Biochimica et Biophysica Acta, 614 (1980) 46—62
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BBA 69026

CHARACTERIZATION OF THE MULTIPLE FORMS AND MAIN COMPONENT OF DEXTRANSUCRASE FROM *LEUCONOSTOC MESENTEROIDES* NRRL B-512F

MIKIHIKO KOBAYASHI * and KAZUO MATSUDA **

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Miyagi 980 (Japan)

(Received October 23, 1979)

Key words: Dextransucrase; Leuconostoc mesenteroides NRRL B-512F

Summary

Multiple forms of dextransucrase (sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase EC 2.4.1.5) from *Leuconostoc mesenteroides* NRRL B-512F strain were shown by gel filtration and electrophoretic analyses. Two components of enzyme, having different affinities for dextran gel, were separated by a column of Sephadex G-100. The major component voided from the Sephadex column was treated with dextranase and purified to an electrophoretically homogeneous state. The purified enzyme had a molecular weight of 64 000—65 000, p*I* value of 4.1, and 17% of carbohydrate in a molecule. EDTA showed a characteristic inhibition on the enzyme while stimulative effects were observed by the addition of exogenous dextran to the incubation mixture. The enzyme activity was stimulated by various dextrans and its K_m value was decreased with increasing concentration of dextran. The purified enzyme showed no affinity for a Sephadex G-100 gel, and readily aggregated after the preservation at 4°C in a concentrated solution.

Introduction

Leuconostoc mesenteroides NRRL B-512F is well known to produce a dextran which is useful in pharmaceutical and fine chemical industries [1]. The B-512F dextransucrase (sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) has been characterized to produce dextran having rather homog-

* To whom correspondence should be addressed.
** Present address: Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, U.S.A.

eneous molecular weight in high yield. In the earlier studies, the general properties and reaction mechanism of B-512F dextransucrase have been discussed [2].

Since dextransucrases of the *Leuconostoc* species were induced by sucrose, a significant amount of dextran was synthesized concomitantly during the enzyme production. Moreover, the complex formation between enzyme and dextran was so tight that it could not be separated by various chromatographic procedures. Thus, the enzyme-dextran complex was considered to be one of the reasons why the multiple forms of dextransucrase were detected as in the case of streptococci [3]. Although B-512F dextransucrase was believed to be a single form of enzyme, the association with dextran might give somewhat different forms of enzyme. Our previous paper described the dextransucrase system of the B-1299 strain [4—6] which produced dextran containing high proportions of α-1,2-branched linkages and was composed of multiple forms of the enzyme [4]. Moreover, highly aggregated form of extracellular enzyme was mainly responsible for the formation of dextran having a similar structure as that produced by the cultured cells [7].

The mechanism for branch formation is one of the most interesting points to consider in the biosynthesis of dextrans. Robyt and Walseth [8] substantiated the acceptor reaction theory proposed by Ebert and Schenk [9] to explain the formation of branch points. However, the synthesis of different types of branch, i.e., α-1,2-, α-1,3- and α-1,4-glucosidic linkages, which were detected occasionally in the same dextran molecule, could not be sufficiently explained by the above theory. Furthermore, no particular branching enzyme could be detected from B-1299 dextransucrase system [7]. Therefore, it is important to compare the catalytic activity of several dextransucrases from the representative strains which produce dextrans with different amounts of secondary and branched linkages.

In the present paper, we report the characterization of the multiple forms of B-512F dextransucrase and purification of the major component having the lowest molecular weight. General properties and characteristics of the purified enzyme are also discussed.

Materials and Methods

Materials. L. mesenteroides NRRL B-512F was kindly provided by Dr. A. Jeanes (Northern Regional Research Center, IL, U.S.A.). Preparation of native dextrans from *L. mesenteroides* strain NRRL B-512F and B-1299 has been described [10]. A clinical dextran (molecular weight 75 000 ± 25 000) with 5% α-1,3-glucosidic linkages was provided by Meito Sangyo Co., Japan. Endodextranase preparation from *Chaetomium gracile* was provided by Sankyo Co., Japan [11]. Ampholyte solution (40%, w/v) was obtained from LKB-Produkter, AB, Sweden. Hydroxyapatite was prepared according to the method of Siegelman et al. [12]. Aminobutyl-Sepharose 4B was prepared according to Shaltiel and Er-El [13].

Purification of the extracellular dextransucrase. Production of dextransucrase was carried out according to the method described by Jeanes [14].

Cells were harvested by centrifugation at 8000 × *g* for 20 min at 0 °C and the

culture supernatant was adjusted to pH 5.2 with 1 M NaOH solution. The following purification steps were carried out at 0 to 4°C

(1) A slurry of hydroxyapatite (500 ml in 0.005 M sodium phosphate buffer, pH 6.0) was added to the culture supernatant (3 l). After continuous stirring overnight, the slurry was centrifuged at 6000 × *g* for 10 min and then the supernatant was removed. The slurry was washed twice with 200 ml portions of 0.1 M sodium phosphate buffer (pH 6.0) and then extracted with 0.4 M buffer (300 ml) to desorb the enzyme. The extracted enzyme was dialyzed overnight against water and concentrated about one-tenth of the volume by ultrafiltration on G-10T membrane (Diafilter, Bio-Engineering Co., Japan).

(2) Dialyzed and concentrated enzyme was applied to a Sephadex G-100 column (2.7 × 22 cm) previously equilibrated with 0.02% sodium azide solution and eluted with the same solution. After the elution of low molecular weight proteins, the column was washed with two-fold column volume of azide solution and then eluted with 0.5% of clinical dextran in azide solution. The fractions containing enzyme activity were pooled and concentrated with a collodion bag (Sartorius membrane filter, collodion-bag 12).

(3) Endodextranase from *C. gracile* (74 units; spec. act. 7.4 units/mg protein) was added to the concentrated enzyme solution and the mixture was incubated at 4°C for 7 days, and then dialyzed overnight against 0.01 M acetate buffer (pH 5.2). The dialyzate was applied to a CM-cellulose column (1.2 × 9 cm) previously equilibrated with 0.01 M acetate buffer. The dextransucrase activity was passed through the column, on which endodextranase was retained. The fractions containing the enzyme activity were concentrated with the collodion bag.

(4) The concentrate (5 ml) was applied to a Sepharose 6B column (3.2 × 80 cm), equilibrated with 0.02% sodium azide solution, and eluted with the same solution. The fractions with activity were pooled and concentrated in the same manner as before.

(5) After overnight dialysis against 0.01 M phosphate buffer (pH 6.0), the sample was applied to an aminobutyl-Sepharose 4B column (1 × 18 cm). The column was washed with the equilibrating buffer and eluted with the same buffer containing increasing NaCl concentrations (linear gradient, 0—0.15 M). The fractions with activity were pooled, dialyzed overnight against the above buffer and then lyophilized. In this manner, no significant loss of the activity was observed for several months at 4°C.

Analytical methods. Proteins were determined by the method of Lowry et al. [15] or by absorbance at 280 nm. Reducing sugar was measured by the Nelson-Somogyi method [16,17]. Total carbohydrate was determined by the phenol-sulfuric acid method [18] and represented as D-glucose.

Enzyme assays. In the standard assay for dextransucrase, 0.05 ml of a suitably diluted enzyme solution was incubated with 0.2 ml of sucrose (125 mg/ml) in 40 mM acetate buffer (pH 5.2) at 30°C for 10 min. The reactions were stopped by the addition of 0.2 ml of 1 M NaOH, followed by the dilution with water (0.55 ml). Samples (1.0 ml) of the inactivated reaction mixture were used for the determination of reducing sugars by the Nelson-Somogyi method. One unit of the dextransucrase activity was defined as the quantity that produced reducing sugars equivalent to 1 μmol of fructose per min under the above conditions.

Endodextranase activity was quantitatively assayed by incubating 0.5 ml aliquots of dextranase solution with 0.5 ml of 1% solution of clinical dextran in 40 mM acetate buffer (pH 5.2) at 30°C. After incubation, the reaction was stopped by the addition of 1.0 ml of the alkaline copper reagent and assayed for reducing sugar as described above.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out according to Maurer [19], using the gel system No. 6 with 7.5% acrylamide as described previously [4]. Enzyme activity in the gel was detected by incubating the gel in the solution containing 5% sucrose in 40 mM acetate buffer (pH 5.2) for 3 h at 30°C. The location of enzymes was visualized by the formation of opalescent bands of dextran. Moreover, gels were scanned with Beckman spectrophotometer ACTA III equipped with densitometric apparatus using a 345 nm filter.

The heterogeneity of dextransucrase was examined with gel electrophoresis by the method of Hedrick and Smith [20] using 5.0 to 11.0% gels. The sodium dodecyl sulfate (SDS) gel electrophoresis was performed as described previously [5].

Gel isoelectric focusing was performed according to the procedure described by Righetti and Drysdale [21] using gradient of pH 3.5—5.0 Ampholine. Gels were cooled to 4°C and pre-electrophoresed for 10 min. The current was set at 1 mA per column and the focusing was terminated after 4 h. Gels were cut into 2 mm segments to measure the pH gradient and then assayed for the enzyme activity by incubating them with sucrose [4].

Analysis of sugar component of the purified enzyme preparation. The purified enzyme was completely digested with 15 units of endodextranase at 40°C for 20 h and applied to a CM-cellulose column (1.2 × 9 cm) as described above. The breakthrough was collected and lyophilized. This sample was applied to a Bio-Gel P-4 column (1 × 11 cm) and eluted with water. Total sugar was determined as described above. Paper chromatography was carried out on Toyo No. 50 filter paper by the descending method with a solvent system of ethyl-acetate/pyridine/water (6 : 3 : 1, v/v) and sugar spots on the chromatograms were detected by alkaline silver nitrate [7].

Enzymatic synthesis of dextran and its structural analysis. Synthesis of dextran with purified B-512F enzyme and structural analysis of the products were carried out as described previously [7].

Results

Multiple forms of B-512F dextransucrase

Extracellular dextransucrase of *L. mesenteroides* B-512F gave two peaks of enzyme activity on a Sepharose 6B column (Fig. 1a). The main peak was voided from the column and a significant amount of dextran was eluted from the same position. The second peak had a much smaller molecular weight than the main peak and contained a lesser amount of dextran. When the first peak was pooled and subjected to a column of Sepharose 2B, the enzyme activity was further separated into four or five peaks (data not shown). The first two peaks were eluted with a large amount of sugars. These results suggested that high molecular weight enzyme might contain dextran molecules and form a highly

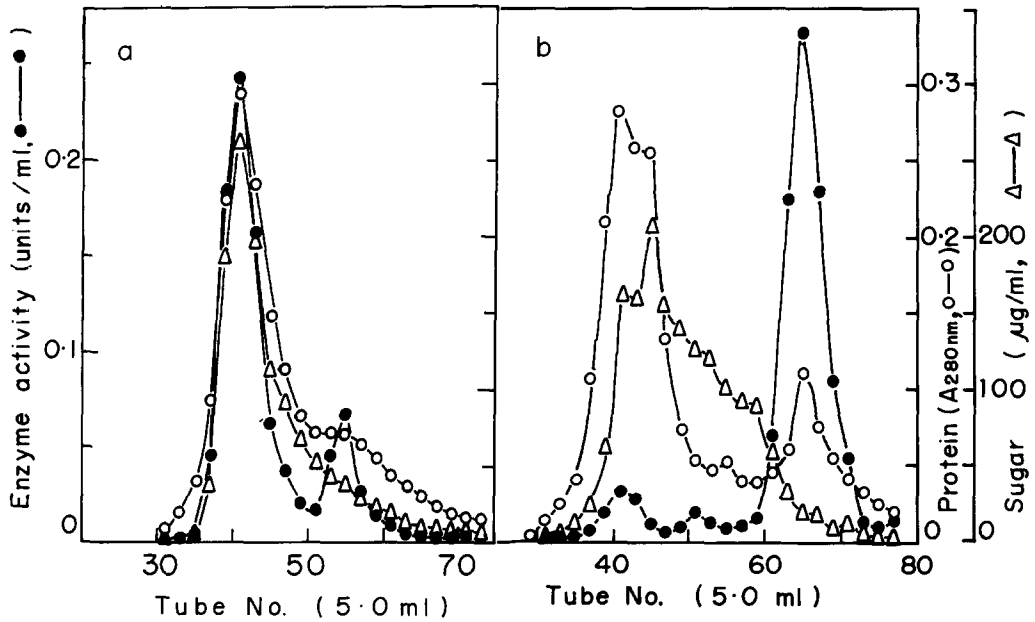

Fig. 1. Elution pattern of the B-512F dextransucrase from a Sepharose 6B column. ●, dextransucrase activity; ○, protein; △, total sugar. a, crude enzyme; b, dextransucrase treated with endodextranase (see Table I, step 4).

aggregated structure or an enzyme-dextran complex.

Furthermore, the multiplicity of dextransucrase was examined by polyacrylamide-gel electrophoresis with various gel concentrations. Besides three enzyme components with faster mobility, which could be visualized by the deposition of dextran produced in the gel during the incubation with 5% sucrose, a broad band of enzyme having a higher molecular weight was detected at the top of the gels (data not shown). When the logarithm of the relative mobility (R_m) of active bands was plotted as a function of the gel concentration, a linear relationship was observed (Fig. 2). The faster moving two enzyme components I and II gave a parallel line (slope 6.15) which showed that these enzymes differed

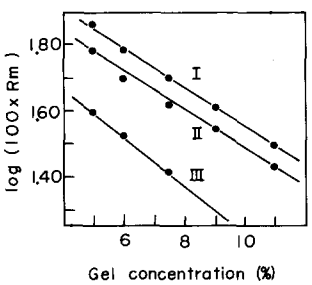

Fig. 2. Effect of gel concentrations on the mobility of B-512F dextransucrase. The negative slopes of each line are: I, 6.15; II 6.15, III, 7.89.

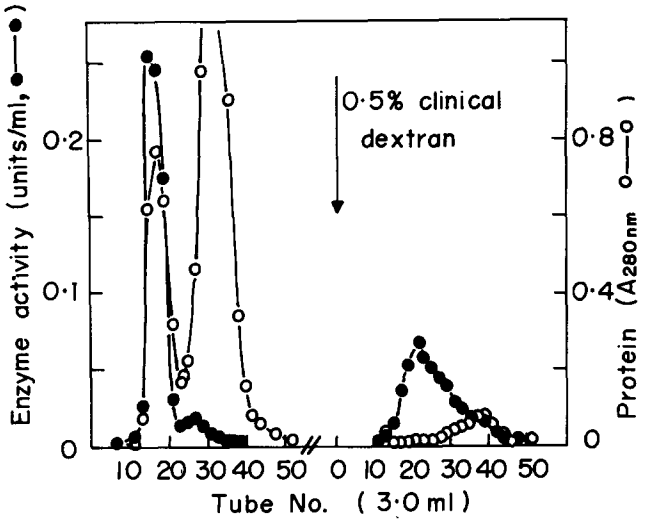

Fig. 3. Elution pattern of the B-512F dextransucrase from a Sephadex G-100 column. ●, dextransucrase activity; ○, protein. The column was eluted with 0.02% sodium azide solution, followed by the same solution containing 5 mg/ml clinical dextran (arrow).

in net charge but were the same in the molecular size. According to the method of Hedrick and Smith [20], the molecular weight of enzymes I and II were estimated to be 65 000 and that of enzyme III (slope 7.89) to be 100 000. These results demonstrated that the extracellular dextransucrase of *L. mesenteroides* NRRL B-512F was not homogeneous but could be separated into several components by gel filtration and gel electrophoresis.

Heterogeneity of B-512F dextransucrase was also demonstrated by affinity chromatography using a Sephadex G-100 column (Fig. 3). The first peak of enzyme activity was mainly eluted at the void volume, while the second peak could be eluted with 0.5% clinical dextran. Therefore, these two components showed different affinities against the dextran gel of Sephadex G-100 and were clearly distinguished from each other.

Effect of endodextranase digestion on the multiple forms

The results above suggested that endogenous dextran molecule might be responsible for the multiple forms of B-512F dextransucrase. Therefore, the effect of endodextranase, which could completely hydrolyze the B-512F native dextran to glucose and isomalto-oligosaccharides, on the enzyme (hydroxyapatite step) was examined. As shown in Fig. 4, the immobile broad band of dextransucrase activity was decreased according to the incubation time proceeded and the main activity peak was shifted from the component III to the components I and II. As the component II activity gave a rather broad band of product dextran, the scanning pattern showed almost a single peak of activity with a small shoulder (Fig. 4, at 30 and 60 min). For a large scale of preparation, the G-100 void volume fraction was treated with dextranase for 7 days at 4°C to minimize the heat inactivation effect during the incubation.

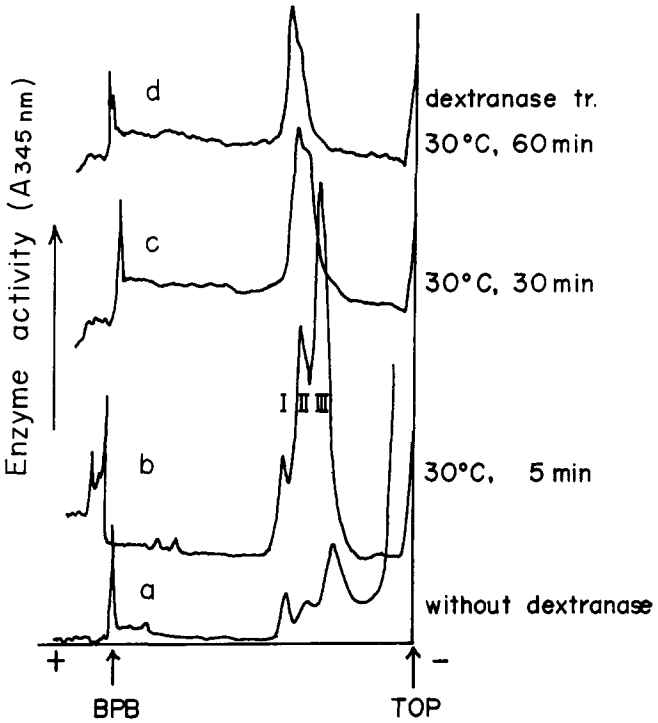

Fig. 4. Densitometric scanning patterns of the B-512F dextransucrase treated with endodextranase. Polyacrylamide gel electrophoresis was performed with 7.5% acrylamide monomer. a, dextransucrase not treated with dextranase; b, c and d, dextransucrase previously incubated with endodextranase for 5, 30 and 60 min at 30°C, respectively. After the electrophoresis, the gels were incubated with 5% sucrose in 40 mM acetate buffer as described in the text. BPB, bromophenol blue.

After removal of the added dextranase by CM-cellulose column adsorption, the enzyme activity was eluted at the low molecular weight region of a Sepharose 6B column (Fig. 1b).

Purification of B-512F dextransucrase

A typical purification procedure of the main component of B-512F dextransucrase is summarized in Table I. The enzyme desorbed from hydroxyapatite gel, was applied to an affinity column of Sephadex G-100 and the major part of the enzyme was eluted at the void volume fraction (Fig. 3). After the digestion with endodextranase and subsequent removal of the dextranase activity by the CM-cellulose column, the enzyme fraction was chromatographed on a Sepharose 6B column (Fig. 1b). The enzyme activity was eluted at the lower molecular weight region where the standard clinical dextran (molecular weight 75 000 ± 25 000) was also eluted. The aminobutyl-Sepharose column was effective for the preparation of dextransucrase having no contaminated dextran (Fig. 5). The relative and specific activity of the purified enzyme were 2672-fold and 72.1 units/mg protein, respectively (Table I).

The purified dextransucrase gave a single protein band coincided with the

TABLE I

PURIFICATION OF B-512F DEXTRANSUCRASE

Enzyme activity was measured in the presence of 5 mg/ml of B-512F native dextran.

	Purification step	Total units	Protein (mg)	Sugar (mg)	Spec. act. (units/mg)	Yield (%)	Purification (-fold)
1	Broth sup. (3 l)	276	10 156	14 700	0.027	100	1
2	Hydroxyapatite	155	661	455	0.235	56	9
3	Sephadex G-100	100	473	318	2.114	36	78
4	Dextranase tr. CM-cellulose	28.0	9.36	72.6	2.996	10	111
5	Sepharose 6B	5.96	1.03	8.46	5.791	2.2	214
6	Aminobutyl-Sepharose 4B	5.41	0.075	0.099	72.133	1.9	2672

enzyme activity upon polyacrylamide gel electrophoresis in 7.5% gel (Fig. 6a, b). SDS-polyacrylamide gel electrophoresis also confirmed the purity of enzyme (Fig. 6c). Moreover, a single peak of enzyme activity was obtained by isoelectric focusing using the Ampholine gradient with pH 3.5—5.0 (Fig. 7).

Physicochemical properties of B-512F dextransucrase

The molecular weight of the protomer enzyme, which corresponded to the component II in mobility, R_m 0.43 (Fig. 2), was calculated to be 65 000 by the Hedrick-Smith plot. SDS-gel electrophoresis gave a molecular weight of 64 000 for the purified enzyme. Isoelectric point of this enzyme was at pH 4.1 (Fig. 7).

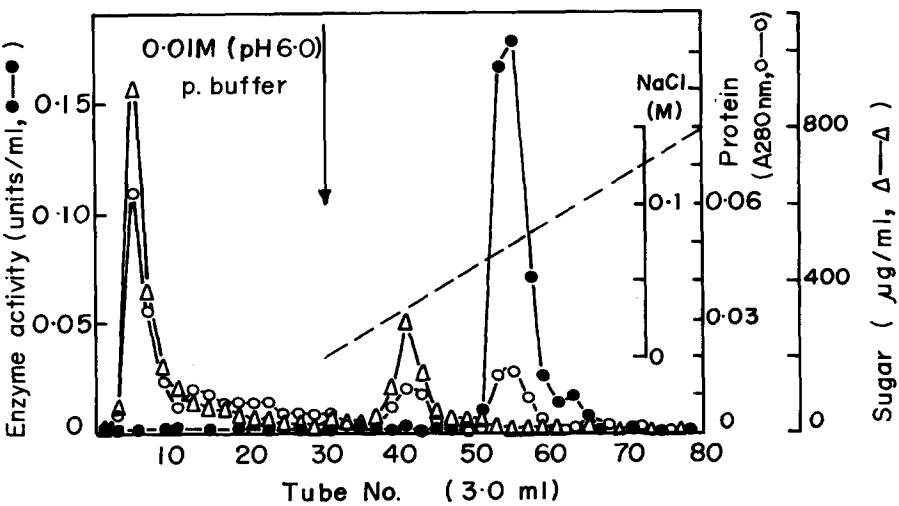

Fig. 5. Elution pattern of the B-512F dextransucrase from an aminobutyl-Sepharose 4B column. ●, dextransucrase activity; ○, protein; △, total sugar; ------, concentration of NaCl in 0.01 M phosphate buffer (pH 6.0).

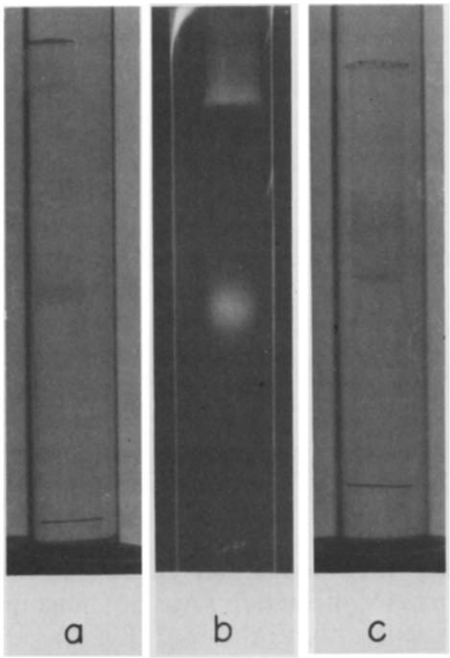

Fig. 6. Polyacrylamide gel electrophoresis of the purified B-512F dextransucrase. The purified enzyme (20—25 μg) was subjected to gel electrophoresis (7.5% gel) in Tris-barbital buffer (pH 7.5) for 120 min, applying 2.5 mA per column. Protein was stained with 0.25% Coomassie brilliant blue (a) and enzyme activity in the gel was detected (b) as described in the text. The enzyme was subjected to SDS-polyacrylamide gel electrophoresis (10% gel) for 100 min applying 8 mA per column. Protein band was stained (c) as described above.

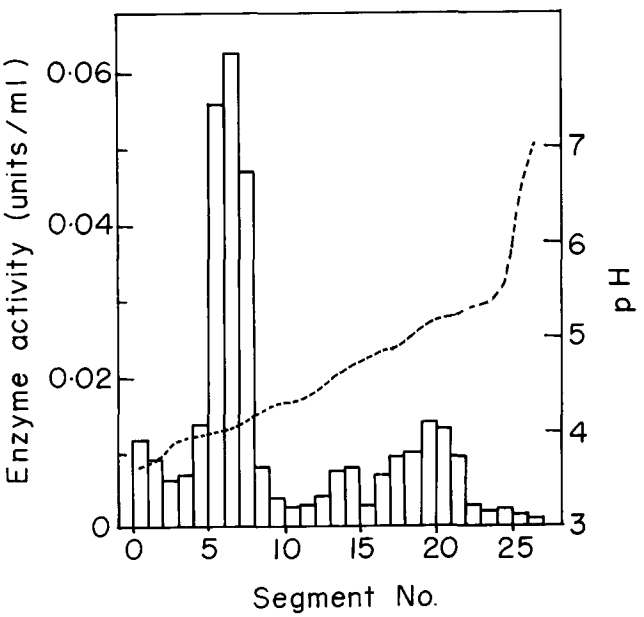

Fig. 7. Isoelectric focusing in a polyacrylamide gel column (4.0% gel) of the purified dextransucrase in the pH range between 3.5 and 5.0. Enzyme activity was assayed using 2-mm segments of the gel by incubating with the usual assay system under optimum conditions.

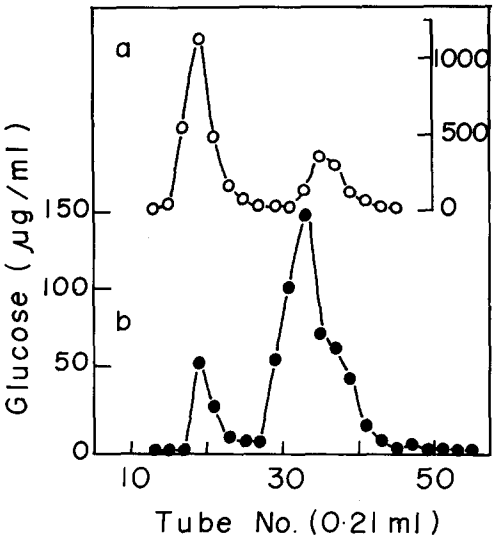

Fig. 8. Elution pattern of the fragments from purified dextransucrase completely digested with endodextranase from a Bio-Gel P-4 column. Total carbohydrate was determined by the phenolsulfuric acid method [18]. (a) Standard sugars; mixture of the B-1299 soluble dextran and D-glucose. (b) After the dextranase digestion, added dextranase was removed by a CM-cellulose column and the concentrated reaction mixture was applied to the column.

After complete digestion of the purified enzyme preparation with endodextranase and subsequent removal of dextranase by CM-cellulose column, the digest was chromatographed on a Bio-Gel P-4 column (Fig. 8). The void volume fraction (16%) was clearly separated from the hydrolyzed low molecular fraction (84%) which was composed of glucose and isomalto-oligosaccharides. Therefore, the purified protomer enzyme was still associated with 28 μg/ml of dextran, i.e., the enzyme was a glycoprotein containing a sugar moiety of 17.4%. Dextran in the purified enzyme preparation seemed to originate not from the contamination but from the so called enzyme-dextran complex which was linked at the active sites.

General properties of the purified dextransucrase

The optimum pH and temperature of the purified enzyme were pH 6.0 and 30°C, respectively (Fig. 9a, b). On the contrary, the crude enzyme from the Sephadex G-100 chromatography step, contained more than 10 mg/ml of dextran, had an optimum pH at 5.0—5.5 which was identical with the values reported by other workers [2]. The presence of dextran in the enzyme solution gave also a remarkable effect on the stability of purified enzyme. Although the enzyme was stable at 4°C for 24 h in the pH range 6.0—9.0 with 4 mg/ml dextrans, the stable range was narrowed at pH 7.0—9.0 without dextran in the solution (Fig. 9c). However, the thermal stability of enzyme was decreased by the addition of exogenous dextran. After incubation of the enzyme at 40°C for 10 min, 80% of activity was lost while the enzyme without dextran held 70% of its initial activity (Fig. 9d). In both stability experiments, approx. 40% of

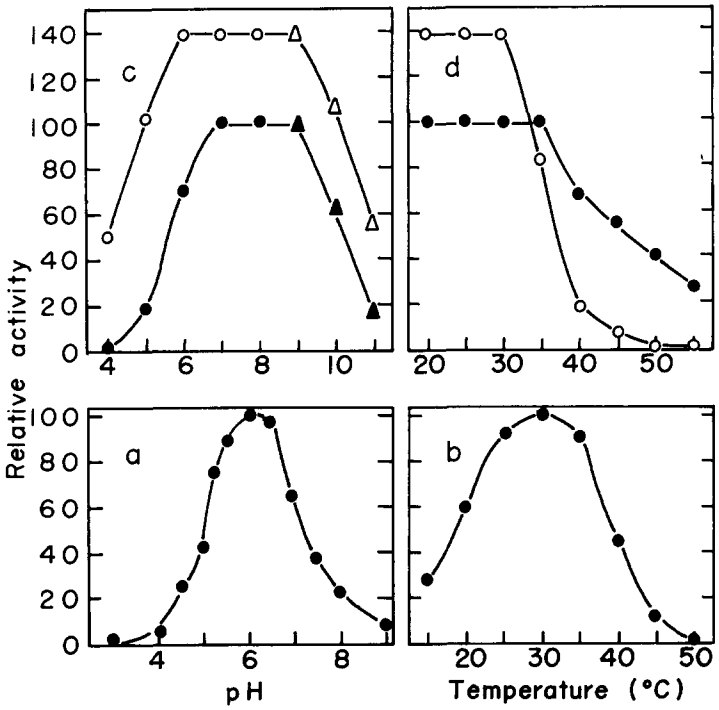

Fig. 9. Effects of pH and temperature on the activity and stability of the purified B-512F dextransucrase. •, Enzyme activity assayed without dextran; ○, enzyme solution was treated with 4.0 mg/ml clinical dextran. (a) pH-activity curve: the assay temperature was 30°C. (b) temperature-activity curve: the enzyme activity was assayed for 10 min at pH 6.0. (c) pH-stability curve: after being kept at various pH values for 24 h at 4°C, each enzyme solution was adjusted to the optimum pH and the residual activity was measured by the standard method. (d) thermal stability curve: after incubation for 10 min at the indicated temperature, the remaining activity was assayed by the standard method. Circles, McIlvaine buffer; triangles, borate buffer.

TABLE II

EFFECTS OF METAL IONS AND CHEMICAL REAGENTS ON THE B-512F DEXTRANSUCRASE ACTIVITY

Final concentration of metal ions and chemical reagents were 1 mM and 0.5 mM, except for *p*-chloromercuribenzoate (0.05 mM). B-512F native dextran (4 mg/ml) was preincubated with the enzyme at 30°C for 10 min.

Compound	Remaining activity (%)		Compound	Remaining activity (%)	
	without dextran	with dextran		without dextran	with dextran
None	100	136	Iodoacetic acid	56	56
$CaCl_2$	111	124	*N*-Ethylmaleimide	79	113
$CuCl_2$	7	4	*p*-Chloromercuribenzoate	88	110
$MgCl_2$	97	125	Phenylmercuric acetate	6	8
$FeCl_2$	83	119	Sodium thioglycolate	105	130
$FeCl_3$	20	0	L-Cysteine	81	118
$CoCl_2$	100	112	Sodium dodecyl sulfate	118	182
$MnCl_2$	38	52	*o*-Phenanthroline	95	103
			EDTA	8	96

stimulation was observed when the enzyme was incubated with dextran at the stable range of pH and temperature.

As shown in Table II, the purified dextransucrase was activated by 1 mM Ca^{2+} and strongly inhibited by Cu^{2+}, Fe^{3+} and Mn^{2+} in this order. Although both phenylmercuric acetate and EDTA (0.5 mM) inhibited the purified enzyme to the same extent, the addition of exogenous dextran particularly protected the enzyme against inactivation caused by EDTA. The addition of Ca^{2+} and Co^{2+} to the EDTA-treated enzyme could restore the activity (data not shown).

Effect of various dextrans on the enzyme

The purified dextransucrase was activated by the addition of exogenous B-512F native dextran (Fig. 10) and its activity reached a maximum at 3 mg/ml dextran (data not shown). Clinical dextran, dextran T-10 and B-1299 native dextran stimulated the activity to a similar extent (data not shown). However, the crude enzyme preparation containing more than 10 mg/ml of dextran was not affected by added dextran as reported previously [8].

As shown in Table III, K_m values of the enzyme decreased with decreasing temperature of incubation as reported by previous workers [9]. In the presence of various dextrans, the Michaelis constants became much smaller than that obtained by incubation at 30°C without dextran. Although no apparent correlation between K_m values and the molecular weight of added dextran was observed, higher concentration of dextran gave smaller K_m values as shown in the case of B-512F native dextran (Table III), and this was consistent with our kinetic analysis of the direction of chain elongation [10].

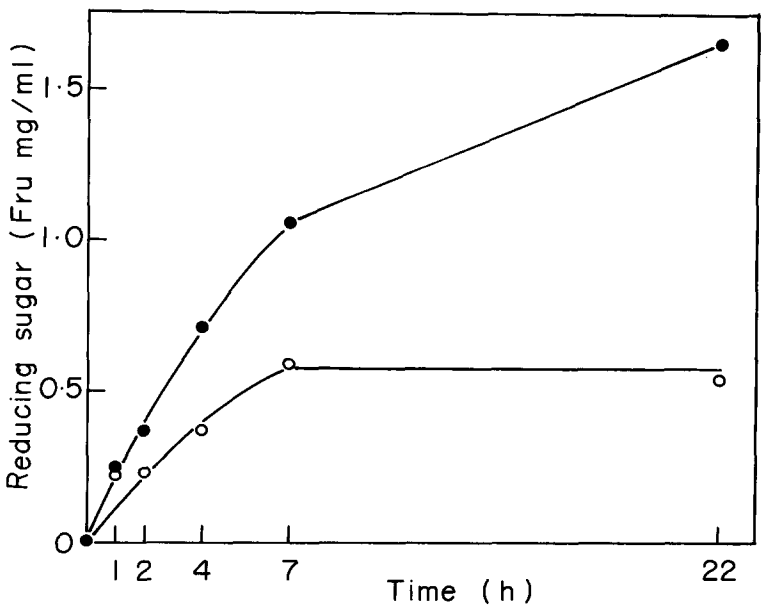

Fig. 10. Effect of exogenous dextran on the B-512F dextransucrase activity. Enzyme activity was assayed in the presence (●) and absence (○) of B-512F native dextran (10 mg/ml).

TABLE III

K_m VALUES AT VARIOUS TEMPERATURES AND WITH PRIMER DEXTRANS

B-512F, B-512F native dextran; Clinical, clinical dextran; T-10, standard T-10 dextran; B-1299, B-1299 soluble dextran. Each dextran was preincubated with the enzyme at 30°C for 10 min.

Temperature (°C)	K_m (mM)	Dextran (mg/ml)		K_m (mM)
20	18	B-512F	1.6	36
30	46	B-512F	6.2	24
40	585	Clinical	4.0	15
		T-10	10.5	26
		B-1299	1.7	15

Product analysis

The purified enzyme was incubated with 10% sucrose for 20 h at 25°C and the reaction was terminated by heating for 10 min in a boiling water bath. The reaction mixture was then chromatographed on a Sepharose 6B column. As shown in Fig. 11, only a small amount of product was eluted at the void volume (V_0) and the remainder was detected at the inner volume (V_i) of the column. The ratio of carbohydrate eluted at the position of V_0 and V_i suggested that purified B-512F enzyme had very low catalytic activity to synthesize polymer dextran, transferring activity of dextransucrase. Paper chromatographic analysis of the V_i fraction showed that this fraction composed

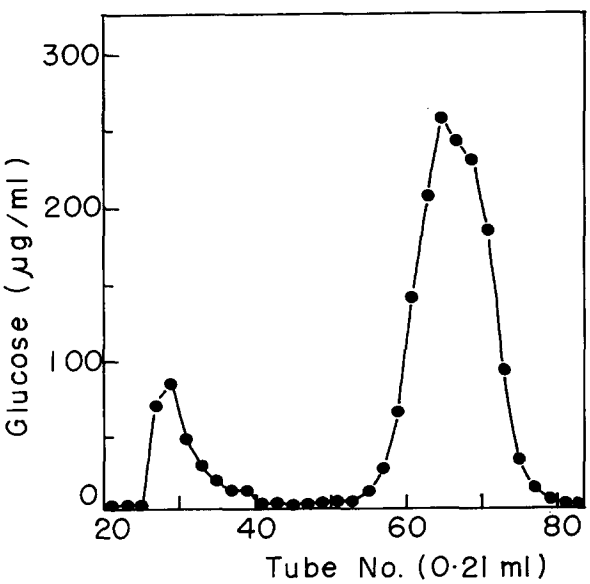

Fig. 11. Gel filtration of the reaction product with B-512F dextransucrase. The reaction mixture contained 200 mg of sucrose, 40 mM McIlvaine buffer (pH 6.0), and purified dextransucrase (0.5 units/ml) in the final volume of 2.0 ml. After incubation at 25°C for 20 h, the reaction was terminated by heating for 10 min at 100°C. The digest (0.5 ml) was applied to a Sepharose 6B column (1 × 20 cm) and eluted with 0.02% sodium azide solution.

TABLE IV

PRODUCTS OF THE REACTION OF PURIFIED DEXTRANSUCRASE ON VARIOUS SUBSTRATES

The reaction mixture contained 2% substrate in 40 mM McIlvaine buffer, pH 6.0 (25 μl) and purified dextransucrase (0.05 units, 25 μl). Clinical dextran (500 μg/50 μl water) or water was added as indicated in the table. After incubation for 20 h at 25°C, the reaction was terminated by heating for 10 min at 100°C. The reaction products were examined by paper chromatography.

Substrate	Products
IM_4	None
B-512F native dextran	None
Sucrose *	Fru, Glc > $IM_{4,5}$ (trace)
Sucrose plus clinical dextran	Fru, Glc > Leucrose, $IM_{4,5}$ (trace)
Raffinose	Fru, Melibiose > Glc > Leucrose (trace)
Raffinose plus clinical dextran	Fru, Melibiose >> Leucrose

IM_4, isomaltotetraose; IM_5, isomaltopentaose; Fru, fructose; Glc, glucose; raffinose, β-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside; melibiose, β-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside; leucrose, α-D-glucopyranosyl-(1 → 5)-D-fructopyranoside.
* A small amount of substrate sucrose remained in the digest.

mainly of fructose, glucose and trace amounts of isomaltotetraose and isomaltopentaose besides sucrose (Table IV). Although the substrate, sucrose, disappeared when clinical dextran was added to the reaction mixture, the amount of glucose formed was almost unchanged. Moreover, native dextran B-512F and isomaltotetraose were not susceptible to the purified enzyme (Table IV). An appreciable amount of glucose was detected when raffinose was incubated with the enzyme; however, no glucose had been formed during the incubation with added dextran. Therefore, the purified enzyme was completely free from any glucosidase and dextranase activities. Levansucrase and invertase could also be ruled out since the enzyme activity was stimulated by the addition of dextran (Fig. 10).

The V_0 fraction of the Sepharose 6B column, which was hydrolyzed with endodextranase gave a degree of hydrolysis (D_H) of 20%, whereas other standard dextrans of the B-512F type gave D_H values of 27—28% (Table V). However, the enzymatically synthesized dextran showed a much higher sus-

TABLE V

SUSCEPTIBILITY OF VARIOUS DEXTRANS TO ENDODEXTRANASE

Each dextran (5 mg) was digested with endodextranase (6 units/ml of 40 mM McIlvaine buffer, pH 5.2) at 40°C for 3 h. Reducing sugar was determined by the Nelson-Somogyi method.

Dextran	Degree of hydrolysis (% of glucose)
Synthesized dextran	20
T-10 dextran	25
Clinical dextran	28
B-512F native dextran	27
B-1299 soluble dextran	2

ceptibility to dextranase than the highly branched B-1299 dextran (2%); and glucose, isomaltose and isomaltotriose were detected from the hydrolyzate by paper chromatography (data not shown). Therefore, the product dextran has an analogous structure to the B-512F type of dextran.

Aggregation of the purified dextransucrase

The purified dextransucrase was voided from a Sephadex G-100 column and showed no affinity for the dextran gel in contrast to the component I (data not shown). Furthermore, aggregation of the purified enzyme, which occurred during storage of the enzyme in a concentrated solution, was demonstrated by gel filtration on the Sepharose 6B. Although the aggregated enzyme was eluted at tube Nos. 37—47 (data not shown), the dextranase-treated enzyme was eluted at the elution volume of the same column (Fig. 1b, tube Nos. 61—71) as described above.

Discussion

The extensive purification of dextransucrase from *L. mesenteroides* was first reported by Ebert and Schenk and the molecular weight of this enzyme was determined to be 280 000 by ultracentrifugation [9]. More recently, Robyt and Walseth have also shown the presence of a higher molecular weight enzyme which was voided from a Bio-Gel A-5m column and transformed into a low molecular form by treatment with dextranase [8]. Therefore, multiple forms of B-512F dextransucrase were analyzed by gel electrophoresis according to the Hedrick-Smith procedure [20]. As shown in Fig. 2, two faster-moving components, enzyme I and II, were classified into charge isomer groups, and the molecular weight of these enzymes was calculated to be 65 000.

Affinity chromatography of streptococcal dextransucrase on a column containing insoluble glucan from cariogenic bacteria has been reported by McCabe and Smith [22]. When the crude enzyme was chromatographed on a Sephadex G-100 column, one component having high affinity for the Sephadex gel could be well separated from the other component, which was eluted at the V_0 fraction from the column (Fig. 3).

The major enzyme component was eluted at the V_0 of not only the Sephadex G-100 but also the Sepharose 6B column and the carbohydrates detected at V_0 were attributable to dextran, which was synthesized during the growing of the culture. The scanning pattern showed that the enzyme I and II were predominant after 30 min incubation with dextranase and the high molecular weight component was not detected (Fig. 4). The transformation into the low molecular weight form was also confirmed by gel filtration on Sepharose 6B (Fig. 1b). These results indicated that the dextran molecule together with the enzyme could be removed by endodextranase digestion and consequently the protomer forms of the enzyme with low molecular weight were obtained.

Molecular weight of the protomer enzyme was estimated to be 64 000 by electrophoresis in SDS-polyacrylamide and this value closely corresponded to the value obtained from the Hedrick-Smith procedure (Fig. 2). In addition to the above two electrophoretic analyses, isoelectric focusing data also confirmed

the purity of protomer enzyme which was focused at pH 4.1 (Fig. 7). The p*I* values of streptococcal enzymes ranged from 4.0 [23] to 7.9 [24].

The purified enzyme had 5.41 units of activity, 75 μg of protein and 99 μg of carbohydrate. Most of the carbohydrates, which were contained in the purified enzyme, seemed to be endogenous dextran covalently bound to the enzyme molecule, since they could be eliminated not by mild treatment (7 days at 4°C) but the complete digestion (20 h at 40°C) with endodextranase. On the other hand, the V_0 fraction of the Bio-Gel P-4 column, which remained unsusceptible after the dextranase digestion of the purified enzyme (Fig. 8), would represent the carbohydrate moiety of this enzyme (17.4%).

The purified B-512F enzyme was activated by Ca^{2+} and strongly inhibited by EDTA, and the derepression of EDTA-inhibition by Ca^{2+} was also reported for dextransucrases of various *Leuconostoc* species [6,8,25]. Unlike B-1299 enzyme [5,6], SDS gave no inhibitory effect on B-512F enzyme and rather stimulated the activity in the presence of dextran. The exogenous dextran stimulated the activity regardless of the presence of metal ions and added chemicals, since the levels of increase in activity were not exceeded the control (136%; Table II). A significant stimulative effect by SDS might result from some modification of the tertiary structure of the enzyme by this surfactant.

The activation of dextransucrase by exogenous dextrans could be detected from several streptococcal enzymes [26,27] and *L. mesenteroides* B-1299 enzymes [5,6] which were purified to contain no appreciable amount of dextran. It was suggested that added dextran could also stabilize the enzyme to prevent loss of activity not only during storage but also during long-term incubation (Fig. 10), although some primer effects would occur simultaneous in the latter case.

From the results of product analysis (Fig. 11 and Table IV), it seems that the purified B-512F dextransucrase posssesses an enhanced level of sucrose hydrolyzing activity. The removal of the endogenous dextran might cause certain modification of the active site. In this regard, it is interesting to note that the high molecular weight dextran was shown to be effectively synthesized by the aggregated form of the enzyme [7,8]. Two types of aggregation of dextransucrase had been shown schematically by Germaine and Schachtele [3]. In one type, the monomer enzyme could aggregate to each other such as *L. mesenteroides* B-1299 [6] and *Streptococcus mutans* GS-5 [28]. In the other type, the aggregation was mediated by dextran molecule to which several enzyme photomers could be attached. A high-molecular weight form of B-512F crude enzyme (Fig. 1a) may fall under the latter category. Rapid reaggregation of the purified B-512F enzyme has also been noted by Robyt and Walseth [8], who attributed this aggregation to be the former type.

Acknowledgement

This work was supported by Grant No. 266031 and 366034 from the Ministry of Education, Japan.

References

1 Jeanes, A. (1966) Encycl. Polym. Sci. Tech. 4, 805—825
2 Sidebotham, R.L. (1974) Adv. Carbohydr. Chem. Biochem. 30, 371—444
3 Germaine, G.R. and Schachtele, C.F. (1976) Infect. Immun. 13, 365—372
4 Kobayashi, M. and Matsuda, K. (1974) Biochim. Biophys. Acta 370, 441—449
5 Kobayashi, M. and Matsuda, K. (1975) Biochim. Biophys. Acta 397, 69—79
6 Kobayashi, M. and Matsuda, K. (1976) J. Biochem. 79, 1301—1308
7 Kobayashi, M. and Matsuda, K. (1977) Agr. Biol. Chem. 41, 1931—1937
8 Robyt, J.F. and Walseth, T.F. (1979) Carbohydr. Res. 68, 95—111
9 Ebert, K.H. and Schenk, G. (1968) Adv. Enzymol. 30, 179—221
10 Kobayashi, M. and Matsuda, K. (1978) Carbohydr. Res. 66, 277—288
11 Hattori, J. and Ishibashi, K. (1969) Abstr. Pap., Annu. Meet. Agric. Chem. Soc. Jpn., p. 115
12 Siegelman, H.W., Wieczorek, G.A. and Turner, B.C. (1965) Anal. Biochem. 13, 402—404
13 Shaltiel, S. and Er-El, Z. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 778—781
14 Jeanes, A. (1965) Methods Carbohydr. Chem. V, 118—132
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Nelson, N. (1944) J. Biol. Chem. 153, 375—380
17 Somogyi, M. (1945) J. Biol. Chem. 160, 69—73
18 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350—356
19 Maurer, H.R. (1968) Disc Electrophoresis, pp. 32—110, Walter de Gruyter, Berlin
20 Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155—164
21 Righetti, P.G. and Drysdale, J.W. (1974) J. Chromat. 98, 271—321
22 McCabe, M.M. and Smith, E.E. (1977) Infect. Immun. 16, 760—765
23 Chludzinski, A.M., Germaine, G.R. and Schachtele, C.F. (1976) J. Bacteriol. 118, 1—7
24 Newbrun, E. (1971) Caries Res. 5, 124—134
25 Itaya, K. and Yamamoto, T. (1975) Agr. Biol. Chem. 39, 1187—1192
26 McCabe, M.M. and Smith, E.E. (1973) Infect. Immun. 7, 829—838
27 Chludzinski, A.M., Germaine, G.R. and Schachtele, C.F. (1976) J. Dent. Res. 55, C75—C86
28 Kuramistu, H.K. (1975) Infect. Immun. 12, 738—749