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Comparative study of the efficacies of nine assay methods for the dextransucrase synthesis of dextran

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ABSTRACT

A comparative study of nine assay methods for dextransucrase and related enzymes has been made. A relatively widespread method for the reaction of dextransucrase with sucrose is the measurement of the reducing value of D-fructose by alkaline 3,5-dinitrosalicylate (DNS) and thereby the amount of D-glucose incorporated into dextran. Another method is the reaction with ^{14}C-sucrose with the addition of an aliquot to Whatman 3MM paper squares that are washed three times with methanol to remove ^{14}C-D-fructose and unreacted ^{14}C-sucrose, followed by counting of ^{14}C-dextran on the paper by liquid scintillation counting (LSC). It is shown that both methods give erroneous results. The DNS reducing value method gives extremely high values due to over-oxidation of both D-fructose and dextran, and the ^{14}C-paper square method gives significantly low values due to the removal of some of the ^{14}C-dextran from the paper by methanol washes. In the present study, we have examined nine methods and find two that give values that are identical and are an accurate measurement of the dextransucrase reaction. They are (1) a ^{14}C-sucrose/dextransucrase digest in which dextran is precipitated three times with three volumes of ethanol, dissolved in water, and added to paper and counted in a toluene cocktail by LSC; and (2) precipitation of dextran three times with three volumes of ethanol from a sucrose/dextransucrase digest, dried, and weighed. Four reducing value methods were examined to measure the amount of D-fructose. Three of the four (two DNS methods, one with both dextran and D-fructose and the other with only D-fructose, and the ferricyanide/arsenomolybdate method with D-fructose) gave extremely high values due to over-oxidation of D-fructose, D-glucose, leucrose, and dextran.

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1. Introduction

Dextransucrases [EC 2.4.1.5] are a wide family of enzymes produced by bacteria from over 120 *Leuconostoc mesenteroides*[1,2] and *Streptococcus* sp.[1–4] that synthesize dextrans with a wide variety of structures. Dextransucrases and glucansucrases use sucrose as the high-energy donor of D-glucose for the synthesis of dextran and related glucans. Dextransucrases make up a family of enzymes that synthesize α-(1→6)-linked glucans that have α-(1→3)-linked D-glucose branches and α-(1→3)-linked dextran chain branches. There also are dextrans that have α-(1→2) and α-(1→4) branch linkages,[1–4] depending on the particular bacterial strain, producing a specific dextransucrase. There is a *L. mesenteroides* B-742CB dextransucrase that synthesizes a bifurcated double-comb dextran with single D-glucose units linked α-(1→3) to every D-glucose unit in the α-(1→6)-linked dextran chain, with some double-comb dextran chain branches linked α-(1→3);[2,5] and there are dextransucrases elaborated by *Streptococcus mutans* strains that synthesize a bifurcated single-comb dextran with single D-glucose units linked α-(1→3) to every other D-glucose unit in the α-(1→6)-linked dextran chains, with some single-comb dextran chain branches linked α-(1→3).[2,3] There also are related glucansucrases that synthesize glucans other than dextran, such as mutansucrase [EC 2.4.1.6] that synthesizes a linear α-(1→3)-linked glucan called mutan;[2,3] there is also a glucan that has an alternating α-(1→6)- and α-(1→3)-linked glucan with α-(1→3) branch linkages, called alternan, synthesized by alternansucrase [EC 2.4.1.140], which is produced by *L. mesenteroides* B-1355CB.[2,6]

An early assay method for measuring the activity of dextransucrase was the measurement of the increase in the reducing value that results from the release of D-fructose from sucrose when the D-glucosyl moiety is polymerized into dextran.[7–10] These studies used the alkaline copper methods of Shaffer and Somogyi[11] and Nelson.[12] In 1959, Miller[13] published the alkaline 3,5-dinitrosalicylate method (DNS method) for determining the reducing values of carbohydrates, and in 1980, Lopez and Monsan[14] introduced the DNS reducing value method to assay dextransucrase by measuring

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the reducing value of D-fructose. Monsan and co-workers and others have used the DNS method to assay dextransucrases and related enzymes from 1980 to the present.[14–42]

A filter paper assay, using ^{14}C-labeled substrates was originally described by Bollum[43] for determining DNA synthesis; it was adapted by Thomas et al.[44] for the assay of UDPGlc glycogen synthase [EC 2.4.1.11]. In 1974, Germaine et al.[45] adapted a rapid filter paper assay involving ^{14}C-UL-sucrose for assaying the formation of glucan by dextransucrases and related enzymes, which has been used by Robyt and co-workers.[6,46–52]

In the present study, nine different assay methods for the determination of the amount of dextran synthesized by dextransucrase have been examined. We show that two diverse methods that are widely used: (1) the reducing value methods, especially the DNS method and the ferricyanide/arsenomolybdate method, and (2) the ^{14}C-sucrose rapid filter paper method, gave erroneous results. The reducing value methods gave extremely high values due to over-oxidation of D-fructose, D-glucose, leucrose, and dextran; and the ^{14}C-sucrose filter paper method, gave low values due to the removal of some of the ^{14}C-dextran from the filter papers by methanol washes. We have found that the direct measurement of dextran can be carried out by its precipitation with three volumes of ethanol from the sucrose/dextransucrase digests, followed by dehydration and determination by weighing, or when ^{14}C-sucrose is used, determination by liquid scintillation counting and the specific activity of D-glucose. These latter two methods gave identical numbers for the μmoles of D-glucose incorporated into dextran/min.

2. Experimental

2.1. Materials

2.1.1. Chemicals

[^{14}C]-UL-sucrose (0.1 mCi/mL) was obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA); 3,5-dinitrosalicylic acid, PPO, and PoPoP were obtained from Fisher Scientific Co. (USA); the liquid scintillation cocktail was prepared by dissolving 5 g PPO and 0.1 g of PoPoP in 1 L of toluene. Pure maltodextrins were available in our laboratory from large-scale charcoal–Celite column chromatography. All other chemicals were of the highest grade obtainable.

2.1.2. Enzymes

L. mesenteroides B-512FMC dextransucrase was obtained as previously described.[50,51] The culture filtrate, containing the secreted enzyme was concentrated using an ultrafiltration hollow-fiber cartridge, with a pore size of 0.1 μm (Diaflo polysulfone ultrafiltration hallow-fiber cartridge [H5P100-43] with a 100 kDa molecular weight cut-off in a column 4.5 cm diameter by 63.8 cm length, obtained from Amicon, Inc., Beverley, MA, USA).[51] The enzyme produced by the B-512FMC bacterium was free of invertase and levansucrase and only produced D-fructose, leucrose, and dextran. *L. mesenteroides* FT045B (from Brazil) was obtained after culturing, using the culture conditions for the B-512FMC dextransucrase.[51]

2.2. Methods

2.2.1. Dextransucrase activity/assay methods

2.2.1.1. Dextransucrase/^{14}C-UL-sucrose with dextran precipitation and liquid scintillation counting. The enzyme digest was prepared by the addition of 60 μL of buffer (84–168 mM pyridinium/acetate, pH 5.2) to 165 μL of 300 mM, 0.1 μCi ^{14}C-UL-sucrose, pre-warmed to 20–30 °C for 5 min. Then 25 μL of enzyme, sufficiently diluted 1:10 to 1:1000 with the buffer solution containing 0.1% (w/v) Tween 80 and 1 mM $CaCl_2$[46] was added to the substrate solution, giving 198 mM 0.1 μCi of ^{14}C-UL-sucrose, which was incubated at the desired temperature. Aliquots (50 μL) were taken at 5, 10, 20, and/or 30 min and each was added to 150 μL of 0–20 °C ethanol and mixed with a vortex mixer. The resulting precipitate was allowed to stand in 0 °C ice water for 15–30 min, and then centrifuged at 10,000*g* for 5–10 min. The supernatant was removed and the precipitate was dissolved in 100 μL of ~90 °C water, cooled to ~20–22 °C and then added to 300 μL of 0–20 °C ethanol and mixed with a vortex mixer. This last procedure was repeated, giving three precipitations of dextran. The final precipitate was dissolved in 250 μL of water, with 25 μL aliquots added to 15-mm squares of Whatman 3MM paper, which were dried and placed into 10 mL of a toluene liquid scintillation cocktail for counting for 10 min or 10,000 cpm, whichever came first. The counting efficiency was found to be 76%. The amount of D-glucose incorporated into dextran was obtained from (cpm/μmol of sucrose/2) from which an International Unit (IU) = 1 μmol of D-glucose incorporated into dextran/min/mL was obtained.

2.2.1.2. Dextransucrase/^{14}C-UL-sucrose digests were added to 15-mm Whatman 3MM square papers, washed three times with MeOH, and counted in a liquid scintillation spectrometer. The assay was adapted from Germaine et al.[45] The reaction digest was the same as that described in Section 2.2.1.1, except that 25-μL aliquots were removed and added directly to 15-mm squares of Whatman 3MM papers that were immediately added to 250 mL of MeOH with stirring for 20 min. The papers were then added to another 250 mL of MeOH and stirred for 20 min. This was repeated a third time, and then the papers were dried under an infrared heat lamp and placed into 10 mL of toluene cocktail for liquid scintillation counting for 10 min or 10,000 cpm, whichever came first. The amount of D-glucose incorporated into dextran and the number of International Units of dextransucrase/mL/min were obtained as described in Section 2.2.1.1.

2.2.1.3. Dextransucrase/sucrose digest and the gravimetric determination of dextran. The enzyme digest was prepared by the addition of 1880 μL of buffer (pyridinium acetate, pH 5.2) to 2000 μL of 400 mM sucrose, which was pre-warmed to the desired temperature (30 °C in the experiments for this study). The reaction was initiated by the addition of 120 μL of sufficiently diluted dextransucrase. Aliquots of 500 μL of the reaction digest were taken at various times and added directly to 1000 μL of cold (0–10 °C) anhyd ethanol, mixed, and centrifuged for 15 min at 13,000*g*, using tared centrifuge tubes. The supernatants were removed and the precipitates were dissolved in 500 μL of water, mixed, and added to 1000 μL of anhyd ethanol, mixed, and centrifuged, dissolved in 500 μL of water, and precipitated a third time. The precipitates were treated four times with 1000 μL of acetone and once with 1000 μL of anhyd ethanol, dried under an infrared heat lamp, and weighed. The number of μmoles of D-glucose incorporated into dextran was obtained by (μg of weighed dextran)/162 (the anhyd weight of D-glucose incorporated into dextran), from which the International Unit (IU), μmoles of D-glucose incorporated into dextran/min/mL of dextransucrase, was computed.

2.2.1.4. TLC of dextransucrase/sucrose digest and scanning densitometry to determine dextran. A reaction digest was conducted as described in Section 2.2.1.2; 100 μL of the digest was taken at various times and added to 900 μL of water; then 10 μL aliquots were added by a Hamilton micro-syringe pipette to a Whatman K6 TLC plate for each diluted reaction aliquot, keeping the size of the spot between 2 and 3 mm by adding 1 μL at a time, with drying in between. A standard curve of dextran was prepared using 25–200 ng. The TLC plate was then eluted to the top of the plate, using 85:15 (v/v) acetonitrile–water for two

ascents to remove D-fructose, D-glucose, leucrose, and unreacted sucrose from dextran. The carbohydrates were visualized by dipping the plates into a methanolic solution of 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) concd sulfuric acid. The TLC plate was dried and placed in an oven at 120 °C for 10 min.[52] The densities of the blue-black spots of dextrans at the origin of the TLC plate were measured by a scanning imaging densitometer (Bio-Rad Model GS-670).[52] The amount of dextran formed was determined from a standard curve using 20–200 ng of dextran. The amount of dextran produced in the reaction was used to calculate the International Units of dextransucrase/mL, as in Section 2.2.1.3.

2.2.1.5. Alkaline 3,5-dinitrosalicylic acid measurement of the reducing value produced in a dextransucrase/sucrose digest. The reaction digest was prepared as described in Section 2.2.1.1, but without ^{14}C-sucrose. A 50-μL aliquot of a sufficiently diluted sample (1:10 to 1:1000) from the reaction digest was added to 50 μL of the 3,5-dinitrosalicylic acid (DNSA) reagent (1.0 g DNSA, 0.2 g phenol, 0.05 g sodium sulfite, and 1.0 g sodium hydroxide in 100 mL of water)[13] and mixed. The mixture was heated at 100 °C for 5 min, cooled in an ice bath to 20 °C; 30 μL was added to 100 μL of water in a microtiter plate and mixed. The absorbance was measured at 540 nm in a microtiter plate spectrometer. A standard curve of 0.5–5.0 μmol of D-fructose was prepared, and the amount of D-fructose in the digest was determined, which was the presumed activity of dextransucrase in the sample. If D-fructose was the only reducing carbohydrate produced, an International Unit of dextransucrase activity is the μmoles of D-fructose formed/min/mL of enzyme. This is the method used by Monsan and co-workers for the assay of dextransucrases.[14–42]

2.2.1.6. Precipitation of dextran from a dextransucrase/sucrose digest, followed by measurement of the reducing value of D-fructose by alkaline 3,5-dinitrosalicylic acid. The dextransucrase/sucrose reaction digest was the same as described in Section 2.2.1.1, but without ^{14}C-UL-sucrose, and with the exception that 50-μL aliquots of the digest were added to 100 μL of ethanol at various times, mixed, and centrifuged in 0.75-mL micro centrifuge tubes for 15 min at 13,000*g*. The supernatant was removed and 50-μL aliquots were added to 50 μL of the DNS reagent (the same reagent as described in Section 2.2.1.5) in a 96-well microsample plate. The mixture was heated at 100 °C for 5 min, cooled in an ice bath to 20 °C, and the absorbance was measured at 540 nm in a microtiter plate spectrometer. A standard curve of 0.5–5.0 μmol of D-fructose was prepared, and the amount of D-fructose in the digest was determined and is the presumed activity of dextransucrase in the sample. If D-fructose was the only reducing carbohydrate produced, an International Unit of dextransucrase activity is the μmoles of D-fructose formed/min/mL of enzyme.

2.2.1.7. Precipitation of dextran from a dextransucrase/sucrose digest, followed by measurement of the reducing value of D-fructose by alkaline ferricyanide–arsenomolybdate. The ferricyanide reagent consists of 16 g of anhyd sodium carbonate, 15 g of disodium phosphate heptahydrate dissolved in 85 mL of water and 0.4 g of potassium ferricyanide was added and the solution was diluted to 100 mL.[54] The arsenomolybdate reagent consisted of 2.5 g of ammonium molybdate tetrahydrate in 45 mL of distilled water, with 2.1 mL of concd sulfuric acid, followed by 2.5 mL containing 0.3 g of disodium arsenate; the solution was heated to 55 °C for 30 min, with constant stirring.[54] The dextransucrase/sucrose digest was the same as that given in Section 2.2.1.3. After precipitation of dextran, 15 μL aliquots of the supernatant were added to 1485 μL of water, from which 100 μL aliquots were added to 50 μL of the alkaline ferricyanide reagent. The solutions were heated at 55 °C for 30 min. They then were quickly cooled in cold water, and the contents were partially neutralized by adding 100 μL of 2 N sulfuric acid, followed by mixing until no more gas evolved; 40 μL of the arsenomolybdate reagent was then added, mixed, and 90 μL was added to 90 μL of water, which was added to a microtiter plate. The absorbance was measured at 515 nm in a microplate spectrometer. The International Unit of dextransucrase was computed using a standard curve of D-fructose (3–30 mM). If D-fructose was the only reducing carbohydrate produced, an International Unit of dextransucrase activity is the μmoles of D-fructose formed/min/mL of enzyme.

2.2.1.8. Precipitation of dextran from a dextransucrase/sucrose digest, followed by measurement of the reducing value of D-fructose by copper bicinchoninate. The dextransucrase/sucrose reaction digest was the same as that described in Section 2.2.1.1, but without ^{14}C-sucrose. Also there was the exception that 50-μL aliquots of the digest were added to 100 μL of ethanol at various times, mixed, and centrifuged in 0.75 mL microcentrifuge tubes for 15 min at 13,000*g* to remove dextran. The supernatant was removed and 50-μL aliquots were added to 50 μL of the copper bicinchoninate working reagent[55] in a microsample plate, which was heated at 80 °C for 35 min, cooled for 15 min, and the absorbance was measured at 560 nm in a microsample spectrometer.[55] A standard curve was prepared, using 1–20 μg/mL of D-fructose. If D-fructose was the only reducing sugar produced, an International Unit of dextransucrase activity is the μmoles of D-fructose formed/min/mL of enzyme.

2.2.1.9. Measurement of D-fructose formed in a dextransucrase/sucrose digest, using a Megazyme Assay Kit. The dextransucrase/sucrose reaction digest was the same as described in Section 2.2.1.3. Aliquots of 200 μL of the reaction digest was taken and added to 10 μL of Carrez I, followed by 10 μL of Carrez II to stop the enzyme reaction; 10 μL was added to 990 μL of water. This solution was then analyzed according to the directions in the Megazyme data booklet (Megazyme, D-fructose and D-glucose assay procedure K-FRU 11/05) obtained from Megazyme International, Ireland Ltd., 2005, Bray Business Park, Bray Co., Wicklow, Ireland. If D-fructose was the only reducing sugar produced, an International Unit of dextransucrase activity is the μmoles of D-fructose formed/min/mL of enzyme.

3. Results and discussion

Nine different assay methods for dextransucrase and related enzymes (glucansucrases) have been compared and evaluated for their efficacy. These can be divided into two distinct types: (1) a direct method by isolating the synthesized dextran and the determination of its amount, and (2) an indirect method by measuring the D-fructose that is released from sucrose when the D-glucose moiety of sucrose was polymerized into dextran. The first group consists of four methods that give the amount of dextran produced at any given time; the second group consists of four different colorimetric methods measuring, the amount of D-fructose produced by measuring the reducing value equivalent of D-fructose, and a fifth method that enzymatically measures the amount of D-fructose.

The methods are given in Sections 2.2.1.1–2.2.1.4, and so forth, but here after are designated as Method-1, -2, -3, and -4 and so forth.

3.1. Group I, the direct measurement of the amount of dextran synthesized

In Method-1, ^{14}C-dextran is precipitated from a ^{14}C-sucrose/dextransucrase digest, by adding three volumes of ethanol. This

precipitation is repeated two additional times to remove the ^{14}C-labeled D-fructose, unreacted sucrose, leucrose, and any D-glucose that might be formed. The amount of dextran produced is determined by liquid scintillation counting of the ^{14}C-labeled dextran. Method-2 is that described by Germaine et al.[45] and involves the direct addition of an aliquot of the dextransucrase/^{14}C-sucrose digest to Whatman 3MM paper squares that are washed three times with methanol to remove ^{14}C-labeled D-fructose, unreacted sucrose, leucrose, and any D-glucose formed. The dried paper squares are placed into a toluene cocktail and the amount of dextran is determined by liquid scintillation counting of the ^{14}C-labeled dextran.

Method-3 involves the precipitation of dextran from the dextransucrase/sucrose digest with three volumes of ethanol. The precipitation is repeated two more times, followed by dehydration of dextran with three acetone treatments and one anhydrous ethanol treatment; dextran is then heated under an infrared heat lamp, and the amount of dextran is then weighed.

Method-4 involves a TLC procedure in which an aliquot of the dextransucrase/sucrose digest is added to a TLC plate and then chromatographed to remove the D-fructose, unreacted sucrose, leucrose, and any D-glucose formed. Dextran remains at the origin and is determined by scanning densitometry of the chromatogram.[52,53]

The results of the four methods are given in Table 1 for triplicate determinations of the activity of B-512FMC dextransucrase. Methods-1 and -3 gave the same value of 168 International Units (IU). Method-2 gave a significantly lower value of 105 IU, because the three methanol washes removed dextran from the papers. It also had a relatively high mean deviation. Method-4 also gave a lower value of 151 IU that we believe is due to some difficulties in measuring the densities at the origin of the chromatogram. A fifth method involving a TLC analysis of the D-fructose formed by scanning densitometry was performed only once and is not described here, as it is a long and rather tedious procedure involving a considerable amount of time. It required three ascents with one solvent and two ascents with a second solvent to completely separate D-fructose from D-glucose. This method, however, did give 167 IU, almost identical to 168 IU that was obtained by Methods-1 and -3.

3.2. Group II, analysis of D-fructose in dextransucrase/sucrose digests by four reducing value methods and by an enzymatic method

Method-5 was the measurement of the reducing value by alkaline 3,5-dinitrosalicylic acid reagent (DNS method) directly on aliquots from the dextransucrase/sucrose digest. This method gave a very high IU value of 606, the highest of the nine methods.

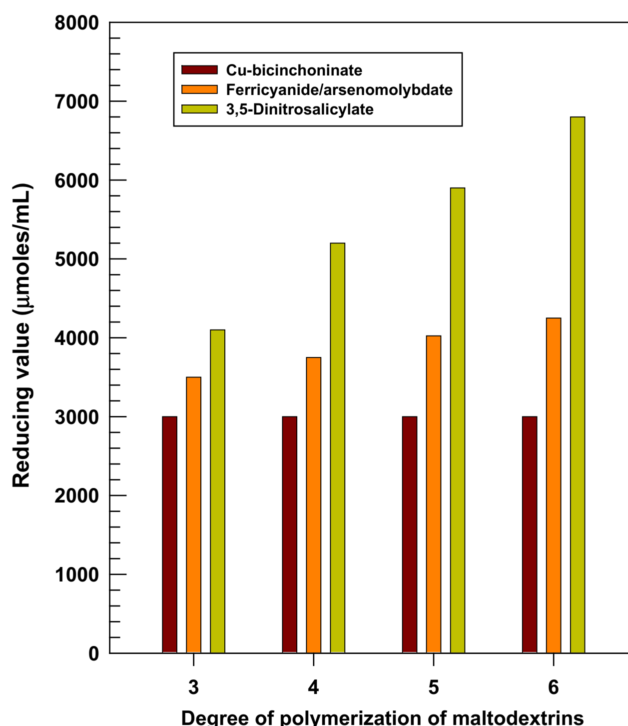

Figure 1. Comparative reducing values of equimolar amounts of maltodextrins of different sizes, maltotriose to maltohexaose, by copper bicinchoninate, ferricyanide/arsenomolybdate, and 3,5-dinitrosalicylate (DNS) methods.

This really was not unexpected, as Robyt and Whelan[56,57] had shown that the alkaline DNS method produces over-oxidation of carbohydrates, monosaccharides and especially oligosaccharides and polysaccharides, as it gives oxidative erosion down the saccharide chain, giving greatly inflated reducing values. Breuil and Saddler[58] confirmed this in 1985. This over-oxidation of equal molar amounts of maltodextrins (maltotriose to maltohexaose) by the DNS method and the ferricyanide method is shown in Figure 1. As the sizes of the oligosaccharides are increased, the amount of over-oxidation is increased. Consequently, the values for the assay by these methods for assaying dextransucrase are greatly inflated, giving 606 IU by the DNS method, and giving 438 U over 168 U obtained by the two methods that determine dextran directly because of the over-oxidation of both D-fructose and dextran. This method consequently gives values that are completely invalid for assays and for kinetic studies of dextransucrases and related enzymes.

Table 1
L. mesenteroides B-512FMC dextransucrase activity measurements using nine different methods

Method[a]	Description of the methods	Experiment 1 (IU[b]/mL)	Experiment 2 (IU[b]/mL)	Experiment 3 (IU[b]/mL)	Mean and mean deviation
1	^{14}C-Dextran precipitation and LSC[c]	168.7	168.8	167.8	168.4 ± 0.4
2	Addition of ^{14}C-dextran to filter paper squares	104.7	116.0	95.3	105.3 ± 7.1
3	Precipitation of dextran, with dehydration, methanol washing and weighing	168.4	170.2	165.5	168.0 ± 1.6
4	TLC and densitometry of dextran	151.1	151.1	150.1	150.8 ± 0.4
5	Alkaline DNS[d] reducing value of dextran digest	606.4	622.0	589.3	605.9 ± 11.1
6	Alkaline DNS[d] reducing value after dextran precipitation	308.4	326.6	287.6	307.5 ± 13.3
7	Ferricyanide/arsenomolybdate reducing value after dextran precipitation	332.2	359.3	368.5	353.3 ± 14.1
8	Copper bicinchoninate reducing value after dextran precipitation	179.5	187.5	183.3	183.4 ± 2.7
9	Enzymatic measurement of D-fructose	148.9	149.0	149.0	149.0 ± 0.0

[a] All assays were run at 30 °C.
[b] IU = International Unit of enzyme activity = 1.0 µmol of D-glucose incorporated into dextran/mL/min.
[c] LSC = liquid scintillation counting.
[d] DNS = 3,5-dinitrosalicylate.

Table 2
L. mesenteroides FT045B dextransucrase activity, determined by Methods 1–3

Method[a]	Description of the method	Experiment 1 (IU[b]/mL)	Experiment 2 (IU[b]/mL)	Experiment 3 (IU[b]/mL)	Mean and mean deviation
1	^{14}C-Dextran precipitation and LSC[c]	1.93	2.04	1.98	1.98 ± 0.04
2	Addition of ^{14}C-dextran to filter paper squares with methanol washing	1.10	1.10	1.11	1.10 ± 0.00
3	Precipitation of dextran, dehydration, and weighing	1.94	2.02	1.98	1.98 ± 0.03

[a] The assays were run at 30 °C.
[b] IU = International Unit = 1.0 μmol of D-glucose incorporated into dextran/mL/min.
[c] LSC = liquid scintillation counting.

Method-6 is the measurement of the reducing value by DNS after the removal of dextran by precipitation with two volumes of ethanol. This method also gave a high IU value of 308 units due to over-oxidation of the D-fructose and leucrose in the digest, which is high but less than that of Method-5, due to the removal of dextran, confirming the over-oxidation of dextran in Method-5. This method, too, is completely invalid for assays and for kinetic studies of dextransucrases, as it is 140 U greater than that of 168 U for the direct methods for determining dextran.

Method-7 is also the measurement of the reducing value of the sucrose/dextransucrase digest after the removal of dextran by precipitation with two volumes of ethanol. The reducing value used in this method was the ferricyanide/arsenomolybdate method[54] that also gave a high IU of 353, with a relatively high mean deviation. This also is due to the relatively high alkaline conditions required for the reduction that give over-oxidation and is unacceptable for assays and kinetic studies.

Method-8 is the measurement of the reducing value by the copper bicinchoninate method[55] after the removal of dextran by precipitation with two volumes of ethanol. This method gave an IU of dextransucrase of 183, which is not nearly as high as that of the two alkaline DNS methods and the ferricyanide/arsenomolybdate method. The copper bicinchoninate method is the most sensitive of any of the known reducing value methods and gave 15 IU greater than the direct measurement of Methods 1 and 3. This must be attributed to some over-oxidation of the D-fructose and D-glucose, and the reducing value of leucrose. The method does not give over-oxidation of oligosaccharides and polysaccharides as shown in Figure 1 and is sensitive enough to give reducing values of polysaccharides that can be used in determining the D.P. and the number-average molecular weights of polysaccharides.[59–61]

Method-9 utilizes an ‘enzyme kit’ obtained from Megazyme that determines the amount of D-fructose. This method gave a relatively low IU value for dextransucrase of 149 IU. This method is similar to that of Method-4 that gave an IU of 151 with values of 19 and 17 IU, respectively, below the values obtained by Methods 1 and 3.

3.3. Assay of a dextransucrase produced by another *L. mesenteroides* strain obtained from Brazil

Because all of the above-described assay methods involved the dextransucrase preparation obtained from *L. mesenteroides* B-512FMC, we also performed methods 1–3 with a second dextransucrase produced by a *L. mesenteroides* strain FT045B obtained from Brazil. The results are given in Table 2, which shows the same relative results of 1.98 IU obtained by Methods 1 and 3 for this dextransucrase, and a lower value of 1.10 IU for Method-2, which is consistent with the results obtained for B-512FMC dextransucrase.

4. Conclusions

It is concluded that the direct measurement of dextran, after precipitation from the dextransucrase digest and re-precipitation two additional times, followed by dehydration (Methods 1 and 3) give the most accurate measurement of the amount of dextran synthesized by dextransucrase, and, therefore, are the methods to be used to obtain an accurate and useful activity of dextransucrase for assays and kinetic studies. These methods can be used for any glucansucrase that synthesizes glucan from sucrose, such as mutan, alternan, comb-dextrans, and so forth. We further conclude that the use of reducing value methods to determine the activity of dextransucrase and related enzymes by measuring the putative reducing value of D-fructose gives highly inflated and unacceptable values due to over-oxidation of D-fructose and other reducing sugars in the digest, as well as dextran if it is allowed to remain in the assay solution. This is especially so for DNS and the ferricyanide/arsenomolybdate methods, both in the presence of dextran and in its absence. The ferricyanide/arsenomolybdate method gave an even higher value for dextransucrase, in the absence of dextran, than did the DNS method. Unfortunately, many laboratories have found the relative ease of using the DNS method for determining the activity of dextransucrases and related enzymes and apparently have not been aware of the problems of over-oxidation by DNS, giving highly inflated assays and kinetics and consequently incorrect measurements.

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