Specific Method for Quantitative Measurement of the Total Dextran Content of Raw Sugar

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A new method for measurement of the total dextran content of raw sugar has been developed which utilizes a combination of enzymic hydrolysis and reverse-phase high-performance liquid chromatography (HPLC). Initially, the dextran is separated from the low molecular weight sugars by precipitation with alcohol to 80% (v/v). Thereafter, enzymic hydrolysis is carried out with *Chaetomium gracile* dextranase and the major product, isomaltose, measured by HPLC. Dextran levels are calculated from the isomaltose content, which is a constant value (68% w/w) for the dextrans found in the sugar industry. With respect to the present enzyme–HPLC method, the alcohol haze procedure underestimates, whereas the Roberts' copper procedure significantly overestimates, dextran levels in raw sugar.

INTRODUCTION

Dextran is the collective name given to a large class of bacterial exopolysaccharides composed exclusively of the monomeric unit α-D-glucopyranose linked predominantly by 1→6 glycosidic bonds. Depending on the microbial source, dextrans exhibit considerable structural variation (Jeanes et al., 1954). Linear dextrans containing up to 97% α, 1→6 glycosidic bonds and highly branched dextrans comprising up to 50% non-α, 1→6 linkages, mainly α, 1→3, have been isolated (Walker, 1978).

Dextran-producing bacteria belong to the genera *Lactobacillus*, *Leuconostoc*, and *Streptococcus* (Jeanes, 1966). Within the cane sugar industry, these bacteria appear to be restricted to *Leuconostoc mesenteroides*, *L. dextranicum*, and some *Lactobacillus* spp. (Foster et al., 1980; Lillehoj et al., 1984; DeStefano and Irey, 1986). Consequently, all highly purified dextrans isolated from sugar cane products (designated cane dextrans) were found to possess a very similar structure, viz. 95% α, 1→6 linkages and 5% branching probability α, 1→3 bonds (Covacevich and Richards, 1977a,b; Foster et al., 1980). Cane dextrans are therefore structurally similar to the dextrans produced by *L. mesenteroides* strain NRRL B-512 (Walker, 1978). However, enzymic studies suggested that the branching pattern in cane dextrans is different from that in the NRRL B-512 dextrans (Inkerman, 1980).

Cane dextrans isolated from deteriorated cane juices and raw sugars possess a weight-average molecular weight of about 5×10^6 (Inkerman, 1980) and are polydisperse (Covacevich and Richards, 1977a,b; Inkerman, 1980; Curtin and McCowage, 1986). Most of the major processing problems associated with dextran, both in the raw sugar factory and in the refinery, are due to this large molecular size. The overall effect on processing is a marked decrease in sucrose recovery, reduced factory and refinery efficiency, and lower quality of the final products (Imrie and Tilbury, 1972). Thus, sugar industries throughout the world are concerned at the presence of dextran.

A number of different methods are available for the analysis of dextran (Curtin, 1988; Sarkar et al., 1990), although none currently holds any official status within the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) (Goodacre, 1986). To date, only the alcohol haze (Keniry et al., 1969) and the Roberts' copper (Roberts, 1983) methods have been widely used within the world's sugar industries. The former is based on the haze developed in a 50% (v/v) ethanol solution following enzymic removal of starch, elimination of interfering salts by ion-exchange resins, and the precipitation of proteins by trichloroacetic acid. The method has been criticized because it is not quantitatively sensitive to dextran of molecular weight (MW) below 10^5, is not specific for dextran, and is not sensitive at dextran concentrations of less than 200 ppm on solids (DeStefano and Irey, 1986; Curtin and McCowage, 1986). Criticisms of the alcohol haze method led to the development of other procedures including that of Roberts (1983). This procedure entails precipitation of oligo- and polysaccharides with ethanol to 80% (v/v), removal of the sucrose by washing with the same ethanol solution, redissolving the precipitate in water followed by "selective precipitation" of the dextran with alkaline copper sulfate, and subsequent quantitation with the phenol–sulfuric acid reagent. Although possessing a greater sensitivity than the alcohol haze procedure, the methodology is more complex and requires the complete removal of sucrose from the dextran precipitate prior to color development. More importantly, recent studies have demonstrated that the Roberts' copper reagent is not specific for dextran (DeStefano and Irey, 1986).

Because of the problems associated with the current analytical procedures outlined above, work was undertaken to develop a quantitative method for total dextran in raw sugar. This paper outlines an analytical procedure which uses a combination of enzymic hydrolysis (to ensure specificity for dextrans) and HPLC separation and quantitation of products to give a specific and quantitative measure of dextran. The results of the enzyme–HPLC method are then compared with the results of the alcohol haze and Roberts' copper methods for the analysis of dextran in a number of raw sugar samples.

MATERIALS AND METHODS

All chemicals were the best available analytical grade reagents and were supplied by Ajax Fine Chemicals (Auburn, Australia) unless noted otherwise. Absolute ethanol (CSR Ltd., Sydney, Australia) was used as supplied or aqueous solutions were prepared by dilution (v/v) with distilled water. Ultrapure ammonium sulfate (enzyme grade) was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Potato starch and (carboxymethyl)cellulose (high viscosity) were purchased from BDH Chemicals Ltd. (Poole, England). Arabinogalactan and

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cellulose were purified from sugar cane by Drs. J. D. Blake and K. E. McNeil, respectively, of the Sugar Research Institute. Pullulan ($\bar{M}_n$ 8.6×10^4) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). The standard sugars α-D-glucose, isomaltose, (*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose), isomaltotriose (*O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→6)-D-glucopyranose), and stachyose (*O*-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) were obtained from the Sigma Chemical Co. (St. Louis, MO). Mixtures of linear (IM_2–IM_6; IM_x refers to a linear oligosaccharide of the isomaltose series containing x D-glucose residues) and branched (B_4, B_5; isomaltotetrose and higher homologues containing a single secondary linkage are indicated by B_x) isomaltooligosaccharides were kindly supplied by Dr. G. J. Walker, University of New South Wales. Dextran T10, dextran T2000, and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Both dextrans and standard sugars were used without further purification. Native dextran (MW ~ 40×10^6), a gift from Dr. K. Williams of Pharmacia, is produced by the NRRL B-512 strain of *L. mesenteroides* and used by Pharmacia to prepare the T-fraction dextrans. Cane dextrans were isolated from the first-expressed juice extracted from deteriorated sugar cane billets obtained from three major sugar growing areas in Australia (Mackay, Tully, and South Johnstone) by precipitation with alcohol (50% v/v). Raw sugars were commercial samples obtained from Australian sugar factories.

Acid-washed Celite was prepared by adding 50 mL of concentrated hydrochloric acid to 50 g of Celite (Hyflo Super-cel; Johns-Manville, Denver, CO) suspended in 1 L of distilled water. The suspension was stirred for 5 min, collected by vacuum filtration on a Whatman No. 42 filter paper, and washed free of acid with distilled water; the cake was dried for 16 h at 104 °C. Mixed bed ion-exchange resin (Amberlite MB-1, BDH) was washed with distilled water followed by acetone and air-dried at room temperature.

Dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11), isolated from the thermophilic fungus *Chaetomium gracile*, was supplied as a partially purified preparation in 50% (v/v) glycerol by Miles-Kyowa (Tokyo) and stored at 4 °C. Lyophilized preparations of another fungal dextranase (*Penicillium* sp., grade 1, chromatographically pure) and a bacterial dextranase (*Bacillus* sp.) were purchased from Sigma and Calbiochem Corp. (San Diego, CA), respectively. The thermostable α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) from *Bacillus licheniformis* (Termamyl 120L) was obtained from Novo Industri A/S (Bagsvaerd, Denmark). The Phadebas amylase test kit was purchased from Pharmacia Diagnostics AB (Uppsala, Sweden).

Dextrans. *Purification.* Native B-512 and cane dextrans were purified from aqueous solution by repeated precipitation with ethanol to 50% (v/v), and the final product was lyophilized to a moisture level of 10–15% (McNeil and Inkerman, 1977). Gas chromatographic analysis [using a combination of the methods of Albersheim et al. (1967) and Harris et al. (1984) and employing *meso*-inositol as the internal standard] indicated that only glucose was present, except in two samples of cane dextran, in which mannose, galactose, xylose, and arabinose were detected in trace amounts (collectively less than 2%).

Moisture Content. The moisture content of dextrans was determined according to the method of Jeanes et al. (1954). Lyophilized samples of dextran were equilibrated with atmospheric moisture by storage for about 3 weeks in a constant-temperature room (20 °C) at 70% relative humidity prior to drying to a constant weight. The dried sample was discarded.

Preparation of Dextran Solutions. Dextrans were dissolved by the addition of small quantities of CO_2-free distilled water to an accurately weighed amount of the lyophilized material with constant stirring to form a homogeneous slurry. The particles were allowed to become uniformly hydrated by standing with occasional stirring of the solution (Jeanes et al., 1954). Additional water was added gradually so as to avoid the presence of an excess while gel masses still remained. After transfer to a suitable container, the sample was autoclaved for 20 min at 100 kPa (121 °C). The sample was allowed to cool to room temperature and the volume made to 250 mL. In the case of some cane dextrans stored over long periods, the above procedure failed to ensure solubilization of all of the lyophilized material. After autoclaving, these samples were centrifuged at 17000*g* for 1 h at 25 °C, and the dextran concentration of the supernatant was determined according to the phenol–sulfuric acid procedure of Dubois et al. (1956). The effectiveness of this step was checked by examination of dust-free solutions in the cell of a light scattering photometer (SOFICA Model 42000 PGD). Dextran solutions were prepared daily. Due to solubility problems, prolonged storage of solutions is not recommended even under refrigeration.

Enzyme Assays. Dextranase activity was determined by measurement of the amount of dye released from Blue Dextran 2000 according to the method of Kow and Khouw (1970), modified as follows: 3 mL of substrate (0.4%, w/v) and 0.4 mL of citrate buffer (250 mM), pH 5.0, were preincubated for 5 min at 45 °C. A 100-μL aliquot of suitably diluted enzyme was added and the reaction mixture incubated for a further 15 min. The reaction was stopped by the addition of 7 mL of ethanol and the solution then laced in an ice bath for 15 min. After centrifugation at 20000*g* for 15 min at 20 °C, the absorbance was read at 630 nm. One unit of dextranase is defined as the amount of enzyme that catalyzes the release of 1 mg of dye complex ($E_{1cm}^{1mg/mL} = 0.965$) in 15 min under the prevailing assay conditions. Specific activity is defined as units mL^{-1} A_{280}^{-1}. The dextranase from *C. gracile* was checked for contaminating activities at 10 times the concentration used for dextran analysis by estimation of the reducing sugars (Dygert et al., 1965) released from the following carbohydrates: maltose, maltotriose, sucrose, potato starch, pullulan, (carboxymethyl)cellulose, sugar cane cellulose, and sugar cane arabinogalactan. A more detailed study with potato starch, isomaltose, and stachyose was carried out by examination of the products of enzymic hydrolysis according to the HPLC procedure described below.

Amylase activity of *B. licheniformis* α-amylase was determined by absorbance measurement at 620 nm of the blue dye released from a blue starch polymer (Phadebas amylase assay) at pH 7.0 and 90 °C. The presence of contaminating dextranase activity in the α-amylase was checked according to the Blue Dextran method described above but assaying at pH 7.0 and 60 and 90 °C using a 10-fold excess of enzyme.

Dextranase. *Preparation.* The commercial dextranase from *C. gracile* was dialyzed against acetate buffer (25 mM, pH 5.0) at 4 °C to remove glycerol. Thereafter, the enzyme was concentrated to an A_{280} ~ 80 by precipitation with ammonium sulfate (80% saturation), dialyzed (in preboiled tubing) against acetate buffer, centrifuged (4 °C, 20 min, 25000*g*) to remove amorphous material, and stored at 4 °C. Alternatively, the enzyme was prepared daily by gel filtration of 4 °C of the commercial enzyme through Econo-Pac 10DG desalting columns (Bio-Rad) equilibrated with 25 mM acetate buffer, pH 5.0.

Purification and Characterization. Preparations of *C. gracile* dextranase were purified according to minor modifications to the chromatographic (CM- and DEAE-cellulose) procedures of Hattori et al. (1969). Chromatofocusing was carried out using Polybuffer ion exchange 94 and amphoteric buffers covering the pH range 8–5 (Pharmacia, 1982). The molecular weight was determined by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) according to the method of Inkerman et al. (1975a). Electrophoretic analysis of the native enzyme was performed according to the method of Inkerman et al. (1975b).

Dextran Analysis. *Preparation of Sugar Solutions.* Raw sugar samples (117.5 g) were dissolved in ~200 mL of distilled water, and the pH was adjusted to 7.2. The solution was autoclaved for 20 min at 100 kPa (121 °C), cooled to room temperature, and made to 250 mL with distilled water. A 50-mL aliquot was equilibrated at 90 °C for 5 min; then 100 μL of α-amylase was added and the solution incubated for a further 15 min.

Isolation of Polysaccharides. Polysaccharides were precipitated at room temperature from 50 mL of raw sugar solution by addition of ethanol to 80% (v/v). Acid-washed Celite (1 g) was added and the solution allowed to stand for 1 h. The precipitate was collected on a Teflon membrane (5-μm pore size, 47-mm diameter)–Celite (0.5 g) pad combination using an all-glass filtration apparatus (Millipore Corp., Bedford, MA). After washing (5 × 25 mL) with an 80% ethanol solution, the polysac-

charides were extracted in distilled water by boiling for 5 min and then filtering the solution through a Whatman No. 44 filter paper. The filtrate was cooled to room temperature and transferred quantitatively to a 100-mL standard flask containing 10 mL of acetate buffer (250 mM, pH 5.0) and 1 mL of the internal standard, stachyose (0.5% w/v).

Enzymic Digestion and Sample Preparation. Dextran solutions were incubated at 45 °C for 4 h with a total of 20 000 units of *C. gracile* dextranase added in three equal aliquots at 0, 20, and 40 min. The reaction mixture was then boiled for 5 min, cooled, and deionized by shaking with 15 g of mixed bed ion-exchange resin for 30 min. The resin was removed by filtration through a 100-mesh sieve and the hydrolysate evaporated to dryness. The hydrolysate was redissolved in 1 mL of distilled water, filtered through a 0.45-μm membrane (Minisart, Sartorius) and stored in 1-mL glass vials (Waters Division of Millipore, Milford, MA) at −20 °C until required for analysis.

HPLC. Separation of the products of dextran hydrolysis was carried out using reverse-phase chromatography according to the method of Ivin et al. (1983). The HPLC system consisted of the following Waters equipment: Model 590 solvent delivery pump; WISP Model 710 B automatic sample injector; R410 differential refractive index detector; radial compression module RCM-100; Maxima 820 chromatography work station. The column was a C_{18} Radial-PAK cartridge (5-μm particle size, 100-× 8-mm) fitted with a precolumn insert (C_{18} Guard-PAK) and pressurized in the RCM-100. The solvent, water, was vacuum filtered (0.45-μm, Millipore) and degassed prior to use. The injection volume was usually 10 μL and the flow rate constant at 0.5 mL/min. A two-point calibration based on peak area integration was determined from duplicate injections of standard sugars covering the concentration range 50–1000 ppm on solids. The response for each standard sugar was shown to be linear over this range. A similar procedure was used for quantitative evaluation of the thawed dextran hydrolysates.

Comparative Studies. Analysis of dextran was carried out according to the enzyme–HPLC, Roberts' copper (Goodacre, 1986; Clark and Godshall, 1988) and alcohol haze (Keniry et al., 1969) methods on 16 Australian raw sugars containing dextran concentrations up to ~400 ppm on solids as measured by the enzyme–HPLC method. An enzymic confirmation step was also incorporated into the alcohol haze procedure (Fulcher and Inkerman, 1974).

RESULTS AND DISCUSSION

Properties and Selection of Dextranase. During preparation of dextranase solutions, the specific activity of the commercial enzyme increased from ~90 to ~225 units $mL^{-1} A_{280}^{-1}$. These preparations appeared as a single band of MW 73 000 on PAGE/SDS, whereas at least five protein bands were observed by PAGE of the native enzyme. Attempts to use the activity strain of Gabriel and Wang (1969) were unsuccessful. Partial separation of these bands was achieved by ion-exchange chromatography using the procedure of Hattori et al. (1969) or by chromatofocusing (p*I* 6–7), but a protein preparation that was homogeneous on native PAGE was not obtained. Subsequently, all experiments were performed using solutions of commercial dextranase prepared as described under Materials and Methods.

At enzyme concentrations 10 times the levels used for dextran analysis, no contaminating activities were detected against any of the carbohydrates listed under Materials and Methods, with the exception of potato starch from which a small amount of glucose was released. Glucose was shown by HPLC analysis to be the sole product of hydrolysis of starch by the dextranase preparations. This exo activity was exhibited by all of the partially separated electrophoretic variants. Under the same conditions, a chromatographically pure dextranase from *Penicillium* sp. also exhibited a similar amount of exo activity against potato starch. These combined studies suggest that the exo activity is due to the dextranase itself rather than an impurity present in these preparations. Commercial dextranase also exhibited no hemicellulose, proteinase, ribonuclease, aminopeptidase, or carboxypeptidase activity (Dr. S. Minato, Sankyo Co. Ltd., private communication, 1982).

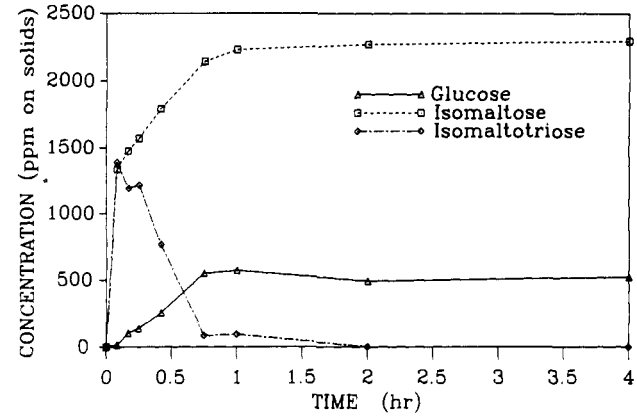

Figure 1. Progress curves for the low molecular weight sugars produced by enzymic hydrolysis of cane dextran (3160 ppm on solids) at pH 5.0 and 45 °C. A total of 20 000 units of *C. gracile* dextranase was added in three equal aliquots at 0, 20, and 40 min.

The fungal dextranase from *C. gracile* was selected because it has been confirmed in our studies that this enzyme hydrolyzes dextrans faster than any of the other enzymes tested (Taylor et al., 1990), and the final breakdown products of dextran, glucose and IM_2, (Hattori et al., 1969; Taylor et al., 1985) are easy to separate and quantitate by the HPLC system used in this study. In contrast, the use of a bacterial dextranase (e.g., *Bacillus* sp.), which did not exhibit activity against starch, was rejected because its hydrolytic products, IM_3, IM_4, and IM_5 (in approximately equal amounts), were more difficult to quantitate (Taylor et al., 1990). An additional consideration is the high cost of using large amounts of these purified enzymes. Commercial batches of *C. gracile* dextranase can be stored at 4 °C for years without significant loss of activity, and concentrated preparations (without glycerol) are also stable when stored at 4 °C, losing about 50% of their activity in 6 months.

Conditions of Enzymic Hydrolysis. Figure 1 shows the progress curves for the release of glucose, IM_2, and IM_3 from cane dextran (3160 ppm on solids) by *C. gracile* dextranase. The level of IM_3 reached a maximum in about 5 min and then decreased as hydrolysis to glucose and IM_2 proceeded. Similar hydrolytic patterns have been reported for a number of other fungal endo-dextranases (Taylor et al., 1990). After 2 h, no IM_3 could be detected; the constant levels of glucose and IM_2 indicate that the enzymic digestion had gone to completion. The selected conditions should ensure complete hydrolysis of cane dextran at the upper limits (~1000 ppm on solids) found in raw sugar within 2 h.

Separation and Identification of Hydrolysis Products. The products of exhaustive hydrolysis of dextran by *C. gracile* dextranase were separated by reverse-phase HPLC. Typical chromatograms for a raw sugar sample (containing a moderate level of cane dextran), a highly purified cane dextran, and native B-512 dextran are shown in Figures 2, 3, and 4, respectively. The results demonstrate that the enzymic digestion products are readily resolved by this simple HPLC method. The order of elution as determined from the retention times (RT) is buffer salts, glucose, IM_2, sucrose, and IM_3 (when

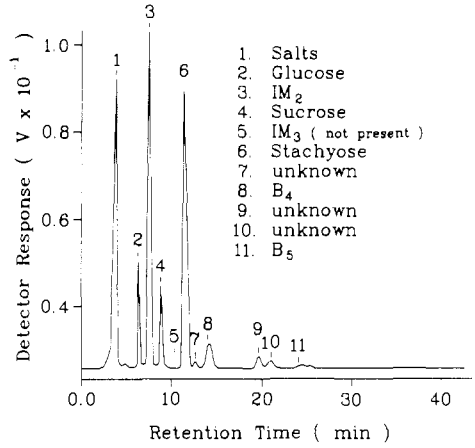

Figure 2. Reverse-phase (C_{18}) HPLC separation of the low molecular weight sugars released after 4 h from the cane dextran (360 ppm on solids) present in a raw sugar by *C. gracile* dextranase under the conditions given in Figure 1 (except for the presence of the raw sugar.

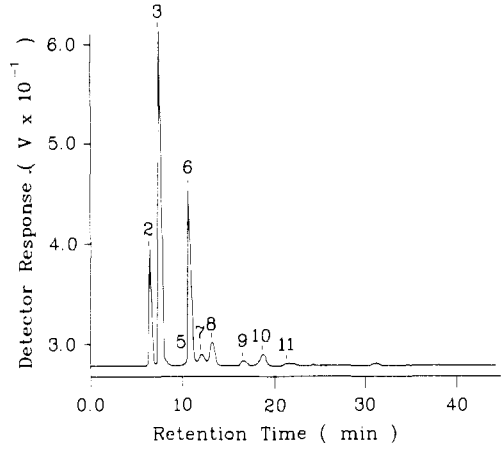

Figure 3. Reverse-phase (C_{18}) HPLC separation of the low molecular weight sugars released after 4 h from cane dextran (~1000 ppm on solids) by *C. gracile* dextranase under the conditions given in Figure 1. For peak assignment, see Figure 2.

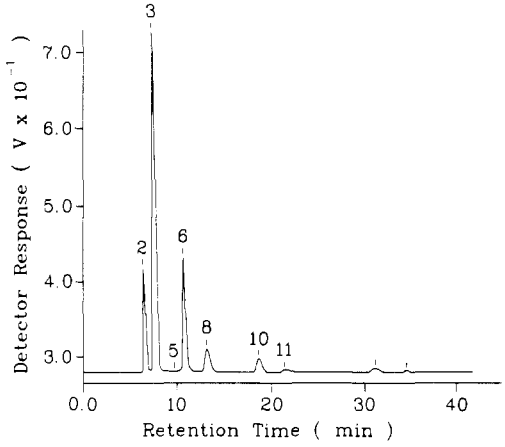

Figure 4. Reverse-phase (C_{18}) HPLC separation of the low molecular weight sugars released after 4 h from native B-512 dextran (~1000 ppm on solids) by *C. gracile* dextranase under the conditions given in Figure 1. For peak assignment, see Figure 2.

present). These are followed by the internal standard stachyose and the higher molecular weight isomaltodextrins. A comparison of the RTs obtained with the linear isomaltodextrins suggests that two (peaks 8 and 11 in Figures 2–4) are probably the branched isomaltodextrins,

Table I. Amounts of Low Molecular Weight Products Obtained from Enzymic Digestion

dextran type	MW[a]	glucose	IM_2	glucose–B_5[b]
T10[c]	1×10^4	14.0	68.8	92
T2000[c]	$\sim 2 \times 10^6$	14.2	69.8	96
native[c] NRRL B-512	40×10^6	14.3	69.2	97
cane[d]	$\sim 5 \times 10^6$	13.6	67.9	94–97

[a] $\bar{M}_w$ determined by light scattering measurements. [b] Summation of all digestion products from glucose to B_5. Due to the lack of appropriate standards, the response factor for IM_2 was used to calculate the amount of low molecular weight isomaltodextrins. Estimated using the appropriate multiplier in eq 1 calculated from the formula $(MW_{sugar} - MW_{water})/MW_{sugar}$. [c] Means of single samples each analyzed three times (T10, native) and tive times (T2000). [d] Mean of duplicate HPLC analyses for five different samples.

B_4 and B_5 (Taylor et al., 1985). Although peak assignment in this region of the chromatogram is difficult due to the lack of appropriate sugar standards, the remaining products (peaks 7, 9, and 10) are also probably branched isomaltodextrins.

The products of enzymic hydrolysis were very similar for cane dextran, dextran T10, dextran T2000, and native B-512 dextran. However, the presence of at least two additional (unidentified) components in cane dextran digests (peaks 7 and 9 in Figures 2 and 3) indicates that the dextrans are not identical. An earlier indication of differences between these dextrans was the report that cane dextrans were hydrolyzed by fungal dextranses at ~70% of the rate observed with the dextran T2000 (Inkerman, 1980). These results show that the products of enzymic hydrolysis are characteristic of the dextran. Therefore, it should be possible to detect different dextrans in a raw sugar sample by the presence of abnormal peaks in the HPLC chromatogram. This potential is adequately demonstrated by the HPLC chromatograms obtained for similar types of dextrans such as cane and native B-512 (Figures 3 and 4). In the case of highly branched dextrans (e.g., from *Streptococcus sobrinus*), the differences in the chromatograms are more marked.

Quantitation of Dextran. A number of dextrans were hydrolyzed with *C. gracile* dextranase and the products measured by HPLC. The results are given in Table I. Most of the dextran (92–97%) was in low molecular weight products, from glucose to B_5. The remainder appeared in unidentified components, presumably branched higher molecular weight isomaltodextrins. Thus, quantitation of dextran is not feasible by summation of the amounts of all components present in enzymic digests. An alternative approach is to use the glucose and IM_2 content. In this regard, the amounts of dextran accounted for by these two components were equal within the experimental error for all dextran samples analyzed (Table I). However, as starch is universally present in raw cane sugars, there is the possibility of elevated glucose levels due to starch hydrolysis (see above). Hence, quantitation of dextran has been based on the IM_2 content.

The amounts of IM_2 produced from a number of different dextrans are shown in Table II. For cane dextrans, the average amount of IM_2 produced was approximately 68%. Dextran concentration in raw cane sugar may be calculated from the IM_2 content using eq 1, where

$$\text{dextran concn} = (IM_2 \text{ concn}/0.68)(324/342) \quad (1)$$

the multiplier, 324/342, makes allowance for the water molecule gained during hydrolysis of the dextran, and the denominator, 0.68, represents the IM_2 content of cane dextran digest.

Table II. Amount of IM_2 Produced from Dextran by Enzymic Digestion

cane dextran	% dextran recovered as IM_2	NRRL B-512 dextran	% dextran recovered as IM_2
A	68.0	T10	67.9, 69.7, 68.8
B	70.1, 68.8		
C	67.2, 67.9	T2000	70.9, 69.3, 69.2, 69.9, 69.5
D	67.8, 66.0		
E	67.9, 67.5	native	67.9, 70.5
av[a]	67.9 ± 1.2	av[a]	69.4 ± 1.0

[a] A statistical analysis (*t* test) demonstrated that the difference between the average is significant at a 0.99 confidence level.

Table III. Effect of Ethanol Concentration on Dextran Levels in Raw Sugar

	dextran[a] obtained at each % ethanol				
sugar	33.3	50.0	66.6	75.0	80.0
A	261	841	954	1038	1035
B	139	770	1000	1041	1021
C	676	656	897	900	911
D	552	616	797	847	855

[a] Dextran levels are calculated from the isomaltose concentration in the hydrolysate according to eq 1. Dextran levels are expressed in parts per million on solids neglecting the water content of raw sugars of ~0.3%. Means of duplicate HPLC analyses.

In the case of dextran T10, dextran T2000, and native B-512 dextran, the average amount of IM_2 produced was approximately 69%. Thus, when quantitation is required for these dextrans, a denominator of 0.69 can be substituted into eq 1.

The types and amounts of enzymic digestion products were constant for five cane dextrans prepared from cane juices obtained from three different sugar-growing districts. The results (Table II) further demonstrate the similarity of the dextrans found in the sugar cane industry (Covacevich and Richards, 1977a,b; Foster et al., 1980; DeStefano and Irey, 1986). In this regard, the dextrans used in this study were produced under field conditions (Foster et al., 1980) and consequently are representative of the type of dextran found in raw sugar.

The amounts of IM_2 found in enzymic digests of dextran T10, dextran T2000, and native B-512 dextran of widely different molecular weights (1×10^4 to 4×10^7) are very similar (column 3, Table I). Therefore, quantitation of dextran is independent of molecular size, at least for dextrans as low as 1×10^4.

Besides being specific for dextran, the other major advantage of this procedure is that it measures the complete range of molecular sizes of dextrans found in raw sugar. Only one component in the hydrolysate, IM_2, needs to be quantitated rather than all of the digestion products. In contrast to the alcohol haze and Roberts' copper procedures, use of this product ensures the specificity of the method. IM_2 is not present in raw sugar, and the other possible source of IM_2 is starch. However, our studies indicate that IM_2 is not produced by the action of *C. gracile* dextranase on starch or any other carbohydrate (except dextran) likely to be present in raw sugar.

Precipitation with Different Concentrations of Alcohol. A number of raw sugar samples were precipitated with varying concentrations of ethanol (33–80%) and the precipitates washed with an 80% ethanol solution; dextran analysis performed. The results (Table III) indicate that ethanol concentrations of 75% or greater were sufficient to precipitate all of the dextran present in raw sugar, i.e., the entire range of molecular sizes of dextran found in raw sugar. The use of 80% ethanol in the present procedure therefore overcomes problems associated with equilibration at lower concentrations (e.g., 50%, as in the alcohol haze test) as well as ensures quantitative recovery of the dextran.

Table IV. Recoveries from Raw Sugar of Added T10, T2000, and Cane Dextrans

	dextran		recovery	
type	added[a]	measured[a,b]	amount[a,b]	%
T10	0	289		
	123	425	136	105.4
	246	566	277	107.4
	369	685	396	102.3
T2000	0	289		
	129	415	126	102.4
	246	547	258	104.9
	387	654	365	98.9
cane	0	225		
	150	373	148	98.7
	300	535	310	103.3
	450	660	435	96.7

[a] Levels in parts per million on solids (see footnote to Table II). [b] The amount of dextran was calculated from the isomaltose concentration according to eq 1.

Recovery of Added Dextran. Cane dextran, dextran T10, and dextran T2000 were added to raw sugars and the recoveries determined from the IM_2 content. The results are presented in Table IV. Recoveries varied from 97 to 107% for dextran T10 and dextran T2000 and from 97 to 103% for the cane dextrans. These quantitative recoveries, especially of the low molecular weight dextran T10, demonstrate that the method gives an accurate measurement of the total dextran content of raw sugars. In this regard, the inclusion of the internal standard, stachyose, allows any losses to be accounted for during the analysis. In comparison, recoveries of dextran T40 and dextran T2000 from raw sugars reported for the Roberts' method are 97–103% (Roberts, 1983; Roberts et al., 1983). For the haze method, recoveries for dextran T40 have been reported to be about 65% (Roberts et al., 1983), and for dextran T2000, recoveries were of the order of 80–100% (Keniry et al., 1969; Roberts et al., 1983).

Repeatability. An indication of the repeatability of the method was obtained by analyzing four raw sugars (containing dextran levels from 300 to 800 ppm on solids) in duplicate on four different occasions. The relative standard deviations were below 3.2% for each sugar. An analysis of variance also indicated that there was no significant difference among the mean values for the four occassions.

Comparison of Dextran Methods. *Alcohol Haze and Enzyme–HPLC.* For most raw sugars analyzed, dextran levels determined according to the enzyme–HPLC method were higher than the corresponding levels measured by the alcohol haze test (Figure 5). Factors likely to contribute to this difference are the use of a higher ethanol concentration (80%) compared to the alcohol haze test (50%), a 1-h period of equilibration rather than the 20 min used in the alcohol haze test, and dextran T2000 as the standard in the alcohol haze test, which leads to underestimation of cane dextran levels by ~10% (Foster et al., 1980). At low dextran concentrations, there is an apparent lack of sensitivity of the alcohol haze test, viz., no dextran is detected below 50 ppm on solids measured according to the enzyme–HPLC method. This lack of sensitivity of the alcohol haze test has been the subject of other comparative studies (Keniry, 1982). The apparent agreement between the two methods at the higher dextran levels

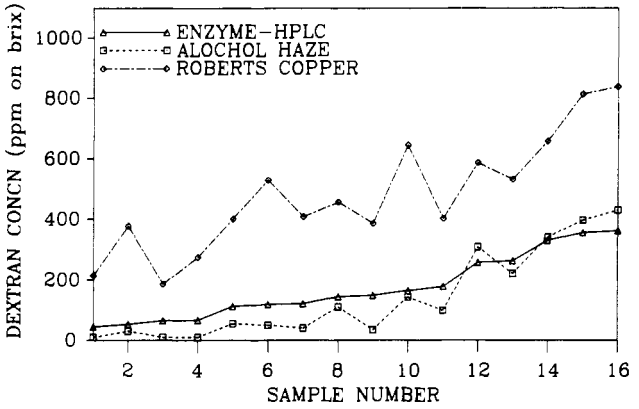

Figure 5. Dextran levels in raw sugar samples measured according to the enzyme–HPLC, alcohol haze, and Roberts' copper methods.

(greater than 200 ppm on solids) is unexpected since in earlier studies with raw sugar samples (containing dextran at 400–1000 ppm on solids) the haze method gave consistently lower values (by 10–30%) than the enzyme–HPLC method. Unfortunately, similar samples were not available for the persent comparative study. After incubation of the raw sugars with dextranase, no significant amount of alcohol haze material was detected. These results indicate that, at least for these sugars, the alcohol haze method is specific for dextran.

Roberts' Copper and Enzyme–HPLC. Dextran values measured according to the Roberts' copper method are significantly higher than those obtained according to the enzyme–HPLC procedure (Figure 5). Both methods have been shown to be independent of the molecular weight of the dextran present in the sample (cf. alcohol haze test). The use of the phenol–sulfuric reagent for quantitation of the dextran in the Roberts' copper method achieves high sensitivity; however, this reagent will react with all carbohydrates present in the mixture, i.e., it is not selective for dextran. For example, at low levels (about 50 ppm on solids by the enzyme–HPLC method), the Roberts' copper method detects dextran in excess of 200 ppm on solids. Presumably this indicates that the Roberts' copper reagent precipitates other polysaccharides besides dextran (DeStefano and Irey, 1986).

CONCLUSIONS

A method has been developed for the measurement of the total dextran content of raw sugar. The first two steps (precipitation with alcohol and enzymic hydrolysis) are pushed to completion, thereby ensuring quantitation, while the third step (HPLC) quantitatively measures IM_2, a specific product of enzymic hydrolysis of the dextran. Any losses during the latter two steps are corrected for with the internal standard, stachyose. The choice of *C. gracile* dextranase is important as many other dextranases do not hydrolyze cane dextrans to the same degree or at the same rate. The method also relies heavily on the restricted type of dextran found in the sugar industry. In this respect, the extensive structural, physicochemical, and microbial studies carried out over the past two decades have confirmed their similarity. Nevertheless, the presence of any unusual dextran should be apparent from the HPLC chromatogram. At the same time, the absence of the IM_3 peak can be used to determine whether the enzymic reaction has reached completion. Overall, the method exhibits greater specificity and sensitivity of measurement than those currently in wide usage in the sugar industry, e.g., the alcohol haze and Roberts' copper procedures. Using the method in its present form, it takes about 2 working days to complete an analysis, although a number of samples can be analyzed concurrently. Future developments include an increase in the sensitivity of the method and a decrease in the time required for analysis. Nevertheless, the general methodology outlined in this paper has the potential to be used to measure dextran of known structural type in any material.

ABBREVIATIONS USED

The abreviation IM_x refers to a linear oligosaccharide of the isomaltose series containing x D-glucose residues. Isomaltotetrose and higher homologues containing a single secondary linkage (1,3-α; 1,4-α; or 1,2-α) are indicated by B_x. As an example, *O*-α-D-glucopyranosyl-(1→6)-*O*-[α-D-glucopyranosyl-(1→3)]-*O*-α-D-glucopyranosyl-(1→6)-D-glucopyranose is abbreviated B_4.

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Registry No. Dextran, 9004-54-0; sucrose, 57-50-1; dextranase, 9025-70-1.