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Structural characterization of a new dextran with a low degree of branching produced by *Leuconostoc mesenteroides* FT045B dextransucrase

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ABSTRACT

This paper offers the physical and chemical characterization of a new dextran produced by *Leuconostoc mesenteroides* FT045B. The chemical structure was determined by Fourier Transform Infrared spectroscopy and ^{1}H Nuclear Magnetic Resonance spectroscopy. The dextran was hydrolyzed by endodextranase; the products were analyzed using thin layer chromatography and compared with those of commercial B-512F dextran. The number-average molecular weight and degree of polymerization of the FT045B dextran were determined by the measurement of the reducing value using the copper bicinchoninate method and the measurement of total carbohydrate using the phenol–sulfuric acid method. The data revealed that the structure of the dextran synthesized by FT045B dextran sucrase is composed of D-glucose residues, containing 97.9% α-(1,6) linkages in the main chains and 2.1% α-(1,3) branch linkages compared with the commercial B-512F dextran, which has 95% α-(1,6) linkages in the main chains and 5% α-(1,3) branch linkages.

1. Introduction

Dextran is an extracellular bacterial homopolysaccharide (Sidebotham, 1974; Monsan et al., 2001), the main chain of which is composed of contiguous α-(1,6)-linked D-glucopyranose residues and branches, stemming mainly side-chains of α-(1,6) glucose units attached by α-(1,3) branch linkages to α-(1,6)-linked chains (Buchholz & Monsan, 2001; Dols, Remaud-Simeon, Willemot, Vignon, & Monsan, 1998; Naessens, Cerdobbel, Soetaert, & Vandamme, 2005; Robyt, 1995; Seymour & Knapp, 1980). Some dextrans have α-(1,2) or α-(1,4) branch linkages, depending on the bacterial source. The exact structure of each type of dextran depends on the degree of branching, involving α-(1,2), α-(1,3) and α-(1,4) linkages (Seymour & Knapp, 1980), which depends on the specific microbial strain and, hence, on the specificity of dextransucrase(s) EC.2.4.1.5 involved (Jeanes et al., 1954). Dextrans can be produced by growing various bacterial strains of *Leuconostoc mesenteroides* and *Streptococcus* species on a medium containing sucrose (Kim, Robyt, Lee, Lee, & Kim, 2003). Dextran production is influenced by a number of factors: physical and chemical properties (Jeanes, 1965), such as solubility, viscosity, specific optical rotation and the content of nitrogen, phosphorus and ash in the medium, which further depends on the specific microorganisms involved (Jeanes et al., 1954). A single enzyme can catalyze the synthesis of several types of dextran linkages, thereby permitting the formation of a branched polymer (Neely & Nott, 1962; Smith, Sahnley, & Goodman, 1994). On the other hand, certain bacterial strains have been shown to produce dextrans of different structures, which have been attributed to the excretion of different dextransucrases (Côté & Robyt, 1982; Figures & Edwards, 1981; Zahley & Smith, 1995). Thus, the structure of each dextran is a characteristic of the specific dextransucrase (Jeanes et al., 1954). The present study discusses the structure of a water-soluble dextran produced by a dextransucrase elaborated by *L. mesenteroides* FT045B. Fourier Transform Infrared (FTIR) spectroscopy, ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and TLC (Thin Layer Chromatography) of oligosaccharides obtained from polymer hydrolysis by endodextranase isolated from *Penicillium* sp were used for the determination of the structure.

The number average molecular weight (MWn) and degree of polymerization (DPn) were determined by the measurement of the reducing value using the copper bicinchoninate method and the measurement of total carbohydrate using the phenol–sulfuric acid method.

Abbreviations: DPn, number average degree of polymerization; FTIR, Fourier Transform Infrared; ^{1}H NMR, ^{1}H Nuclear Magnetic Resonance; MWn, number-average molecular weight; NCBI, National Center for Biotechnology Information; Pyr/Ac, Pyridine/Acetate; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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2. Experimental

2.1. Organism

L. mesenteroides FT045B was isolated from an alcohol and sugar mill plant and was provided by the Microbiology Industrial Process Control Division of Fermentec (Brazil). The nucleotide sequence was submitted to the GenBank sequence database (GenBank ID: JF812153.1).

2.2. Enzymes

(a) *L. mesenteroides* B-512FMC dextransucrase (heretofore B-512FMC dextransucrase) was obtained from a constitutive mutant of *L. mesenteroides* B-512FMC (Kim & Robyt, 1994; Zahley & Smith, 1995) which was provided by the Laboratory of Carbohydrate Chemistry and Enzymology, Iowa State University (USA). The culture supernatant was concentrated by passing it through a polysulfone ultrafiltration hollow fiber cartridge (0.1 m × 1.0 m, H5P100-43, Amicon, Inc., Beverly, MA, USA) at a flow rate of 4 L min^{-1} at 21 °C. When the volume of the concentrate was approximately 1 L, small molecules were removed by passing, (10 times) through 500 mL of 20 mM pyridine/acetic acid buffer, pH 5.2, containing 0.04% PVA 10 K and 1 mM $CaCl_2$. The concentrated enzyme was then collected and the hollow fiber cartridge was washed with approximately 400 mL of the buffer which was added to the concentrate (Kitaoka & Robyt, 1998). B-512FMC dextransucrase activity was 89 IU mL^{-1}, as determined by the ^{14}C-sucrose assay (Vettori, Mukerjea, & Robyt, 2011), in which 1.0 IU = 1.0 μmol of α-D-glucose incorporated into dextran per min.

(b) *L. mesenteroides* FT045B dextransucrase (hereinafter FT045B dextransucrase) was obtained from *L. mesenteroides* FT045B. The growth medium for *L. mesenteroides* FT045 B was sucrose (40 g L^{-1}), yeast extract (20 g L^{-1}), K_2HPO_4 (20 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), $MgSO_4$ (0.2 g L^{-1}), NaCl (0.01 g L^{-1}), $FeSO_4$ (0.01 g L^{-1}) and $MnSO_4$ (0.01 g L^{-1}). The strain was stored at −20 °C in a cryogenic solution until preparation of the inoculum for fermentation. The fermentation inoculum accounted for up to 5% (v/v) of the total volume of the medium. The microorganism was transferred from the stock culture to the same medium and was grown for 18 h at 25 °C and 150 rpm. The culture supernatant was obtained by centrifugation for 30 min at 13,000 × *g* and stored at 4 °C during the execution of the experiments. FT045B dextransucrase activity was 2 IU mL^{-1}, as determined by the ^{14}C-sucrose assay described above.

(c) Dextranase from *Penicillium* sp was obtained from Sigma [lyophilized powder, 10–25 units mg^{-1} of solid]; one unit releases 1.0 μmole of isomaltose (measured as maltose) per min at pH 6.0 and 37 °C using dextran as substrate. The powder was dissolved in a buffer solution (about 4 mL of 20 mM Pyr/Ac, pH 5.5 for 1 mg of powder) to obtain 5 U mL^{-1}.

2.3. Dextran reference

The B-512F dextran was purchased from Sigma Chemical Co.

2.4. Dextran production

The enzyme digest solution was prepared with the addition of 40 mL of buffer solution (20 mM Pyr/Ac, pH 5.2) to 50 mL of 400 mM sucrose and preheated to 30 °C for 10 min. The enzyme reaction was started by adding 10.0 mL of the dextransucrase (2 U mL^{-1}) produced by *L. mesenteroides* FT045B and incubated at 30 °C for 33 h (2 conversion periods). At the end of the reaction 200 mL of cold ethanol was added to precipitate the synthesized dextran and the solution was kept at 4 °C for 24 h. The precipitated dextran was centrifuged at 13,000 × *g* for 30 min and dissolved in 100 mL of water. After complete solubilization, the solution was cooled in ice for 10 min. The dextran was precipitated again with the addition of 200 mL of ethanol, which was mixed and centrifuged for 30 min at 13,000 × *g*. Solubilization and precipitation were repeated once more to ensure that there was no remaining fructose or sucrose. The dextran was treated five to seven times with acetone (30 mL) and once with anhydrous ethanol (30 mL) and then placed into a vacuum oven at 40 °C for 18 h.

2.5. The number-average molecular weight (MWn) and degree of polymerization (DPn)

The MWn and DPn (Jane & Robyt, 1984) of the dextran were determined by the measurement of the reducing value using the copper bicinchoninate method and the measurement of total carbohydrates using the phenol–sulfuric acid method (Fox & Robyt, 1991); DPn = [(total carbohydrates in μg of D-glucose)/(reducing value in μg of maltose)] × 1.9; MWn = [(DPn) × 162] + 18.

2.6. Dextran hydrolysis by endodextranase

The dextran (10 mg) was dissolved in 960 μL of buffer solution (20 mM Pyr/Ac, pH 5.5); an aliquot of 40 μL of *Penicillium* sp endodextranase (5 U mL^{-1}) was added and incubated at 37 °C for 14 h. Aliquots of 100 μL were taken at 10, 20, 30, 40, 50, 60 and 840 min. Five μL of concentrated trifluoroacetic acid (TFA) were added and the aliquots (final concentration of 6.14 M TFA) were heated to 50 °C for 10 min to stop the reaction.

2.7. Thin layer chromatography

An aliquot of 1 μL of each sample and of the isomaltodextrin and maltodextrin standards was applied 15 mm from the bottom of a thin layer chromatography (TLC) plate (Whatman 5K) using a 5 μL Hamilton microsyringe pipette. Each spot size was kept between 2 and 3 mm distant from each other by adding 1 μL at a time, with intervals for drying. The plate was irrigated with three ascents to the top at 20–22 °C using, 85:20:50:70 (v/v/v/v) acetonitrile/ethyl acetate/1-propanol/water. After development, the carbohydrates were visualized by dipping the TLC plate into an ethanol solution containing 0.5% (w/v) α-naphthol and 5% (v/v) H_2SO_4. After air drying, the TLC plate was placed into an oven at 120 °C for 10 min. The densities of the carbohydrate spots on the TLC plate were determined using an imaging densitometer (Bio-Rad densitometer, Model GS-670) (Robyt & Mukerjea, 1994; Robyt, 2000).

2.8. ^{1}H NMR spectroscopy

One milligram of dextran from B-512F and 1 mg of dextran from FT045B were dissolved separately in 0.5 mL of pure D_2O and then placed into 5 mm NMR tubes. ^{1}H NMR spectra were obtained from a Bruker DRX 500 spectrometer, operating at 500 MHz at 25 °C. Acetone (2.22 ppm) was used as the standard for adjusting the ^{1}H NMR chemical shifts.

2.9. Fourier-Transform Infrared spectrometric analysis

The samples of FTIR spectra for the native dextran from B-512F and FT045B were prepared by mixing 150 mg of KBr with 5 mg of each dextran under press.

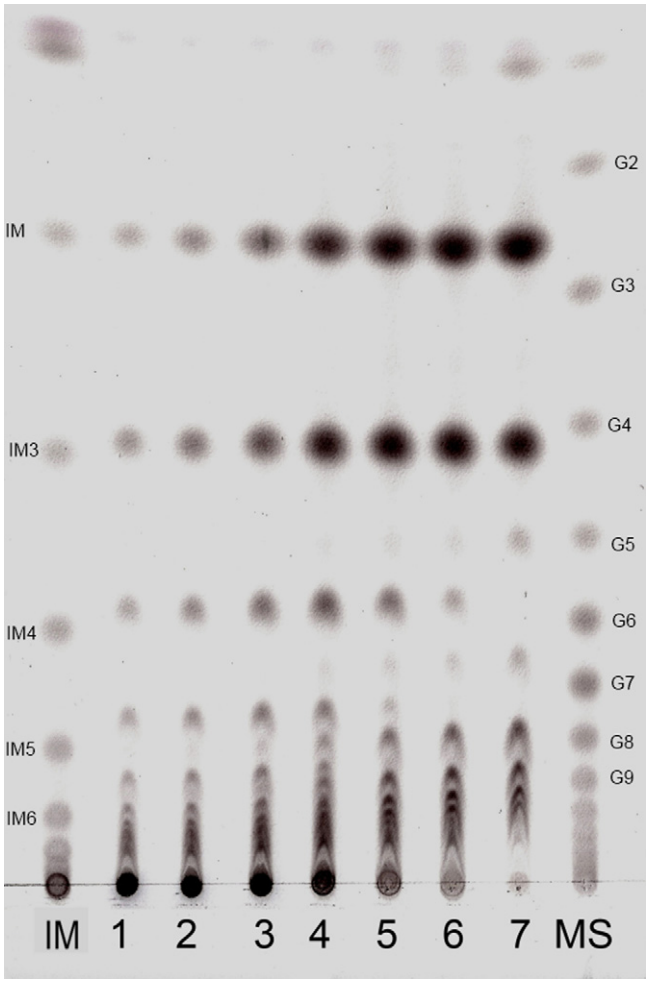

Fig. 1. TLC analysis of hydrolysis of dextran FT045B with endodextranase (5 U/mL) at 37 °C. Lane 1, 2, 3, 4, 5, 6, and 7 refer to samples taken at 10, 20, 30, 60, 90, 120 and 840 min, respectively; IM = isomaltodextrin standards (IM = isomaltose, IM3 = isomaltotriose, and so forth); MS = maltodextrin standards (G2 = maltose; G3 = maltotriose, and so forth).

3. Results and discussion

The dextran produced by *L. mesenteroides* FT045B had a number average molecular weight of 91,536 Da and DPn of 565. Dextran hydrolysis by endodextranase requires a minimum of six or seven non-substituted contiguously linked α-(1,6) glucose residues (Boune, Huston, & Weigel, 1962; Côté & Robyt, 1984; Richards & Streamer, 1978). Thus, the decision was made to make certain that *L. mesenteroides* FT045B was producing a real dextran with predominantly α-(1,6)-glucopyranosidic linkages within the main chains prior to carrying out the other characterization methods (Buchholz & Monsan, 2001; Dols et al., 1998; Seymour & Knapp, 1980).

The mixture of oligosaccharides obtained from FT045B dextran hydrolysis were applied to TLC (Fig. 1) and densities were determined to find the quantitative amount of each product by scanning imaging densitometry, as described by Robyt and Mukerjea (1994). The limit of hydrolysis by endodextranase was expressed as the apparent conversion into isomaltose. The major products after 840 min of hydrolysis with *Penicillium* sp dextranase (Fig. 1, lane 7) were isomaltose (33.2%) and isomaltotriose (24.6%). These were derived from the linear regions of the α-(1,6) glucan backbone chains. Moreover, the branched oligosaccharides, representing significant branches in dextran, branched isomaltotetraose (0.4%), isomaltopentaose (3.9%) and higher branched isomaltodextrins

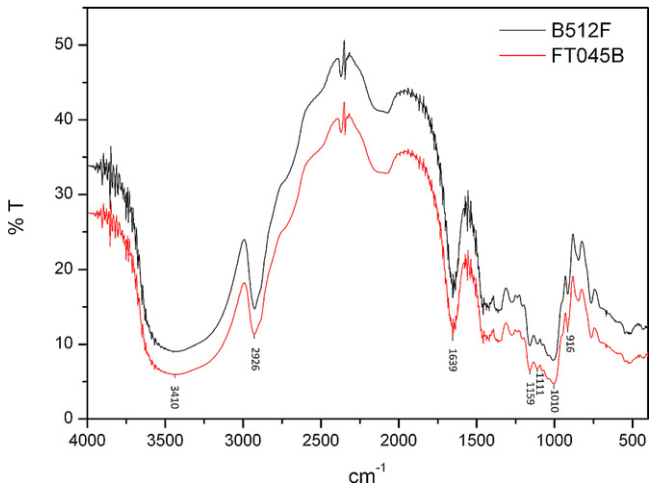

Fig. 2. FTIR spectra for commercial dextran from B512F from FT045B.

were obtained, indicating FT045B dextran branching. The type of the branching was then confirmed by NMR as α-(1,3) linkage.

The type of linkages and the functional groups of the commercial dextran from *L. mesenteroides* NRRL B-512F and dextran from FT045B were characterized by FTIR spectroscopic analysis. Fig. 2 shows the FTIR spectra of commercial dextran from *L. mesenteroides* B-512F and dextran from FT045B. Both dextrans gave very similar spectra, indicating a similar structure, although with different degrees of α-(1,3) branch linkages. Dextran from FT045B and B-512F contain α-(1,6) linkages, as demonstrated by the peak at 1010 cm^{-1}, which characterizes the considerable chain flexibility present in dextran around this type of glycosidic bond (Shingel, 2002). The α-glycosidic bond is also confirmed by the peak at 916 cm^{-1} and the band at 1159 cm^{-1} caused by covalent vibrations of the C—O—C bond and glycosidic bridge. The peak at 1111 cm^{-1} was due to the vibration of the C—O bond at the C-4 position of the glucose residue (Shingel, 2002). The hydroxyl stretching vibration of the polysaccharide caused the band in the region of 3410 cm^{-1} and the C—H stretching vibration caused the band in the region of 2926 cm^{-1} (Capek et al., 2011; Liu, Lin, Ye, Xing, & Xi, 2007). The band in the region of 1639 cm^{-1} was due to bound water (Park, 1971). The α-(1,6) linkages were further confirmed by ^1H NMR analysis.

The ^1H NMR spectra afford compelling evidence for the main structural features of dextran. The individual assignments derived from the present experiments and those reported in the literature are displayed in Table 1.

Fig. 3 displays the ^1H NMR spectra of the native dextran from *L. mesenteroides* B-512F and FT045B in D_2O. The results show that the dextran from *L. m.* B-512F and *L. m.* FT045B have a similar structure, although the α-(1,3) branch linkages and α-(1,6)

Table 1
Assignments of individual signals in ^1H NMR spectra of the native dextran from B-512F (used as standard) and from FT045B dextran.

	B-512F (reported values)	B-512F (experimental)	FT045B (experimental)
	^1H (ppm)[a]	^1H (ppm)	^1H (ppm)
C1—H	4.99	4.91	4.90
C2—H	3.6	3.50	3.51
C3—H	3.76	3.65	3.63
C4—H	3.52	4.45	3.42
C5—H	3.88–4.04	3.85	3.85
C6—H	3.88–4.04	3.93	3.95
C6′—H	3.81–3.86	3.70	3.70

[a] Gil et al. (2008).

Table 2
Different linkage structures found in all dextran series and reported in literature.

		Percent of glycosidic linkages[a]				
		α-1,6	α-1,3	α-1,3 branch	α-1,4 branch	α-1,2 branch
Leuconostoc mesenteroides						
FT 045B	S[b]	97.9[e]		2.1[e]		
B-512F	S	95		5		
B-742	L[c]	87			13	
B-742	S	50		50		
B-1355	L	95		5		
B-1355	S	54	35	11		
B-1299	L	66		7		27
B-1299	S	65				35
B-1498	S	50	50			
B-1501	S	50	50			
Streptococcus downei						
Mfe28		12	88			
Mfe28		90	10			
Streptococcus mutans						
GS5		13	87			
GS5		15	85			
GS5		70	30			
6715	S	64		36		
6715	I[d]	4	94	2		

[a] Adapted from Monsan et al. (2001).
[b] S = soluble.
[c] L = low soluble.
[d] I = insoluble.
[e] Determined in this study.

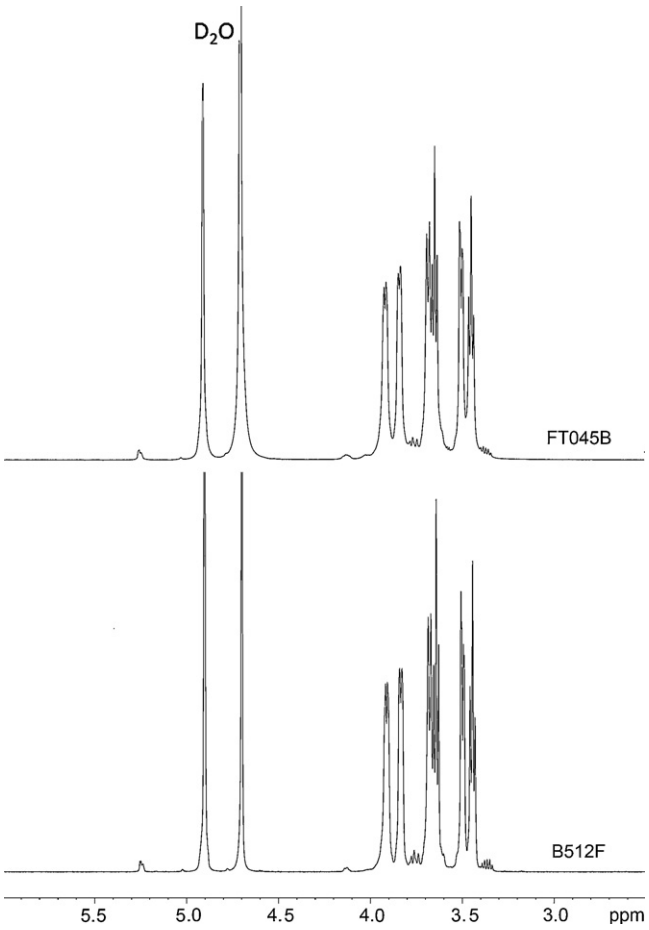

Fig. 3. ^{1}H NMR spectra of native dextran from *Leuconostoc mesenteroides* B512-F and FT045B in D_2O.

linkages differ in terms of percentage. Dextrans of different structures can be produced by specific dextransucrases, each excreted by a certain bacterial strain (Zahley & Smith, 1995). Table 2 displays the different linkage structures found in the dextran series produced by *Leuconostoc mesenteroides*, *Streptococcus downei*, and *Streptococcus mutans* as well as the comparison between FT045B dextran, with 97.9% α-1,6 linkages and 2.1% α-1,3 branch linkages, and B-512F dextran, which has about more than twice as many α-1,3 branch linkages (5%).

The three signals that appear centered at ~5.25 ppm (not shown) represent important structural characteristics of α-(1,3)-linked-D-glucosyl residues. The peak at 4.90 ppm is due to the H-1 of the α-(1,6) glucosyl residues of the main chain (Purama, Goswami, Khan, & Goyal, 2009; Seymour, Knapp, & Bishop, 1979). The integration of signals 5.25 and ~4.90 gives the percentage of α-(1,3)-linked D-glucosyl and α-(1,6)-linked D-glucosyl linkages, respectively (Table 2).

The FTIR and ^{1}H NMR spectral analyses confirmed that the polysaccharide produced from *L. mesenteroides* FT045B is an α-(1,6) low-branched dextran, with 2.1% α-(1,3) branch linkages, giving an average chain length of $(1/2.1) \times 100 = 48$ glucose units between branch linkages, with 97.9% α-(1,6) linkages. The commercial B-512F dextran from Sigma exhibits 5.0% branching, giving $(1/5) \times 100 = 20$ glucose units between branch linkages, which is a significant difference. There are 21 branch linkages for every 1000 glucose units of FT045B dextran and 50 branch linkages for every 1000 glucose units of B-512F dextran, indicating a significant difference in structure. B-512F dextran has 5% α-(1,3) branch linkages with an average chain length of 20 glucose units and FT045B dextran has 2.1% linkages with an average chain length of 48 glucose units, which is more than twice that of B-512F dextran. Branching, however, can also occur in different amounts and different distributions, such as (1) random distribution on the main and/or secondary chains, (2) regular distribution on every other glucose unit in the main chains, or (3) on every glucose unit in the main chains and (4) different branch chain lengths, depending on the amount of branching. Thus, there may be many different dextran structures that have yet to be identified and characterized and that may have different properties to be determined in further studies. FT045B dextran is

one of these, in which the branching is less than half that of the B-512F commercial dextran or any other dextran that has been characterized so far. This produces a chain length that is more than twice the length (48 *vs.* 20 glucose units, for FT045B dextran and B-512F dextran, respectively). This increased chain length should give different chemical and physical properties, in comparison to other dextrans.

The discovery of new dextrans that have different structures and, hence, potentially different properties, resulting from different structures, is important with regard to potential novel applications and the potential improvement in or alterations to previously established applications.

4. Conclusions

The present experiments demonstrated that the dextran produced by *L. mesenteroides* FT045B has a structure similar to that of *L. mesenteroides* B-512F dextran, but with 97.9% α-1,6 linkages and 2.1% α-1,3 branch linkages, as compared to B-512F dextran, which has more than twice as many α-1,3 branch linkages (5%). The FT045B dextran has an average branch chain length of 48 D-glucopyranose residues/molecule, as compared to B-512F dextran, which that has an average branch chain length of 20 D-glucopyranose residues/molecule.

The structure of the FT045B dextran, with 2.1% branching, is obviously not linear and would have 12 branch linkages per 565 D-glucose residues in a dextran with a MWn of 91,536 Da, as determined in the present study.

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