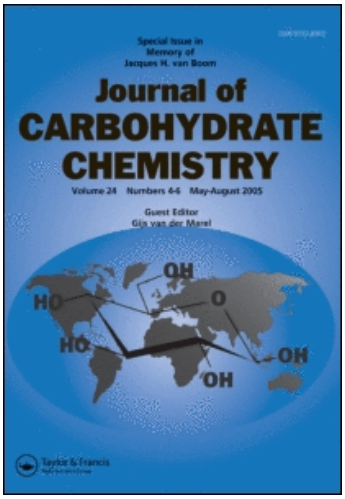

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Molecular Weight Characterization and Structural Properties of Controlled Molecular Weight Dextrans Synthesized by Acceptor Reaction using Highly Purified Dextransucrase

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MOLECULAR WEIGHT CHARACTERIZATION AND STRUCTURAL PROPERTIES OF CONTROLLED MOLECULAR WEIGHT DEXTRANS SYNTHESIZED BY ACCEPTOR REACTION USING HIGHLY PURIFIED DEXTRANSUCRASE

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ABSTRACT

Controlled molecular weight dextrans were synthesized using a highly purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F in a multi-step process. Maltose was used as acceptor for the first reaction step. The purified product obtained at a given reaction step was used as acceptor for the next reaction step. Dextrans of molecular weights ranging from 1,000 to 100,000 were thus obtained with a good yield (80 %). After purification, the molecular weight distribution of the products was characterized using size exclusion chromatography coupled with low angle laser light scattering (LALLS). Polydispersity of the products was shown to be similar to that of commercial dextrans.

^{13}C NMR spectra and enzymatic hydrolysis data are consistent with the fact that the enzymatically synthesized dextrans are

essentially composed of α(1->6) linkages. It was observed that controlled molecular weight dextrans were less branched than commercial products obtained by acidic hydrolysis of high molecular weight dextrans.

INTRODUCTION

Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (EC 2.4.1.5.) is a glucosyltransferase which catalyzes the synthesis of dextran, a polyglucan composed of α(1->6) linkages (95 %) and of α(1->3) linkages (5 %), from sucrose.[1] When other sugars are added to the reaction mixture, the enzyme can also transfer glucosyl residues from sucrose to the free hydroxyl groups of sugars which act as acceptors.[2-9] Several authors determined the acceptor efficiency of various mono- and disaccharides according to their ability to divert the glucosyl residues from dextran to form oligosaccharides and to their effect on the reaction rate.[2-9] Among disaccharides, maltose and isomaltose have been demonstrated to be the most efficient acceptors.[2,8] After reaction with maltose, a series of oligosaccharides containing α(1->6) linkages and a molecule of panose (6-0-α-D-glucosylmaltose) at their reducing end is produced. Low molecular weight (LMW) dextrans obtained from acidic hydrolysis of high molecular weight (HMW) dextrans or from the acceptor reaction with maltose were also shown to act as acceptors and to present a better efficiency than the initial accceptors, maltose or glucose.[3,4,6,9-11] The application of dextrans to the medical and analytical fields most often requires LMW dextrans: for example, clinical dextran used as blood plasma substitute presents an average molecular weight of 70,000. Presently, such products are prepared from acidic hydrolysis of HMW dextrans followed by alcohol precipitations. They present a low polydispersity and a degree of linearity around 95 %.

In order to synthesize controlled molecular weight (CMW) dextrans having the same properties as clinical dextran, Paul *et al.* studied more precisely an acceptor reaction with maltose and with maltose reaction oligodextrans.[10,11] They used for this purpose a highly purified dextransucrase produced by fed-batch culture of *Leuconostoc mesenteroides* NRRL B-512F and purified by phase-

partition.[12,13] The sucrose/acceptor ratio (S/A) was shown to be a very important parameter, which allowed control of the molecular weight and the polydispersity of the products. With optimized reaction conditions, it was thus possible to synthesize dextrans with molecular weight ranging from 500 to 20,000.[10]

In the present paper, we describe the enzymatic synthesis of CMW dextrans in a multi-step process, using maltose as initial acceptor. Purified LMW dextrans obtained after each enzymatic step were used as acceptors. The structural characteristics of enzymatically synthesized dextrans were further compared to those of commercial dextrans.

RESULTS AND DISCUSSION

Dextran Synthesis

In order to study the different properties of LMW dextrans produced in a multi-step process, we carried out acceptor reactions according to the following scheme:

Step 1:
sucrose + maltose --------> LMW dextran-1 + fructose
Step2:
sucrose + LMW dextran-1 --------> LMW dextran-2 + fructose
Step 3:
sucrose + LMW dextran-2 --------> LMW dextran-3 + fructose
Step 4:
sucrose + LMW dextran-3 --------> LMW dextran-4 + fructose

The optimization of the reaction conditions for enzymatic steps 1 and 2 was described by Paul *et al.*.[10] The molecular weight average ($M\overline{W}$) of LMW dextrans 1 and 2 was equal to 1,500 and 3,800 respectively. We determined the effect of the S/A ratio on the efficiency of the synthesis of LMW dextrans produced at steps 3 and 4. The corresponding yields of LMW dextrans, HMW dextrans and leucrose (5-O-α-D-gluco-β-D-fructopyranose, fructose acceptor reaction product) are given in Tables 1 and 2. LMW dextran yields are very close to 80 % (for each reaction step). These values confirm the results of Hehre and show that LMW dextrans are more and more efficient acceptors, as their molecular weight is increasing.[4]

TABLE 1
Step 3: Synthesis Yield of Dextransucrase Reaction Products

$\overline{Mw}$ of acceptor	Sucrose/ Acceptor (w/w)	Yield of products (%)		
		LMW dextrans (a)	HMW dextrans (b)	Leucrose (c)
3,800	3	91	2.8	1.5
3,800	5	86	3.9	2.3
3,800	10	86	5.6	3.2

(a) calculated as the ratio of:

$$\frac{\text{LMW dextrans (g/L)}}{\text{(acceptor (g/L) + 0.474 sucrose (g/L))}},$$

(b) calculated as the ratio of:

$$\frac{\text{HMW dextrans (g/L)}}{\text{(acceptor (g/L) + 0.474 sucrose (g/L))}},$$

(c) calculated as the ratio of:

$$\frac{\text{leucrose (g/L)}}{\text{sucrose (g/L)}}$$

TABLE 2
Step 4: Synthesis Yield of Dextransucrase Reaction Products

$\overline{Mw}$ of acceptor	Sucrose/ Acceptor (w/w)	Yield of products (%)		
		LMW dextrans (a)	HMW dextrans (b)	Leucrose (c)
9,800	3	83	2.8	8
9,800	5	83	3.5	9
9,800	20	79	4.0	11

(a), (b), (c): see foot-notes Table 1

Although HMW dextran and leucrose production remains limited, it is directly related to the S/A ratio and increases with it. Leucrose yield is increased at step 4 of the synthesis.

Molecular weight average determination and structural analyses were carried out for one product from step 3 and one product from step 4.

LMW dextran-3 was obtained with LMW dextran-2 ($M\overline{w}$ acceptor = 3,800) at S/A ratio = 3. It was purified as described in the "Experimental" section, and more than 80 % of the original product was recovered after purification. The polymer product has a purity of 93 %. Impurities are fructose and leucrose.

LMW dextran-4 was synthesized with LMW dextran-3 ($M\overline{w}$ = 9,800) at S/A ratio = 5. The use of ultrafiltration (membrane cut-off: 100,000) and isopropyl alcohol precipitation allowed elimination of HMW dextrans and any traces of leucrose and fructose, but LMW dextrans were recovered with lower yield (50 % of the original materials).

Molecular Weight Average Determination

Molecular weight averages of enzymatically synthesized LMW dextran-3 and dextran-4 and those of Dextran T-10® and Dextran T-70® purchased from PHARMACIA were determined using the same analysis system (Table 3). LMW dextran-3 and commercial Dextran T-10® have similar molecular weight values. Polydispersity of LMW dextran-3 is quite satisfactory when compared to that of Dextran T-10®.

The higher polydispersity observed for LMW dextran-4 when compared to Dextran T-70® can be attributed to inadequate purity. In fact, LALLS analysis shows the presence of molecules of very high molecular weight (above several millions) which were not eliminated during purification. On the other hand, a very weak response of the refractometer was recorded at the elution volume of these molecules (corresponding to the void volume of the column: Fig. 1-b). Values obtained without taking into account these impurities are closer to the values found for Dextran T-70® (Table 3).

TABLE 3
Molecular Weight Averages of LMW Dextrans

LMW dextrans	$\bar{M}w$	$\bar{M}n$	Poly-dipersity, $\bar{M}w/\bar{M}n$	Intrinsic viscosity, mL/g
Dextran-3	9,540	8,595	1.1	9.3
Dextran-4	85,690 (115,270)*	65,660 (66,030)*	1.3	23.0
T-10®	9,030	6,990	1.3	8.9
T-70®	64,140	51,140	1.2	26.0

* Values in brackets are calculated by taking into account impurities of high molecular weight (see Fig.1)

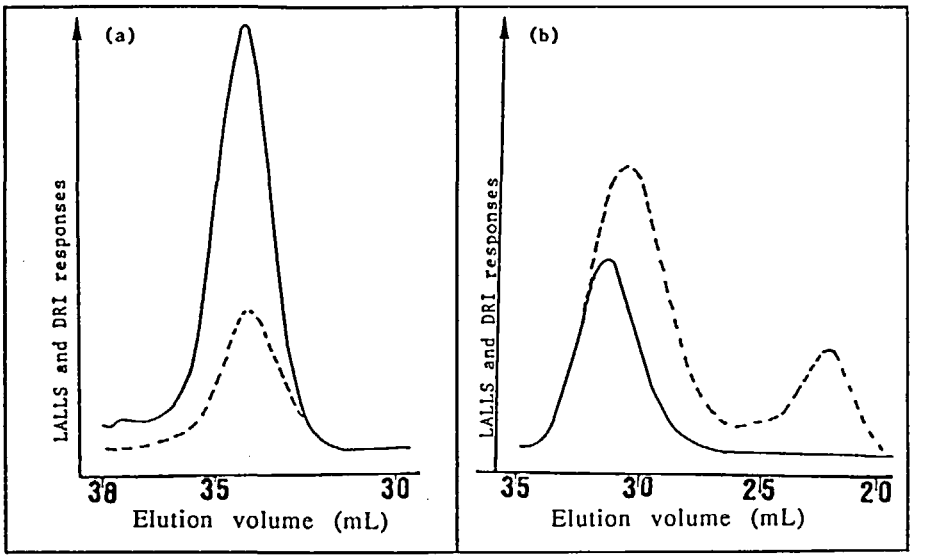

FIG. 1 Size exclusion chromatography of LMW dextrans:
(a) LMW dextran-3, (b) LMW dextran-4. Both LALLS (---) and DRI detector (___) responses were recorded. Two SHODEX OH PACK B-803 and SHODEX B-804 columns were connected in series. Sodium nitrate (0.1 M) was used as eluent at a flow rate of 1 mL/min.

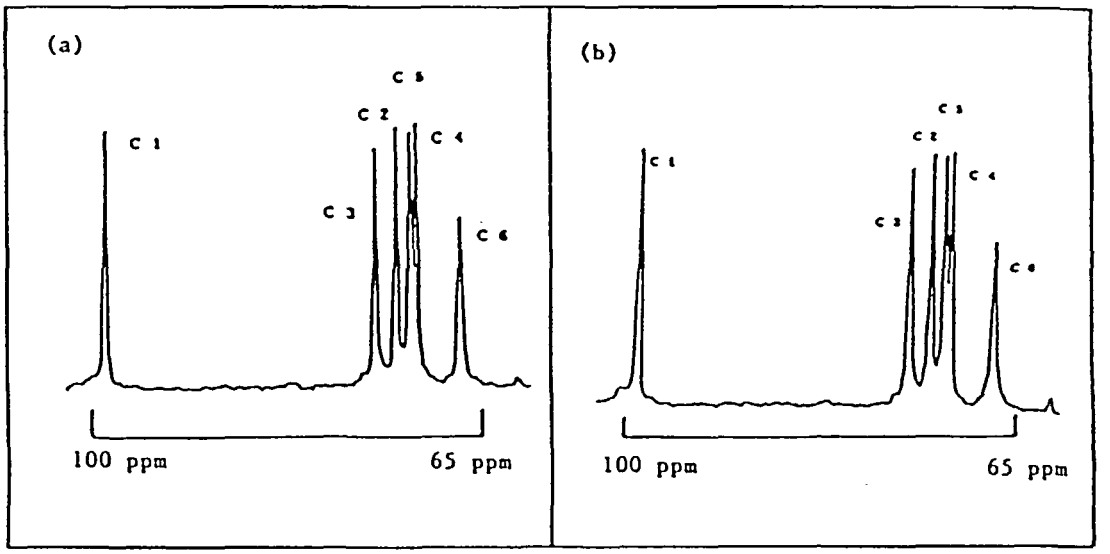

FIG.2 ^{13}C NMR spectra of dextrans:
(a) LMW dextran-4, (b) Dextran T-70®

^{13}C NMR Spectroscopy

Each reaction product was analysed by ^{13}C NMR spectroscopy. The corresponding spectra (Fig. 2) present the chemical shifts usually observed for linear dextrans at 98.7, 74.7, 72.5, 71.2, 70.7, 66 ppm.[14]

Endodextranase Analysis

HPLC chromatograms (Fig. 3 and 4) of the hydrolyzates show the distribution of the oligosaccharides present in the reaction mixture after 60 hours of hydrolysis. The degree of polymerization is inferior to 8 for all the products (Fig. 4). It proves that dextrans have been extensively hydrolyzed. The oligosaccharides which do not belong to the isomaltooligosaccharide series (B_4, B_5, B_6,...) were identified by showing that their retention times do not correspond to those of standard isomaltooligosaccharides (Fig. 3(d) and 4(d)).
The proportion of each product of the reaction mixture after 60 hours of hydrolysis was determined by measurement of the peak area (Table 4).

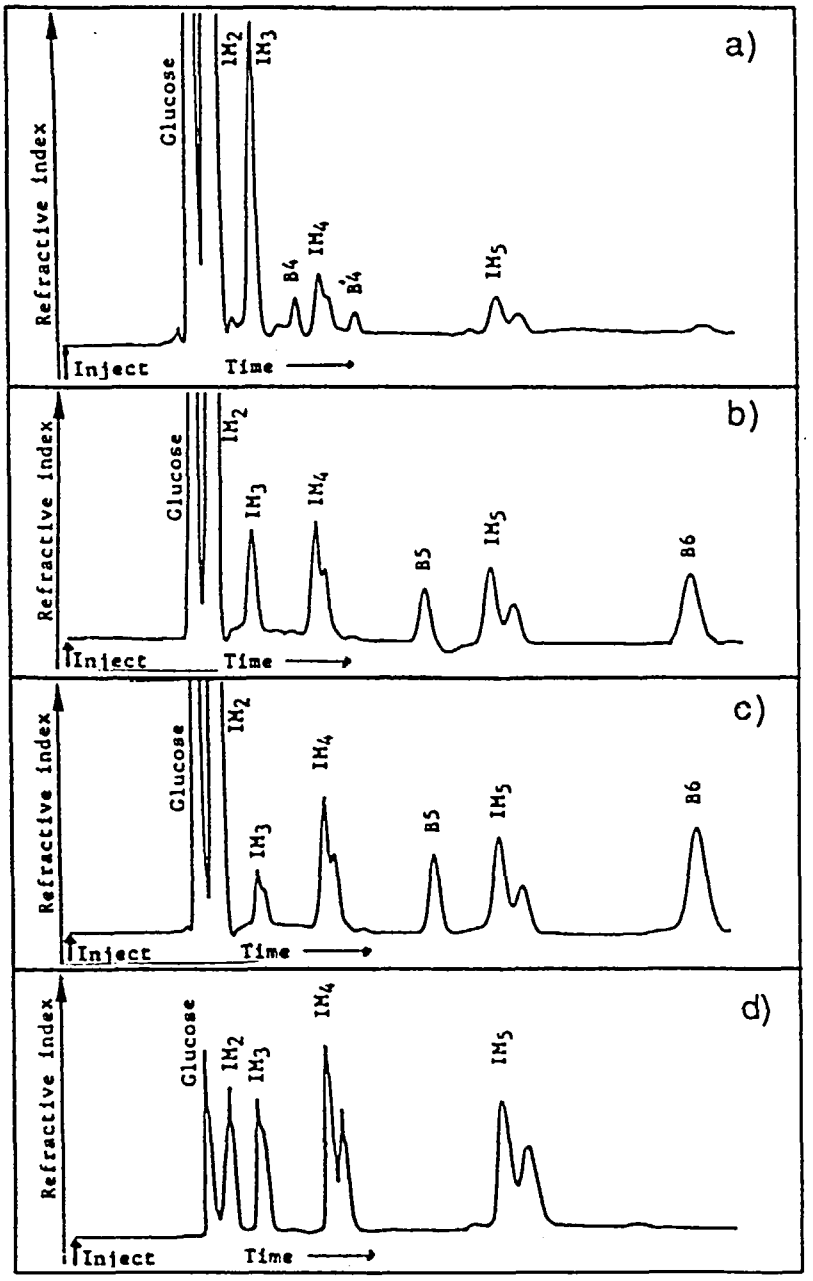

FIG.3 HPLC chromatograms of LMW dextran hydrolyzates

Hydrolysis of LMW dextran-3 (a), LMW dextran-4 (b) and Dextran T-70® (c) was carried out using endodextranase (SIGMA) for 60 hours . Analyses were conducted on a C18 column using water as eluent. The products were identified by comparison with standard isomaltooligosaccharides (d). IMn series represents isomaltooligosaccharides, Bn series represents branched products.

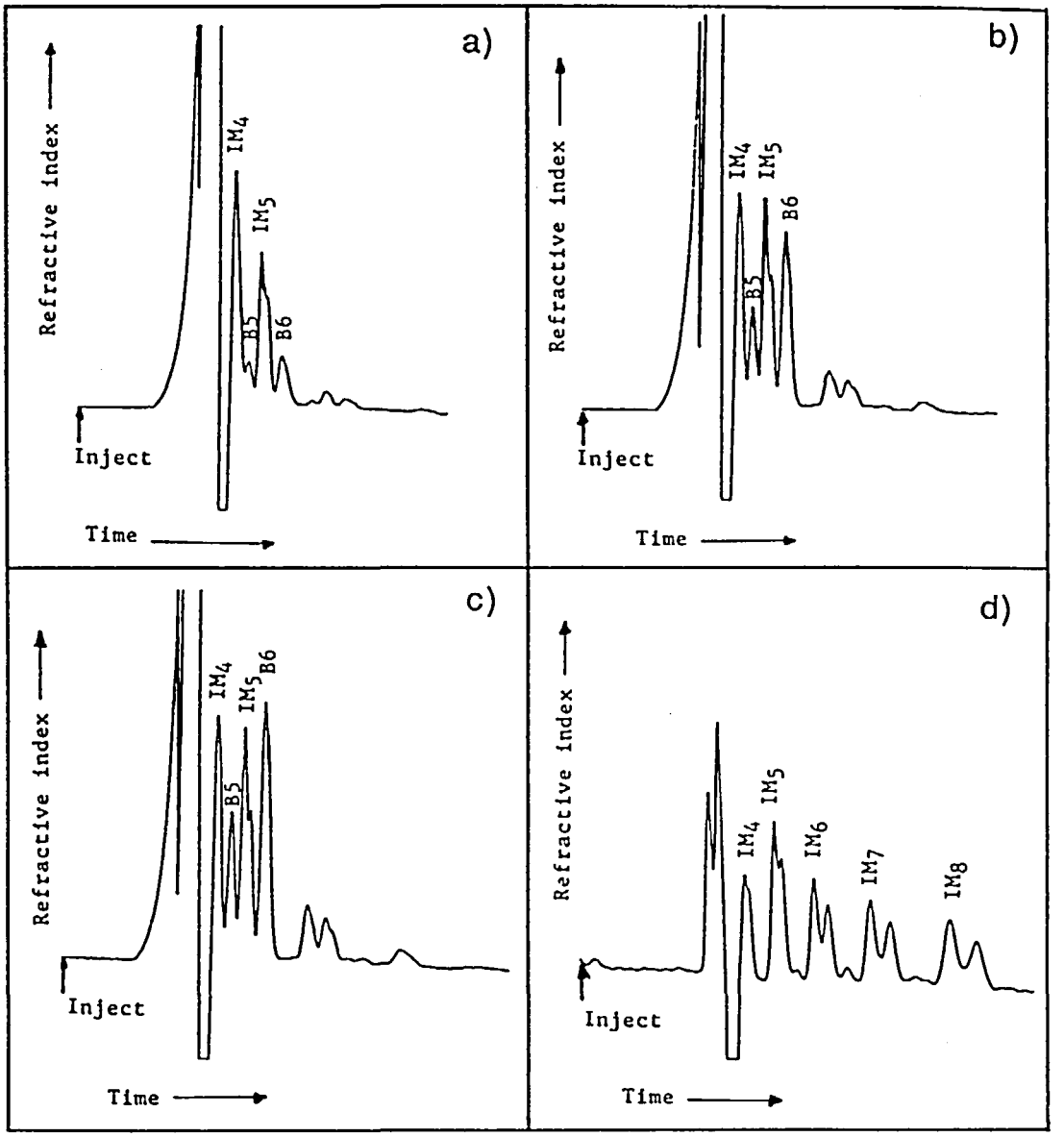

FIG. 4 HPLC chromatograms of oligosaccharides of d.p. higher than 4 obtained after 60 hours of endodextranase hydrolysis.

LMW dextran-3 (a), LMW dextran-4 (b), Dextran T-70® (c), standard isomaltooligosaccharides (d). The products were identified by comparison with standard isomaltooligosaccharides (d). IMn series represents isomaltooligosaccharides, Bn series represents branched products. Analyses were conducted on a C18 column using a mixture of water/methanol (96/4, v/v) as eluent.

TABLE 4
Distribution of Oligosaccharides after 60 Hours of Hydrolysis of LMW dextrans

LMW Dextrans	Yield of products (%)[a]									
	G[b]	IM_2	IM_3	B_4	IM_4	B'_4	B_5	IM_5	B_6	DP> B_6
Dextran-3	17.8	68.4	5.8	0.6	1.9	0.4	0.0	2.9	1.1	1.1
Dextran-4	20.5	62.3	2.4	0.0	3.4	0.0	1.5	3.9	3.6	2.4
T-10®	20.3	63.1	2.1	0.0	3.7	0.0	1.9	3.6	3.4	1.8
T-70®	19.7	60.7	1.8	0.0	4.4	0.0	2.1	4.2	4.8	2.4

(a) The percentage yield of each product was determined by measurement of their area divided by the total area of the chromatogram.
(b) Glucose

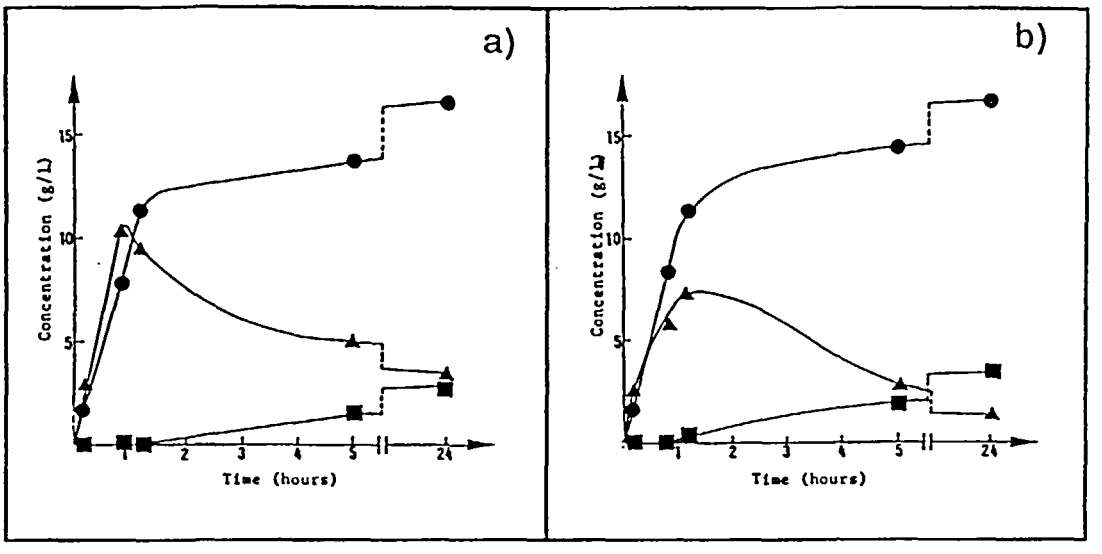

FIG.5 Production of glucose, isomaltose (IM_2) and isomaltotriose (IM_3) during hydrolysis of LMW dextran-3 (a) and Dextran T-10® (b) with endodextranase.

(■) glucose; (●) IM_2 ; (▲) IM_3. The concentration of the products was measured by HPLC analysis using a C18 column.

Fig. 5 shows the production of glucose, isomaltose and isomaltotriose during the hydrolysis of LMW dextran-3 ($M\overline{w}$ = 9,500) and Dextran T-10®. During the first hour of reaction, the production of isomaltotriose is higher for LMW dextran-3 than for Dextran T-10®. The hydrolysis profile of dextran-4 (not shown) is similar to the profile of Dextrans T-10® and T-70®. The differences observed between the enzymatically synthesized dextrans and the commercial ones decrease when the molecular weight increases.

According to the results previously obtained by Tsuchiya *et al.* and Hehre, LMW dextrans can be used as acceptors for subsequent synthesis reactions catalyzed by dextransucrase.[3,4] These LMW dextrans (ranging from 4,000 to 10,000 daltons) are very efficient acceptors. In fact, with a S/A ratio from 3 to 10, we obtained synthesis yields higher than 80 %. The yield of leucrose and HMW dextrans was about 10 %.

As it was already shown by Paul *et al.* for the first step of LMW dextran synthesis, the S/A ratio is the key parameter of the reaction.[10] It affects both the molecular weight and the polydispersity of the product. When it reaches a too high value, the production of leucrose and HMW dextrans increases (Tables 1 and 2). S/A ratios inferior to 5 are suitable for LMW dextran synthesis. In addition, narrow molecular weight distributions are obtained. The polydispersity is close to that of commercial dextrans ($M\overline{w}/M\overline{n}$ of LMW dextran-3 =1.1, $M\overline{w}/M\overline{n}$ of LMW dextran-4 = 1.3; Table 3).

^{13}C NMR spectra of the enzymatically synthesized dextrans represent the six typical chemical shifts of glucopyranosyl residues linked by α(1->6) osidic bonds, showing that LMW dextrans contain at least 95 % of α(1->6) linkages (Fig. 2). Endodextranase hydrolysis confirms these results. The percentage of residual oligosaccharides, which do not belong to the isomaltooligosaccharide series, are 3.2 %, 7.1 %, 7.5 % and 9.3 % for LMW dextran-3, Dextran T-10®, LMW dextran-4 and Dextran T-70® respectively (Table 4).

It was shown by Taylor *et al.* that two series of branched oligosaccharides were produced after the enzymatic hydrolysis of dextrans from *L. mesenteroides* NRRL B-512F.[15] These oligosaccharides were shown to contain α(1->3) branching linkages.

Consequently, we can assume that oligosaccharides B5 and B6, and higher molecular weight products, which do not belong to the isomaltooligosaccharide series, contain α(1->3) linkages (they are observed both in the Dextran T-70® and LMW dextran-4 hydrolyzates). Oligosaccharides B'_4 and B_4 only appeared after hydrolysis of LMW dextran-3. As they do not belong to the isomaltooligosaccharide series , they probably present α(1->3) and /or α(1->4) linkages in addition to the α(1->6) linkages. The α(1->4) linkage corresponds to the reducing end of the enzymatically synthesized dextrans, which is constituted by a maltosyl residue. In fact, α(1->4) linkages represent 1.7 % of the total linkages in a dextran of $M\overline{w}$ 10,000 and 0.2 % in a dextran of $M\overline{w}$ 80,000.

However, the yield of branched products is higher for dextrans obtained by acid hydrolysis than for enzymatically synthesized dextrans of quite similar molecular weights. An increase in the production rate of isomaltose and isomaltotriose is observed during the hydrolysis of LMW dextran-3, when compared to Dextran T-10®. This is consistent with a higher linear structure for LMW dextran-3.

We must also notice that the yield of the branched products increases with the molecular weight of CMW dextrans (Table 4). These results demonstrate that *L. mesenteroides* NRRL B-512F dextransucrase transfers specifically the glucosyl residues from sucrose on the primary hydroxyl group of LMW dextran acceptors. However, when the molecular weight of the acceptor increases, the accessibility of the primary hydroxyl group on C-6 at the non-reducing end of the acceptor could be more and more difficult. The enzymatic transfer then occurs more often on the C-3 secondary hydroxyl group of any glucosyl residue of the dextran acceptor.

Intrinsic viscosities of Dextran T-10® and LMW dextran-3 are very close. Values obtained for LMW dextran-4 and Dextran T-70® are respectively 23 and 26 mL/g. The intrinsic viscosity of LMW dextran-4 is smaller than that of Dextran T-70®, although LMW dextran-4 presents a higher molecular weight than Dextran T-70®. If there is any influence of the high molecular weight impurities of LMW dextran-4 on its intrinsic viscosity, it may be very low. However, those impurities should contribute to increase

the intrinsic viscosity of LMW dextran-4 and consequently reduce the difference observed between LMW dextran-4 and Dextran T-70®. In addition, LMW dextran-4 is less branched than Dextran T-70®. The lowest viscosity of LMW dextran-4 could be attributed to a difference in the length of branching between LMW dextran-4 and Dextran T-70®. Kuge *et al.* reported that long-chain branchings are more efficient in reducing the intrinsic viscosity than short chain ones.[16] This fact suggests that Dextran T-70® and LMW dextran-4 may present significant differences in their secondary structures and particularly in the length of their branching.

As a conclusion, the enzymatic synthesis of dextrans using a multi-step process can be envisaged for the production of highly linear CMW dextrans with a good reaction yield. The structural characteristics of such products are quite satisfactory compared to existing clinical dextran products.

EXPERIMENTAL

Dextransucrase Production and Purification

Dextransucrase was produced by fed-batch culture of *L. mesenteroides* NRRL B-512F as previously described.[10,12] Final culture dextransucrase activity was 8.8 U/mL. After cell removal by centrifugation (20 min, 4 °C, 10,000 g), extracellular dextransucrase was purified by aqueous two-phase partition between the dextran present in the supernatant and polyethyleneglycol ($M\overline{W}$ = 1,500) added to the culture supernatant.[13]

Dextransucrase was obtained in the dextran-rich phase in a concentrated, highly purified form. Successive phase-partition steps resulted in an enzyme preparation having an activity of 1.5 U/mg of dextran and 83 U/mg of protein.

One Unit (U) is defined as the amount of enzyme that catalyzes the formation of one µmole of D-fructose per minute at 30 °C, pH 5.2, in the presence of 100 g sucrose/L.

Dextran Synthesis

Reactions were carried out at 20 °C in 20 mM sodium acetate buffer, pH 5.2, with dextransucrase (0.25 U/mL) and sucrose (100 g/L) . Various amounts of purified dextran acceptors were added to the reaction mixture. After complete sucrose consumption, reaction was stopped by heating at 80 °C during 30 min. The solutions were then analyzed by size exclusion chromatography with micro-Bondagel E-linear columns (MILLIPORE-WATERS) using water as eluent. From the elution chromatograms, it was possible to measure the amount of HMW dextrans which is eluted in the void volume of the columns. Reverse-phase chromatography with a C18 bonded silica column (MILLIPORE-WATERS) was used to determine the concentration of leucrose synthesized. All HPLC analyses were performed with a system consisting in a M 6000-A pump, a U6K injector and a R-410 differential refractometer, all purchased from MILLIPORE-WATERS.

Dextran Purification

From the first to the third step of the enzymatic synthesis, fructose was eliminated by passing the dextran solution through a strongly acidic ion exchange resin containing Ca^{++} ions. At the fourth step, LMW dextrans were separated from fructose by ultrafiltration using AMICON hollow fiber system with a membrane cut-off of 10,000. Ultrafiltration (membrane cut-off: 100,000) or isopropyl alcohol precipitation (38 %, v/v) were used to eliminate HMW dextrans and dextransucrase.

Determination of Molecular Weight Distribution

The molecular weight distribution of LMW dextrans was determined by coupling size exclusion chromatography to low angle laser light scattering (LALLS) detector. Two SHODEX OH PAK B-803 and SHODEX B-804 columns were connected in series to a LALLS KMX detector (which gave the absolute molecular weight) and a IOTA differential refractive index (DRI) detector. Both LALLS and DRI responses were recorded. For fixed intervals, the absolute molecular weight (Mi) and concentration (Ci) were determined using data from both detectors (DRI and LALLS). Then, the

molecular weight averages and the polydipersity were calculated in the conventional manner:

$$M\bar{n} = \frac{\Sigma Ci}{\Sigma Ci/Mi} \qquad M\bar{w} = \frac{\Sigma Ci.Mi}{\Sigma Ci} \qquad P = M\bar{w}/M\bar{n}$$

The columns in series presented a void volume of 23.6 mL and a total volume of 39.6 mL. 250 µL of samples were injected, sample concentrations varying from 1 to 5 g/L. Sodium nitrate O.1 M was used as eluent.

^{13}C NMR Spectroscopy

LMW dextrans (50 to 100 g/L) were dissolved in deuterium oxide. Spectra were recorded at 30 °C using a BRUCKER AM 300 spectrometer at 75 MHZ (spectral width:15 KHz; acquisition time : 0.54 sec; number of transients: 1,000 to 2,000). Chemical shifts are expressed in ppm relative to acetone in water.

Endodextranase Hydrolysis

Endodextranase from *Penicillium sp.* was purchased from SIGMA (D 1508, grade I). The reaction mixture contained purified LMW dextrans (25 g/L), dextranase (1 U/mL) in 20 mM sodium acetate buffer, pH 5.4. Hydrolysis reactions were conducted at 37 °C. During hydrolysis, the oligosaccharides produced were analyzed by reverse-phase chromatography using water or a mixture of water and methanol (96/4, v/v) as eluent. Isomaltooligosaccharide standards were produced by dextransucrase using isomaltose as acceptor, their linearity was demonstrated by performing ^{13}C NMR spectra.

Viscosity Determination

Intrinsic viscosity measurements were carried out using a VISCOSIMATIC M8 capillary viscosimeter, at 30 °C. The diameter of the capillary was 0.46 nm. The flow time of the solvent (0.1 M sodium nitrate) was equal to 253 sec.

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