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FORMATION OF A DEXTRAN / ß-CYCLODEXTRIN POLYMER BY IMMOBILIZED LEUCONOSTOC MESENTEROIDES IN CALCIUM ALGINATE BEADS

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ABSTRACT

Dextran was produced using immobilized cells of L. mesenteroides ATCC 10830 (NRRL B-512F) in 3% calcium alginate beads in absence and presence of 1% (w/v) ß-cyclodextrin (ß-CD). Information about the molecular structure of the produced polymers was obtained using size exclusion chromatography and on-line low angle laser light scattering (SEC/LALLS) detection. Addition of ß-CD to the dextran production medium led to a drastic change in the structure of the produced dextran. Branching of the produced polymer in presence of ß-CD was drastically increased and possibly produced a star-like polymer containing ß-CD in the center with different dextranyl residues attached to its primary or secondary hydroxyl groups.resolution.

INTRODUCTION

Dextransucrase (DS, EC 2.4.1.5.) of Leuconostoc mesenteroides ATCC 10830 (NRRL B-512F) produces a low branched dextran polymer containing about 95% α-D-(1----> 6) bonds and 5% α-D-(1----> 3) bonds, which is highly soluble in water. The side chains of the dextran obtained from this bacterium contain 2 or more glucose residues attached by α-D-(1---> 6) bonds(1). The reaction mechanism of dextransucrase (DS) involves two identical fixation sites of polymeric D-dextranyl (primer) and of monomeric D-glucosyl (acceptor). The monomeric glucosidic acceptor is transferred by nucleophilic attack (of C_6-OH, C_3-OH, C_2-OH or C_4-OH) at the reducing end of the growing dextran chain. This chain remains covalently bonded to the enzyme. Chain termination occurs by release of dextran from the enzyme when, instead of glucose, fructose (produced during the reaction) or another sugar binds at the acceptor side and is transferred to cap the growing chain. Maltose, isomaltose, lactose, panose, nigerose, cellobiose, sorbose, raffinose, galactose, xylose, mannose and melibiose or a sugar derivative, (e.g. P-nitrophenyl α-D-glucopyranoside and methyl ß-D-glucoside) can act as an acceptor for terminating the dextran chain(2,3).

Sucrosephosphorylase (disaccharide glucosyl transferase, EC 2.4.1.7.) is an intracellular enzyme known to be produced by only six microorganisms including L. mesenteroides. This enzyme catalyzes the hydrolysis of sucrose to glucose and fructose via a reversible reaction. This enzyme can also transfer the glucosyl moiety to inorganic phosphate, thereby forming glucose-1-phosphate (G-1-P). Fructose is thus produced simultaneously by such glucosyl transfer from sucrose to phosphate. Besides sucrose, G-1-P and glucosylfluoride can also donate the glucosyl moiety. Although phosphate is the best known glucosyl accepter, sucrosephosphorylase

can also transfer the glucosyl moiety of sucrose to other sugars (e.g. fructose, sorbose, xylose, arabocetose, rhammulose, arabinose), to alcohols (e.g. cis-and trans-1,2-cyclohexandiol, methanol, ethanol and ethylene glycol), and even to water[4,5].

Cyclodextrins (CDs) are torus shaped compounds, the secondary hydroxyl groups on the C_2 and C_3 atoms of the glucose units being located in one side of the torus, while the primary groups on C_6 are positioned on the opposite side of the torus[6]. Either the primary hydroxyl groups on C_6 or the secondary hydroxyl groups on C_2 and C_3 are available as points of structural modification without the danger of blocking or eliminating the "central void", which remains available for the accommodation of the guest molecules[6]. Although hundreds of chemically modified derivatives are known, and some are already marketed, none or very little information is known about the biologically derivatized CDs[7,8]. In 1986, Koizumi et al.[9] reported the isolation of three branched cyclodextrins from the mother liquors of a large scale preparation of the unbranched CDs produced from Bacillus ohbensis cyclomaltodextrin glucanotransferase (CGT). These compounds were linked with α-(1---->6) linkage with C_6 of the cyclic oligosaccharide.

Recently, we reported about using size exclusion chromatography on-line with low angle laser light scattering (SEC/LALLS) detection for the study of molecular weight distribution and branching frequency of dextran produced by free and in calcium alginate immobilized cells[10]. SEC/LALLS is regarded as one of the most important analytical techniques in polymer characterization[11-14].

This paper deals with the molecular structure characterization of dextran produced by L. mesenteroids ATCC 10830 (NRRL B-512F) immobilized in calcium alginate beads in the absence and presence of β-CD as detected by size exclusion chromatography and on-line low-angle laser light scattering.

MATERIALS AND METHODS

Microorganism and fermentation conditions :

The dextran-producing strain L. mesenteroides ATCC 10830 (NRRL B-512F) was immobilized in 3% calcium alginate beads and a fed-batch dextransucrase (DS) production cycle for 24 hours was performed. Then, the sucrose concentration was raised to 10% (w/v), pH was adjusted to 5.2 ± 0.1, temperature was controlled at 30°C and a dextran production cycle by the immobilized cells was performed for 24 hours[15-17]. For formation of dextran-β-CD, β-CD was added to the sucrose solution in fed-batch DS production cycle and in batch dextran production cycle in a final concentration of 1% (w/v). Fermentations were performed in a IL-bubble column containing 200 g of beads and 800 ml of medium[10,15,16]. After fermentation, the culture broth was centrifuged at 4000 xg at 4°C for 10 minutes and the dextran in the supernatant was recovered by precipitation with 2 volumes of ethanol. The precipitated dextran was purified 3-4 times by dissolving in sterile demineralized water and reprecipitation with 2 volumes of ethanol. Purified dextran was dried under vacuum at room temperature.

Size exclusion chromatography and on-line low-angle laser light scattering (SEC-LALLS) detection :

Theoretical background of the analysis, instruments, columns, elution system and performance of the analysis were the same as described previously [10,13,14].

RESULTS

1- Determination of molecular weight distribution (MWD) :

Figures 1A-C compare the SEC/LALLS chromatograms collected from

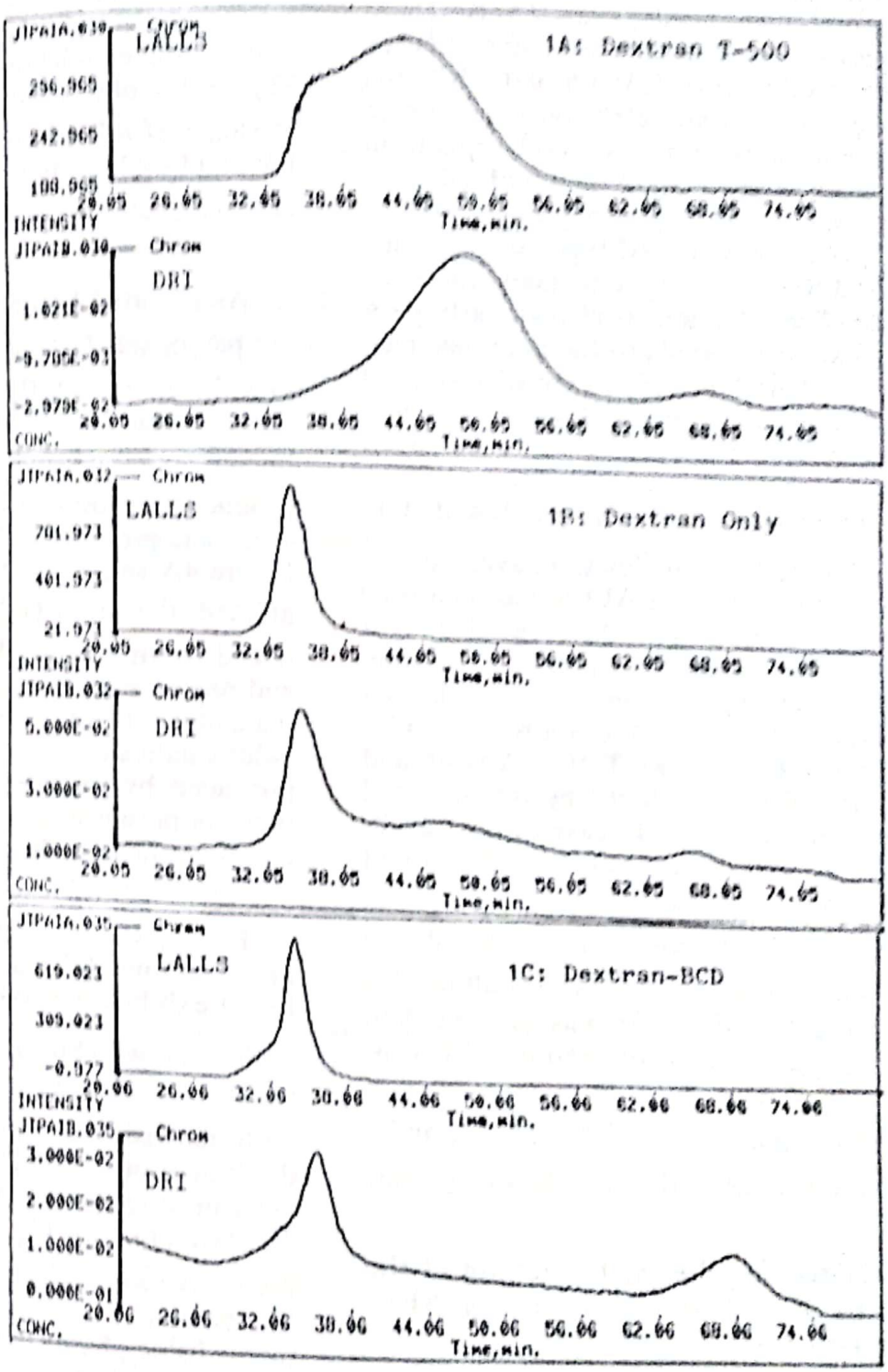

Figure (1): SEC/LALLS chromatograms as detected by differential refractive index (DRI) detector and low angle laser light scattering (LALLS) detector for dextran T-500 (A), and dextran produced by immobilized L mesenteroids ATCC 10830 (NRRL B-512F) in calcium alginate beads in absence (B) or in presence of β-CD (C).

DRI and LALLS detectors of dextran T-500, as well as dextran and dextran/ß-CD produced by immobilized cells in calcium alginate beads, respectively. These figures give the intensity of responses measured in both LALLS and DRI cells as a function of retention time. From these figures, a fairly good separation was obtained via LALLS and DRI responses, especially through the LALLS. In the three analyzed types of dextrans, LALLS show only one peak for each case. The DRI detects also an early peak almost corresponds to the peak detected by the LALLS, but in case of produced dextran or dextran/ß-CD, a late peak was also obtained in each case. This delayed peak was more intense in presence than in absence of ß-CD (Figures 1B and 1C).

Through the analysis, an average molecular weight via LALLS was measured and registered. The differential weight fractions (molecular weight distribution, MWD) were plotted vs. the log molecular weights. Figures 2A-C show such relationship for dextran T-500, dextran and dextran/ß-CD produced by immobilized cells, respectively. In case of dextran T-500, a main peak was obtained at about $2x10^5$ daltons. Also, MWD of dextran produced by immobilized cells are shown in a main peak around $5x10^7$ daltons, but a wide left shoulder was also evident (Figure 2B). MWD of dextran/ß-CD was, however, represented in two main peaks, a small peak around 10^6 daltons, and a major peak around $8x10^8$ daltons (Figure 2C).

2- Determination of branching of the produced polymers using SEC/LALLS:

SEC/LALLS system is also applicable for the study of branched polysaccharides[10,13,14]. The Log M_W vs. elution volume (EV) of the standard dextran T-500 as well as the produced dextran and dextran/ß-CD by immobilized cells were analyzed by SEC/LALLS and are shown in Figure 3. Figure 3 indicates that a reverse linear relationship exists between Log M_W and EV for both dextran T-500 and dextran produced by immobilized cells in absence of ß-CD. Figure 3 shows also a complex relationship between Log M_W vs. EV of dextran/ß-CD. A sigmoidal shape of relationship exists between EV and Log M_W. In the three cases there is a wide separation between the three lines.

As reported before[10,13,14], branching parameters $G^{-1}{}_V$ and G_M were determined (Figures 4A-B, and Table 1). Using the standard dextran T-500 as a linear dextran, values $G^{-1}{}_V$ of dextran produced by immobilized cells in absence and presence of ß-CD are given in Figure 4A and Table 1. In the range examined, the mean $G^{-1}{}_V$ of dextran produced by immobilized cells in absence and presence of ß-CD are about 7.675 ± 0.8 and 282.6 ± 47.7, respectively. These values indicate that a dextran molecule produced by immobilized cells in absence or presence of ß-CD is about 7.7 or 282 times heavier than an equivalent hydrodynamic volume of dextran T-500.

Figure 4B shows that dextran produced by immobilized cells in absence of ß-CD exhibits a maximum G_M at low molecular weights (about 2×10^6 daltons). Then, G_M values steady decrease with increasing the molecular weight of dextran produced by the immobilized cells in absence of ß-CD. Also, Figure 4B shows that the G_M values of dextran/ß-CD give a complexed relationship with log molecular weight. At least three distinguishable phases in Figure 4B are evident. Phase 1 includes a group of polymer with a very low G_M values at low molecular weights which slightly decrease with increasing molecular weights. Phase 2 includes a polymer which has high G_M values which decrease sharply with increasing the molecular weights. Phase 3 includes a polymer

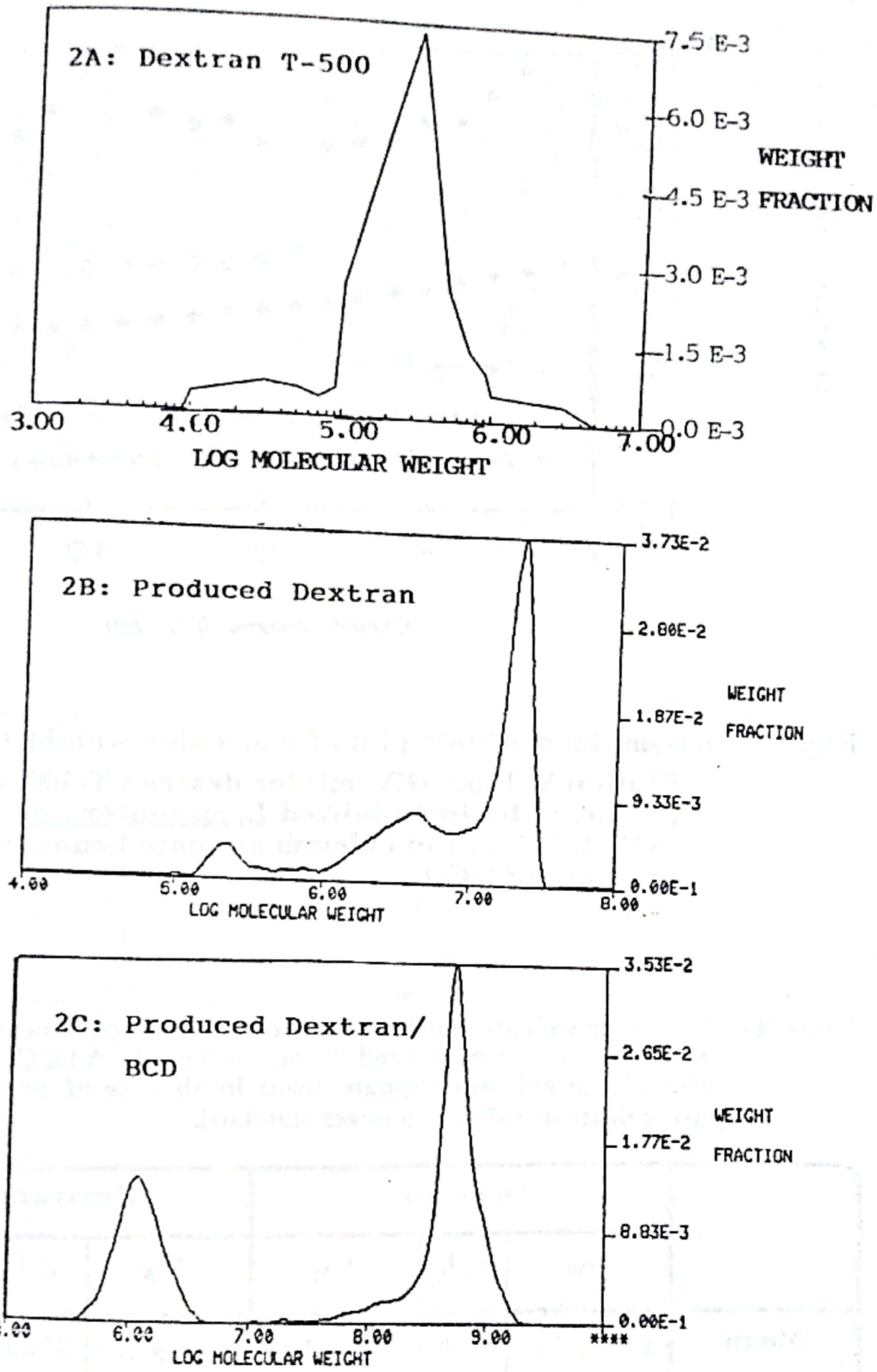

Figure (2): Differential weight fraction plot detected by SEC/LALLS for dextran T-500 (Total injected mass = 180 µg, A), and dextran produced by immobilized L. mesenteroids ATCC 10830 (NRRL B=512F) in calcium alginate beads in absence (Total injected mass = 69.13 µg, B) or in presence (Total injected mass = 45.5 µg, C) of ß-CD.

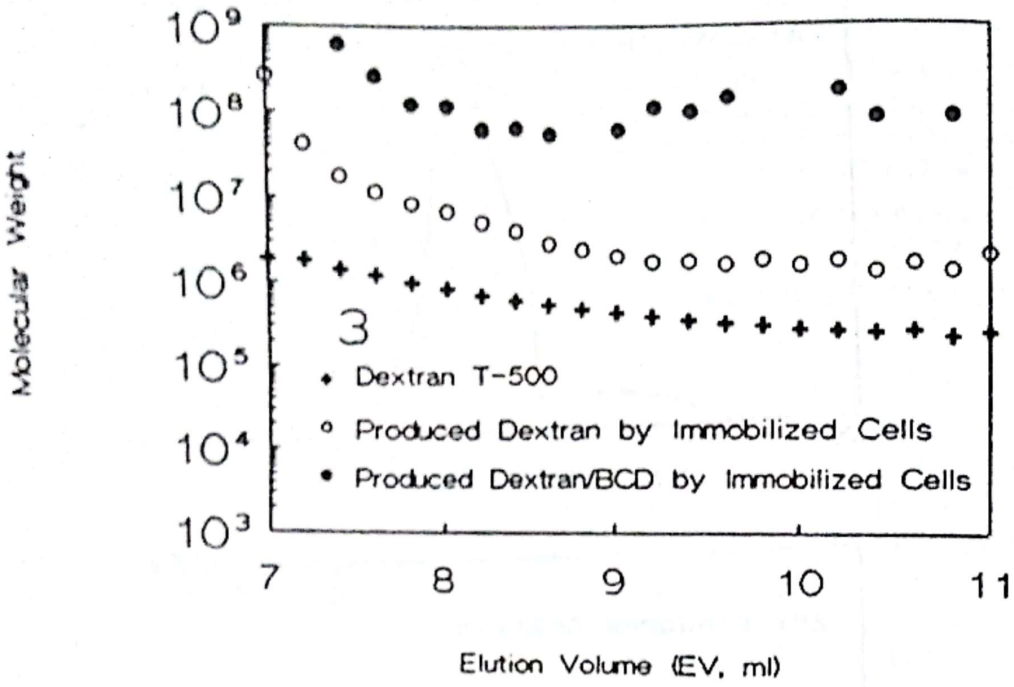

Figure (3): Semi-logarithmic plot of molecular weight (Log M_W) vs. Elution Volume (EV, ml) for dextran T-500, and dextran produced by immobilized L. mesenteroids ATCC 10830 (NRRL B-512F) in calcium alginate beads in absence or presence of ß-CD.

Table (1): **Molecular weights and branching distribution parameters for dextran produced by immobilized L. mesenteroids ATCC 10830 (NRRL B-512F) in calcium alginate beads in absence or presence of ß-CD using dextran T-500 as a linear standard.**

	Dextran			Dextran/ß-CD		
	M_W	$G\text{-}I_V$	G_M	M_W	$G\text{-}I_V$	G_M
Mean	1.84×10^7	7.68	6.27×10^{-2}	1.47×10^8	282.6	4.6×10^{-4}
S.D. (*)	5.78×10^7	3.65	2.76×10^{-2}	1.49×10^8	178.5	3.8×10^{-4}
S.D. (**)	1.26×10^7	0.8	6.02×10^{-3}	3.98×10^7	47.7	1.0×10^{-4}

(*) SD = Standard deviation and (**) SE = Standard error were calculated using slide Write plus soft ware program

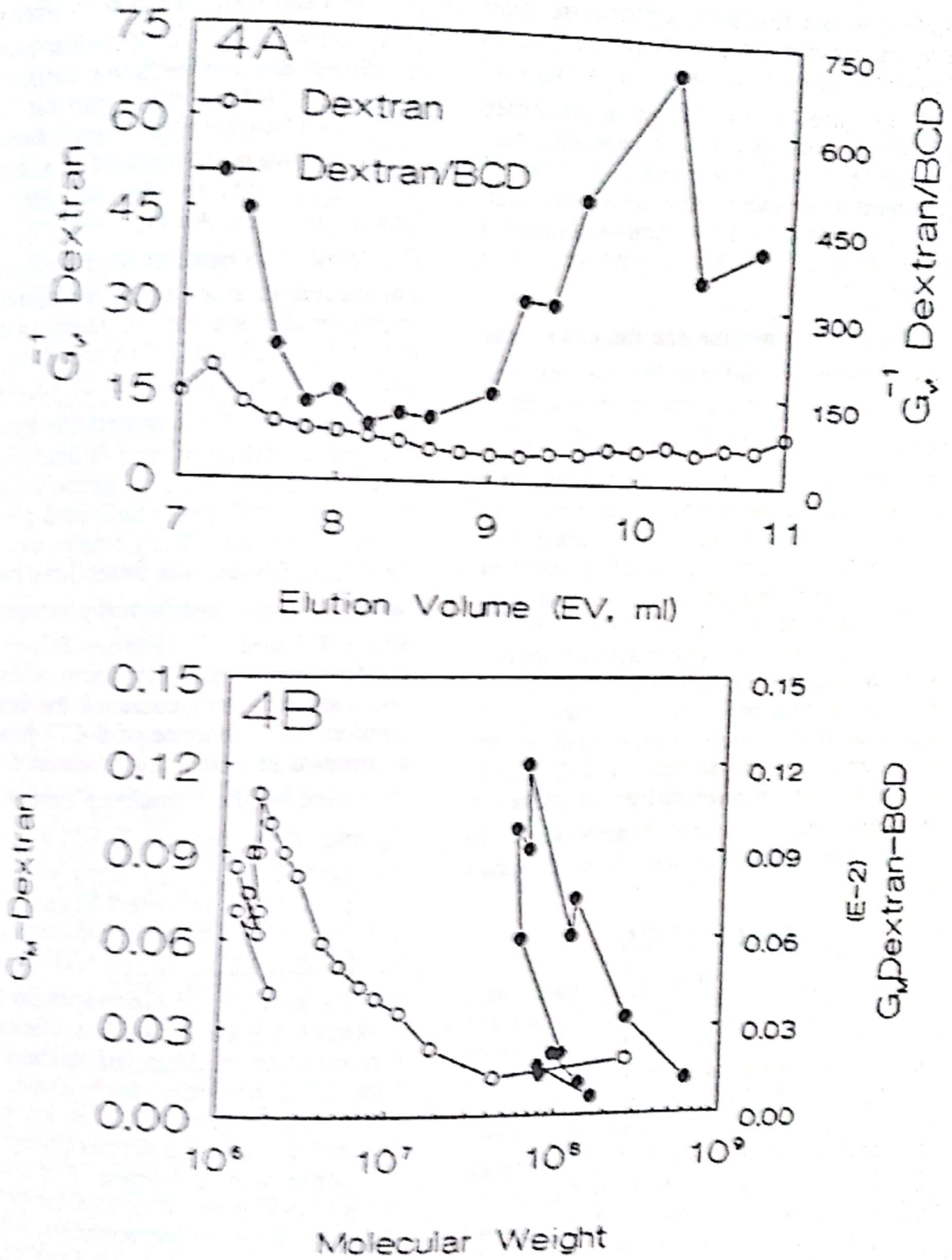

Figure (4): Plot of the branching parameters (G^{-1}_V vs. Elution Volume, A) and (G_M vs. molecular weight; semilog plot, B)for dextran produced by immobilized L. mesenteroids ATCC 10830 (NRRL B-512F) in calcium alginate beads in absence and presence of β-CD using dextran T-500 as a linear standard.

which possesses high G_M values at low molecular weights with a moderate drop with increasing the molecular weights. It has to be noted that the scale of G_M values in Figure 4B for dextran is 100 times of that of dextran/ß-CD. Shape and performance of the relationship of phase 3 polymers are more or less similar to that of dextran produced by immobilized cells in absence of ß-CD and represented in Figure 4B.

Table 1 shows that the mean G_M values of dextran produced by the immobilized cells in absence and presence of ß-CD are in average $6.27 \times 10^{-2} \pm 6.02 \times 10^{-3}$ and $4.57 \times 10^{-4} \pm 1.01 \times 10^{-4}$, respectively. These values represent the ratios of branched (product) and linear (dextran T-500) mean-square radii of gyrations. Dextran produced by immobilized cells in absence of ß-CD exhibits a mean-square radius of gyration approximately 0.06 the value of an equivalent molecular weight of dextran T-500 (Table 1). On the other hand, dextran produced by immobilized cells in presence of ß-CD possesses a mean-square radius of gyration of approximately 4.6×10^{-4} the value of an equivalent molecular weight of dextran T-500.

DISCUSSION

Aqueous SEC/LALLS has been applied in determining molecular weight distribution and branching frequencies of dextran produced by immobilized L. mesenteroides ATCC 10830 (NRRL B-512F) in calcium alginate beads in presence and absence of ß-CD. The presented data show that presence of ß-CD in the dextran fermentation brought significant changes in the structure of the produced polymer. Considering the standard dextran T-500 a linear polymer, the represented data show that produced dextran by immobilized cells is about 7.7 times heavier than dextran T-500 at the same elution volume. Interestingly, incorporation of ß-CD in the fermentation medium accompanied by an average increase in the weight of dextran of about 36 times (G^{-1}_V values of dextran in absence to presence of ß-CD) heavier than normally produced dextran with the same hydrodynamic volume. In comparison of dextran T-500 with the produced dextran/ß-CD, the same volume of the latter is about 282 times heavier than the former linear polymer. As reported by Yu and Rollings[13], when the degree of branching increases at a common elution volumemolecular weight increases as detected by LALLS due to an increase in the scattering centers in the molecule. Yu and Rollings[13] compared the molecular weight distribution and branching parameters of amylose (0% branched), amylopectin (4-5% branched) and glycogen (10% branched). They found that using SEC/LALLS and the branching parameter G^{-1}_V, amylopectin and glycogen were about 4-5 and 15-20 times heavier than the linear amylose, respectively. In comparison, dextran produced by immobilized cells in absence of ß-CD possesses a percentage branching of about 6-9% as indicated by the branching parameter G^{-1}_V and using dextran T-500 as a linear standard. This value is in a good agreement with the reported branching frequencies (5%) of the produced dextran by L. mesenteroides ATCC 10830 (NRRL B-512F)[2]. Correspondingly, addition of 1% (w/v) ß-CD to the dextran fermentation medium led to the production of a polymer with about 282% branching frequencies. This value could not be true, unless a drastic change in the molecule would happen. For example, the comb-like branching of the produced dextran through fermentation with few glucose units in each branch[2,3] becomes a star-like branching. Formation of star-like branches from oligosaccharides leads to formation of multiple scattering centers in the molecule and to increases in both size-average molecular weight (W_Z) and number-average molecular weight (W_n)[13,14].

The proposed hypothesis of formation of a star-like polymer of dextran/ß-CD by the immobilized L. mesenteroides ATCC 10830 (NRRL B-512F) was also based on the calculation of the branching parameter G_M (the ratio of the meansquare radii of gyration between the linear and branched polymers). As reported by Yu and Rollings [13], amylopectin with 4-5% branches and glycogen with 10% branches possess G_M values of 0.05 and 0.01 compared with the linear amylose, respectively. Our results show that mean G_M value of dextran produced by immobilized cells is 0.067 ± 0.006 which can indicate a branching frequency of about 7% as above proposed by calculation of G^{-1}_V. For the formed dextran/ß-CD, the mean G_M value is about 0.0005 which is inversely proportional with the branching frequency. Therefore, calculation of G_M for dextran/ß-CD highly supports our proposal in formation of a star-like dextran/ß-CD polymer by the immobilized cells, when ß-CD is added to the dextran production medium. Formation of multiphasic system between G_M and log molecular weight of dextran/ß-CD can be related to changes in number and length of bound dextran units to the OH groups of ß-CD. Therefore, comparatively high (Phase 1), moderate (Phase 2) or low (Phase 3) number of linked dextran units to the OH groups of ß-CD were observed (Figure 4B).

As stated above, experimental observation suggests that cyclodextrin incorporated into dextran during its microbial/enzymatic synthesis from sucrose. We hypothesize that sucrosephosphorylase could play a role by supplying the medium (even in low concentrations) with G-1-P, followed by transfer of the glucosyl moiety from G-1-P to one of the hydroxyl groups in cyclodextrin. If this does indeed occur, a tail-like glucosyl moiety would be formed on the CD molecule. This tail on CD could then act as an acceptor for further glucosyl or dextranyl moieties activated by dextransucrase present in high concentration in the fermentation medium. A similar phenomenon was reported by Bergeron et al.[18] and Machida et al.[19], who studied activity of fatty acid synthetase in the presence of CD and their alkyl derivatives. They reported that cyclodextrin binds first with palmitoyl-CoA before binding the mycobacterial polysaccharides or lipopolysaccharides.

The formation of such dextranyl-ß-cyclodextrin (dextran/ß-CD) polymers by the cultures of L. mesenteroides might open the door for production of a novel group of polymers, containing cyclodextrin linked to dextran. A combination of both molecules in a single polymer could find wide applications in chromatographic separation technology and many other medical and pharmaceutical uses. Such new polymers will contain more than one pore size, the fixed primary lipophilic cavities in cyclodextrins, secondary cavities between the cyclodextrin molecules, and tertiary cavities formed by the three dimensional structure of the dextran moieties in the polymer. The pore size of either the secondary and/or the tertiary cavities can be further chemically modified (e.g. epichlorhydrin treatment) to give a Sephedex-like polymers with cyclodextrin moieties in the same polymer.

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انتاج مبلمر من الدكستران / والسيكلودكسترين بواسطة خلايا الليكونوستك ميزنتروبدس المثبطة فى ألجينات الكالسيوم المكورة

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تم انتاج الدكستران بواسطة الخلايا المثبطة من ليكونوستك ميزينترويدس ATCC 10830 (NRRL B-512F) فى اللجينات الكالسيوم المكورة فى وجود وعدم وجود ١٪ بيتاسيكلودكسترين فى مستنبات التخمر لإنتاج انزيم الدكستران سيكراز ولإنتاج الدكستران. وقد تم الحصول على معلومات عن التركيب الجزيئى للمبلمرات المنتجة بواسطة كروماتوجرافيا التخلص الحجمى المتصل على التوالى بكاشف لأشعة الليزر ذات الزوايا المنخفضة (SEC/LALLS). وقد اثبتت الدراسة أن اضافة البيتاسيكلودكسترين ادى الى تغير هائل فى التركيب الجزيئى للدكستران المنتج. كما اثبتت الدراسة أن درجة التشعب فى المبلمر المنتج قد زادت بصورة هائلة مما أدى الى الاعتقاد بتكون مبلمر ذات تشعب شبيه بالنجمة مع وجود جزئ البيتاسيكلودكسترين فى الوسط متحدا مع جزيئات مختلفة من الدكستران فى الأطراف عن طريق روابط مع مجموعات الهيدروكسيل الابتدائية أو الثانوية لجزئ البيتاسيكلودكسترين.