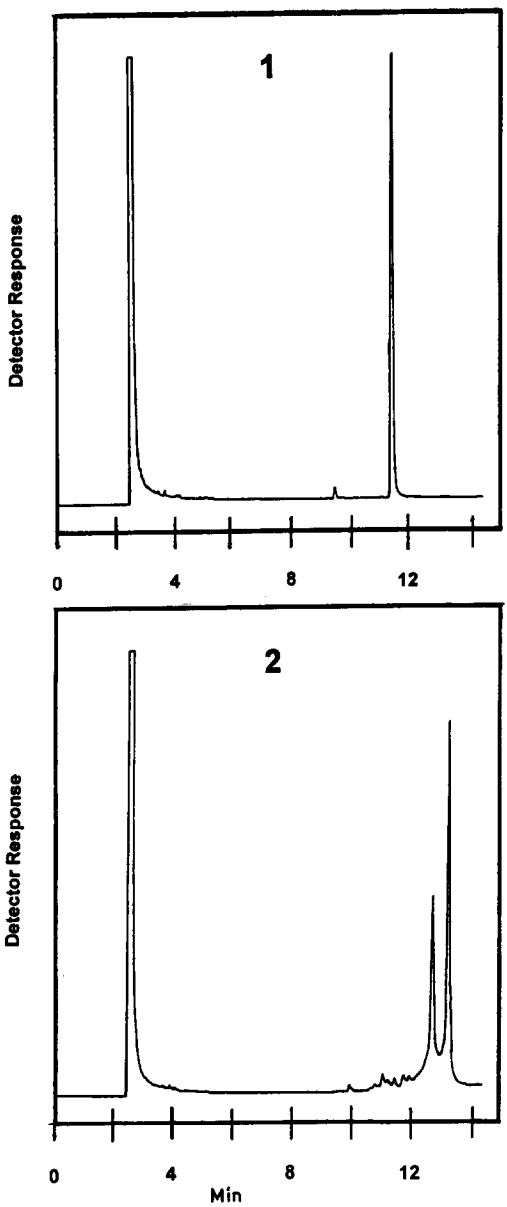

Fig. 2. Equal amounts of authentic trehalose and unknown saccharide **1** (upper panel) and authentic isomaltose and unknown saccharide **2** (lower panel) were analyzed by GLC after trimethylsilyl derivatization as described in Methods.

lysine production process utilizing a proprietary recombinant microorganism, and two (0.5 and 4.5 g/L trehalose) were from an *Aspergillus niger* fermentation producing citric acid.

To delineate the possible source of these disaccharides, the trehalose and isomaltose in the starting material just prior to the addition of the active microorganism was compared

Table 1
Chemical shifts [a] for methine and methylene protons of α,α-trehalose (**1**) and α,β-isomaltose (**2**)

Compound	Residue	Proton chemical shifts (ppm)						
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
1	I or I′	5.191	3.643	3.847	3.444	3.820	3.756	3.856
2	Iα	5.239	3.525	3.705	3.990	3.525	3.697	3.997
	Iβ	4.671	3.252	3.478	3.518	3.638	3.760	3.954
	IIα	4.947	3.554	3.734	3.425	3.734	3.770	3.849
	IIβ	4.954	3.554	3.734	3.425	3.734	3.770	3.849

[a] Obtained at 600 MHz and 298 K in D_2O, referenced to internal acetone at 2.225 ppm (error ± 0.001 ppm).

with the concentrations of these two disaccharides after the fermentation process. The glucose starting material contained isomaltose (3.37 g/L) but no trehalose and the sterilized media just prior to the addition of glucose and the microbial cells contained trehalose (0.01 g/L) but no isomaltose. After the fermentation the main fermentor broth before separation of the cells contained trehalose (0.25 g/L) and isomaltose (1.76 g/L) compared to the liquor after cell separation in which there was found trehalose (0.33 g/L) and isomaltose (1.11 g/L). These analyses demonstrate a 20–30-fold increase in α,α-trehalose after the fermentation process and a two-fold decrease in α,β-isomaltose. These data support the conclusion that the trehalose and not the isomaltose is a co-product of the fermentation process..

2. Experimental

Methods.—The byproducts streams were centrifuged (100*g*, 15 min) and filtered to give a clear liquid that was stored at −20°C in glass containers. Aliquots of each stream were chromatographed on charcoal–Celite according to the procedure of Whistler and Durso [4],

Table 2
Apparent vicinal coupling constants [a] for methine and methylene protons of α,α-trehalose (**1**) and α,β-isomaltose (**2**)

Compound	Residue	Proton–proton coupling constants (Hz)						
		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
1	I or I′	3.8	10.0	9.3	9.9	5.3	2.1	−12.1
2	Iα	2.5	9.7	8.2	8.2	[b]	<2.0	−9.5
	Iβ	8.0	9.1	9.3	9.5	2.0	4.7	−11.3
	IIα	3.8	9.8	9.5	9.5	[b]	3.8	−10.2
	IIβ	3.7	9.8	9.5	9.5	[b]	3.8	−10.2

[a] Error estimated to be ± 0.3 Hz. [b] Obscured by other resonances.

fractions from elution with water and then aq 5% EtOH were collected. Analysis of each fraction by phenol–H_2SO_4 [5] and HPAEC–PAD [6] directed the collection of those fractions containing the unknown sugars. Unknown **1** eluted with water and **2** with 5% EtOH. The fractions were further purified by Bio-gel P2 (−400 mesh) chromatography (1.5×80 cm), eluting with water.

High pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD).—Identification and quantitation of the monosaccharide and oligosaccharide composition of samples was accomplished by HPAEC–PAD using a BioLC system from Dionex. The gradient utilized for the separation of mono- and oligo-saccharides was the following: The first 20 min of the gradient was isocratic at 20 mM NaOH, after which followed a 30 min gradient in which a concentration of NaOAc from 0–500 mM was applied while the concentration of NaOH remained constant at 100 mM. Using this gradient, a standard mixture containing various monosaccharides and the malto-oligsaccharides up to maltoheptaose was conveniently separated. Such a standard mixture was analyzed on a weekly basis in order to calibrate the column and obtain updated response factors for each standard sugar.

Gas–liquid chromatographic analysis.—Trimethylsilyl (Me_3Si) derivatives of disaccharides were prepared by the addition of 200 μL of bis(trimethylsilyl)acetamide–10% Me_3SiCl to the rigorously dried samples. The mixture was heated at 40°C for 30 min. A sample (1–2 μL) of the mixture was analyzed by GLC with an OV-1 capillary column (0.2 mm×30 M) utilizing the following temperature gradient: $T_{0\ min}$ 240°C, $T_{3\ min}$ 240°C, $T_{15\ min}$ 300°C. The carrier gas was He and its flow rate was set to 24 mL/min in order to maintain a split flow of 1 mL/min through the column. Injection and detector ports were maintained at 250°C. The GLC system used was a Hewlett–Packard 5890 equipped with a flame ionization detector.

600-MHz ^1H NMR analysis.—Samples (1–5 mg) were exchanged twice with D_2O before final dissolution in D_2O (100%, Merck). Just before analysis a trace of acetone was added as a reference (2.225 ppm) in a 5-mm NMR tube (Wilmad). Spectra were obtained at 600 MHz at 298 K on a Bruker AMX-600 spectrometer (University of Iowa High Field Nuclear Magnetic Resonance facility) using UXNMR software and a Fourier-transform program (up to 200 scans). Spectral connectivities were obtained using the program COSY-45.

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