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PURIFICATION, AND COMPARISON, OF TWO FORMS OF DEXTRANSUCRASE FROM *Streptococcus sanguis**

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ABSTRACT

A procedure has been developed whereby native and proteolyzed forms of dextransucrase have been purified; it involves gel filtration, and hydroxylapatite chromatography in the presence of 0.10% sodium dodecyl sulfate. This procedure is highly reproducible, and permits ~30% recovery of high purity (94% homogeneous) enzyme as an inactive, SDS complex that can be reactivated by the addition of Triton X-100. The purified enzymes have been compared with regard to amino acid compositions, and isoelectric and catalytic properties. An analysis of the structure of their product D-glucans was also made. Although the structural characteristics of the enzyme forms differ, proteolysis does not cause alterations in their catalytic properties.

INTRODUCTION

Dextransucrase (EC 2.4.15), which appears to play an important role in the formation of dental caries[1–3], is produced by a variety of micro-organisms. Among these are several species of oral *Streptococci*, such as *S. mutans*, *S. sanguis*, and *S. mitis*. The enzyme is a member of a class of D-glucosyltransferases that catalyze the transfer of the D-glucosyl group of sucrose to a growing D-glucan chain.

Several of the organisms appear to produce more than one type of analogous enzyme, and these are distinguished by the structural properties of their D-glucan products. Some of the polysaccharides are water-soluble, whereas others are relatively water-insoluble[4]. The terms GTF(S) and GTF(I) have been used to designate those enzymes responsible for water-soluble and water-insoluble D-glucan formation, respectively. Insolubility is related to a higher content of α-D-(1→3)- than of α-D-(1→6)-glucosidic linkages[4]. Electrophoretic analyses indicated that multiple forms of GTF(I) and GTF(S) exist[5]. Furthermore, distinctly different, multicomponent, GTF patterns have been observed from many of the *S. mutans* serotypes[4–12]. In general, the relationship between these various enzyme forms is not well understood. Some differences in molecular weights have been observed[6], and other

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studies indicated variations in net charge[4,7]. However, these studies were complicated by the fact that major portions of the enzyme remain immobile under nondenaturing, electrophoretic conditions. It has been suggested[13] that this is due to the propensity of the enzyme to form aggregates.

Multiple forms of the enzyme have also been observed for the dextransucrase produced by *S. sanguis*[14]. We recently demonstrated[15] that these forms are the result of aggregation of two different, monomeric units having molecular weights of 174,000 and 156,000, respectively. In addition, it was shown that the monomeric unit having mol. wt. 156,000 was derived from the larger one by proteolytic cleavage. We speculated that some of the multiplicities observed in other strains may be the result of similar proteolysis, or aggregation of monomeric forms of the enzyme[15], or both.

It was not known which characteristics of the enzyme had been altered by proteolysis. Thus, it was the purpose of the present investigation to compare the structural and catalytic properties of the two monomeric forms of the *S. sanguis* enzyme. In order to accomplish this, a purification procedure had to be developed that would reliably yield the two enzyme forms with good recovery and in a high state of purity. We have based the purification on the observation that high concentrations of SDS are required for complete dissociation of the enzyme to monomeric forms; this was accomplished by utilizing the observation of Russell[16,17] that SDS-inactivated GTF can be reactivated by such nonionic detergents as Triton X-100. In fact, he successfully employed[17] SDS during gel filtration for purification of GTFs from *S. mutans*. We now report on the purification of the native and proteolyzed forms of dextransucrase from *S. sanguis* as their inactivated, SDS complexes, and on the comparison of their properties.

RESULTS

Purification. — The aim of the present study was to compare the native (mol. wt. 174,000) and the proteolyzed (mol. wt. 156,000) forms of the enzyme. The strategy was to produce the proteolyzed form at the crude stage, and to purify the two forms independently. A single procedure was found to be effective for purifying both forms. Concentrated, crude enzyme [80 mL (1093 units); see Methods] was permitted to undergo proteolysis by incubation for 6 days at 25° in the presence of 0.01% of chloramphenicol. Under these conditions, 92% of the activity was maintained, and SDS-electrophoretic analysis revealed quantitative conversion of the species having mol. wt. 174,000 to the form[15] of mol. wt. 156,000.

The following purification procedure was applied to either proteolyzed or unproteolyzed, concentrated crude enzyme; it involves the initial disaggregation of the crude enzyme to the respective monomeric units. This was accomplished by the addition of a sufficient volume of 10% SDS to 80 mL of the proteolyzed or unproteolyzed, concentrated crude enzyme, to yield a final concentration of 0.4% of SDS at 10°. The mixtures were gently stirred for 1 h, during which the temperature was allowed to rise to 25°.

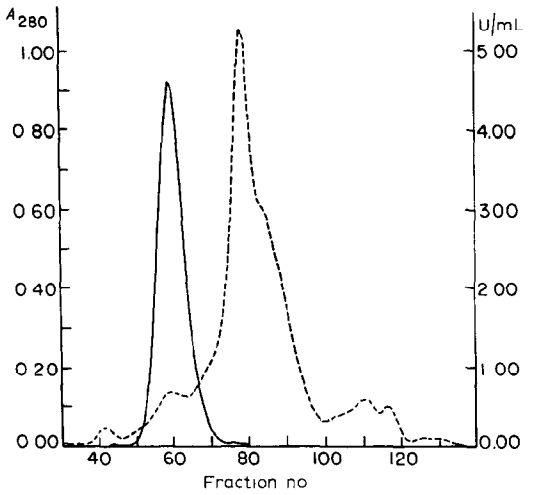

Fig. 1. Gel filtration in 0.1% SDS. Dextransucrase (880 units) that had been inactivated and disaggregated with 0.4% SDS was applied to a column (5.0 × 115 cm) of Sepharose CL-6B that had been equilibrated as described in the text. Elution was conducted as described in the text, and the absorbance (A) at 280 nm (· · · · ·) and enzyme activity (——) of SDS-inhibited enzyme (see Experimental section) of each fraction was measured. The results are plotted against elution volume. The fractions having the highest specific activity (units/A_{280}) were pooled (fractions 55–63).

Gel filtration in 0.1% SDS. The disaggregated enzyme, in 0.4% SDS, was applied to a column (5.0 × 115 cm) of Sepharose CL-6B 200 that had been equilibrated with 0.1M lithium phosphate, 0.1% SDS, pH 6.3, at 4°, with a flow rate of 1.81 mL/min. Elution was continued with the same solution, and fractions were analyzed for enzyme activity (see Methods) and for absorbance at 280 nm. The enzyme was eluted as a single peak (see Fig. 1), which typically represented 85 to 95% of the enzyme activity applied. Although recovery was somewhat lower, similar behavior for the proteolyzed form was observed (the elution profile is not shown). The fractions having the highest ratio of activity/A_{280} were pooled, and used in the subsequent step.

Hydroxylapatite chromatography in 0.1% SDS. The general procedure of Moss and Rosenblum[18] was utilized for chromatography on hydroxylapatite. The pooled enzyme obtained by gel filtration was brought to room temperature and applied to a column (2.5 × 14.4 cm) of hydroxylapatite (30 g) that had been prepared in 0.1M lithium phosphate, pH 6.3, and equilibrated in the same buffer containing 0.1% SDS. After the sample had been applied (at a flow rate of 1.5 mL/min), the column was washed with 1 bed volume of the equilibrated solution. Elution was conducted, at room temperature and the same flow rate, with a linear gradient (900 mL) of sodium phosphate, pH 6.4 (0.1M to 0.5M), containing 0.1% SDS. Fractions were analyzed for enzymic activity (see Methods) and protein by using the Lowry procedure[19]. In addition, the conductivity was measured on 25-μL aliquots that had been diluted to 2.7 mL, and compared to that of a set of phosphate

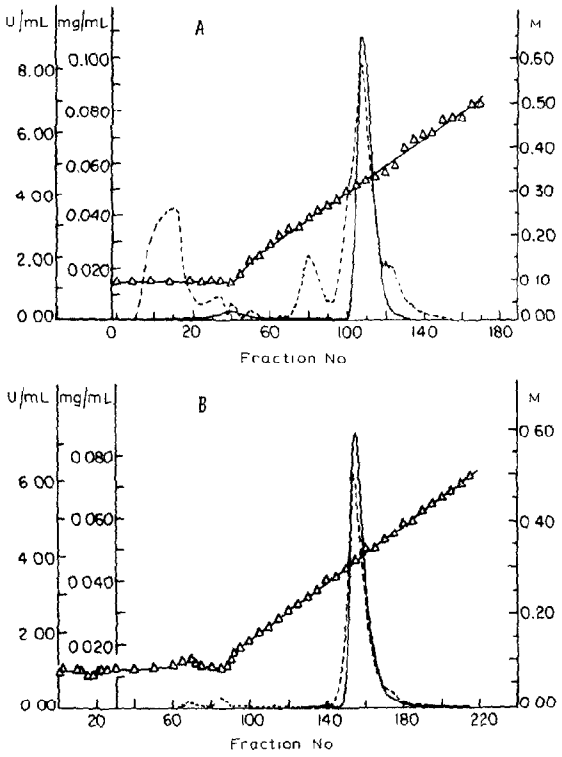

Fig. 2. Hydroxylapatite chromatography in 0.1% SDS. A. The pooled enzyme from Fig. 1 (589 units) was applied to a hydroxylapatite column (2.5 × 14.4 cm) that had been equilibrated as described in the text. Elution was performed as described, using a linear gradient of phosphate buffer in 0.1% SDS. The fractions were assayed for enzyme activity (——); protein, using the Lowry procedure (· · · · ·); and conductivity, which is expressed as molarity of phosphate (△----△). The results are plotted as a function of fraction number. Fractions (105–118) that contained the bulk of the enzyme activity were pooled. B. The pooled enzyme (502 units), after dilution, was aplied to a second hydroxylapatite column, which was identical to that described in A. The same procedures for elution, and measurements of activity, protein, and conductivity were used as described in A.

buffer standards. A single enzyme peak, which was eluted at 0.32M was observed (see Fig. 2A); it regularly accounted for a minimum of 95% of the applied enzyme. SDS-gel electrophoretic analysis of the fractions in the peak (data not shown) indicated that the leading and trailing edges contained substantial proportions of contaminating protein. However, the fractions having the highest specific activity exhibited a single protein component.

In an effort to obtain a larger proportion of the enzyme in a homogeneous form, the bulk of the peak (~90% of the recovered activity) was subjected to rechromatography on a second, but identical, hydroxylapatite column. In order to adsorb the enzyme onto the column, the phosphate concentration was diminished by four-fold dilution with 0.1% SDS. Gradient elution of the column resulted in a

TABLE I

SUMMARY OF PURIFICATION

Sample, and purification step	*S.A.*	*Total units*	*Yield*[a]	*Recovery*[b] *(%)*
A. Native				
Concentrated crude	3.21	880	—	100
Gel filtration/SDS	30.9	758	86.2	86.2
HA/SDS 1 (peak)	93.0	560	95.1	63.6
HA/SDS 2, pool II	106.7	284	56.5	32.3
B. Proteolyzed				
Concentrated crude	3.17	1093	—	100
Incubation[c]	n.d.[d]	1001	91.6	91.6
Gel filtration/SDS	36.4	672	67.1	61.5
HA/SDS 1 (peak)	82.8	533	96.9	48.8
HA/SDS 2, pool II	112.2	305	61.5	27.9

[a]Yield represents the percentage of enzyme activity recovered in the specific step. [b]Recovery is the percentage of the initial enzyme activity recovered in the entire procedure. [c]The concentrated, crude enzyme was converted into the proteolyzed form by incubation for 144 h at 25° in the presence of 0.01% chloramphenicol. [d]n.d. = not determined.

single detectable protein peak (see Fig. 2B) and a 93% recovery of applied enzyme. The fractions comprising the leading and trailing edges had a lower specific activity (S.A.) than the rest of the peak, which had a relatively uniform S.A. of 107 units per mg. The peak was collected in three pools, the leading and trailing edges being designated pools I and III, respectively. Pool II consisted of fractions having the highest S.A., which included the peak maximum and constituted ~60% of the total enzyme recovered in the step.

The purification of the proteolyzed form on hydroxylapatite was quite similar; however, it required a somewhat higher concentration of sodium phosphate (0.36M) to achieve elution. The pooled fractions were dialyzed against 0.1% SDS in 5mM lithium phosphate, pH 6.5, and rapidly frozen by dripping into liquid nitrogen. In this way, the enzyme could be stored at −70° without losses due to freezing and thawing.

The purification of the two forms is summarized in Table I. Overall recovery typically lay between 30 and 35% of the original activity present in the crude enzyme. The specific activity obtained for the native enzyme was ~107 units per mg, whereas that of the proteolyzed form was 112 units per mg. The procedure offered a highly reproducible method for purifying both the native and proteolyzed forms in good yield. It should be pointed out that proteolytic alteration of the native form did not proceed in the presence of SDS, so that it was possible to obtain this form free from the proteolyzed material. SDS gel electrophoresis of the unproteolyzed form at various stages of purification is shown in Fig. 3A. Enzyme from the hydroxylapatite (HA/SDS) pool II was applied at two concentrations to

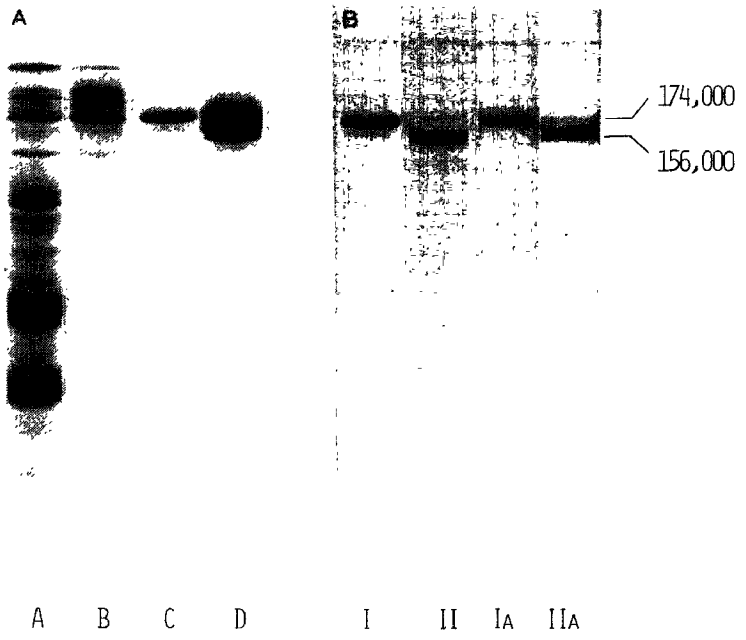

Fig. 3. Analysis of purity by SDS electrophoresis. A. SDS gel-electrophoresis was performed as previously described[14]. Four 6% poly(acrylamide) gels were prepared, and loaded with the following samples: (A) crude concentrate, 62.5 μg of protein, 0.50 unit; (B) the pooled enzyme obtained from gel filtration (Fig. 1), 16.3 μg, 0.50 unit; (C) SDS/HA pool II (Fig. 2B), 5.3 μg, 0.57 unit; and (D) SDS/HA pool II, 26.6 μg, 2.84 units. The gels were stained with Coomassie Blue. B. Comparison of purified native and proteolyzed forms. SDS gel electrophoresis on 6.0% polyacrylamide gels was carried out as previously described[14]. Four gels were prepared, and loaded as follows: I, native enzyme (3.8 μg); II, proteolyzed enzyme (3.6 μg); IA, native enzyme (0.95 μg); and IIA, proteolyzed enzyme (0.9 μg). Following electrophoresis, gels I and II were stained with Coomassie Blue, and IA and IIA were developed to detect enzymic activity by allowing the gels to react in a sucrose (0.1M)–Triton X-100 (1%) solution. The gels were then stained by using the PAS reagent[14].

duplicate gels (C and D) that demonstrated a high degree of homogeneity. A comparison of the two forms is shown in Fig. 3B, in which duplicate gels were stained for protein or enzyme activity. As may be seen, each preparation is highly purified, with the major contaminant being the alternative form of the enzyme. Estimates of the degree of homogeneity, based on densitometric tracings of a series of Coomassie-stained SDS gels, gave values of >94% purity.

Properties. — The two purified forms of dextransucrase were compared with each other in terms of catalytic and structural properties.

Structural properties. Amino acid analyses of the native and proteolyzed forms were conducted as described under Methods, and the results are summarized in Table II. The number of amino acid residues per molecule was calculated, based on molecular weights of 174,000 and 156,000. Both forms are virtually devoid of cysteine. Proteolysis results in the loss of 177 amino acids, of which 114 are polar or ionic.

TABLE II

AMINO ACID ANALYSES

Amino acid	*Average number of residues per chain*		*Difference*
	Polypeptide (mol. wt. 174,000)	*Polypeptide (mol. wt. 156,000)*	
Aspartic acid	243.9 ±6.7 (4)	221.4	22
Threonine	106.1 ±2.2 (4)	86.9	19
Serine	94.8 ±3.0 (4)	71.4	23
Glutamic acid	194.2 ±3.9 (4)	155.5	39
Proline	35.3 ±2.4 (3)	30.9	4
Glycine	120.3 ±1.8 (3)	117.9	
Alanine	125.8 ±1.4 (3)	105.3	21
Cysteine	0.4 ±0.5 (4)	0.0	
Valine	85.3 ±1.0 (3)	77.2	8
Methionine	25.1 ±1.8 (3)	26.4	
Isoleucine	49.7 ±0.6 (3)	45.7	4
Leucine	99.5 ±2.1 (4)	91.9	8
Tyrosine	83.3 ±2.3 (4)	84.5	
Phenylalanine	55.7 ±0.9 (3)	55.5	
Histidine	12.2 ±0.2 (4)	11.6	1
Lysine	125.8 ±1.8 (4)	109.6	16
Arginine	59.0 ±0.6 (4)	54.7	4
Tryptophan	39.0 ±3.0	32	7
Total	1555 ±36	1378	177

It should be pointed out that no constituents other than amino acids were detected. Specifically, amino sugars were not observed, and this implies that the enzyme is not a glycoprotein. This issue was further explored in a more general analysis for neutral sugars, using the sensitive gel-staining technique of Racusen[20]. The procedure, based on a thymol–H_2SO_4 reaction, was calibrated by using electrophoresis of ovalbumin on 8% polyacrylamide–SDS gels. The lower limit of detectability corresponded to 3 ng of D-mannose on a 5-mm gel. No carbohydrate could be detected when 22.5 μg of native dextransucrase was analyzed, and therefore it contained <30 ng. This indicated that the enzyme contained <0.13% of sugar. Thus, one molecule of the enzyme contains, at the most, 1 monosaccharide residue.

The two forms were subjected to isoelectric focusing in order to compare their isoelectric points. Following reactivation, most of the detergent was removed by ultracentrifugation in a gradient of glycerol. Enzyme samples were then subjected to glycerol-gradient isoelectric focusing according to a modification of the method of Weller *et al.*[21]. The results (see Fig. 4) indicated that the native and proteolyzed forms have different isoelectric points (pI). The native form has a pI of 5.14 ±0.07, based on 12 analyses, whereas the proteolyzed form has a pI of 6.25 ±0.19, based on 10 analyses. The higher isoelectric point is consistent with the fact that 61 acidic amino acids are lost during the proteolytic conversion. It may also be

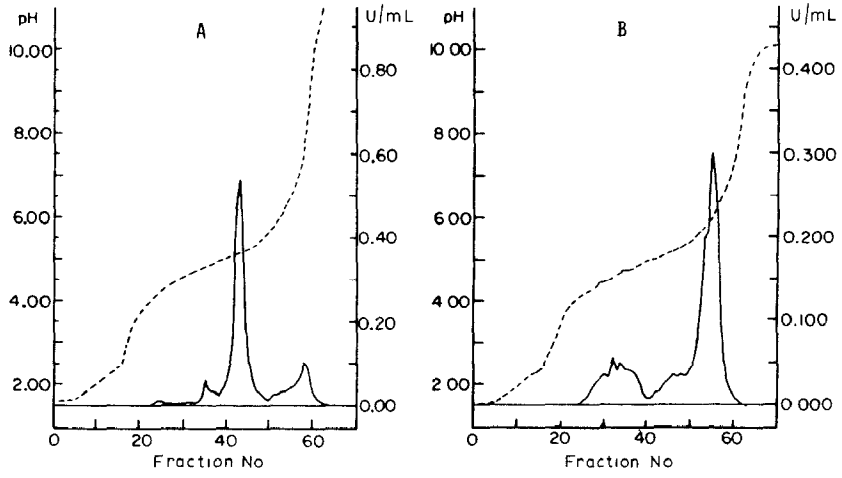

Fig. 4. Isoelectric focusing of native and proteolyzed forms. A. Detergent-free, native enzyme (3.0 units) was subjected to isoelectric focusing as described in the Experimental section The pH (· · · · ·) and enzymic activity (——) were measured in the collected fractions B. All conditions were the same as in A, except that proteolyzed enzyme (1.9 units) was utilized.

TABLE III

CATALYTIC PROPERTIES

Form	*Acceptor*[a] *(mM)*	*Sucrose*[b] *(K_m) (mM)*	*V_{max}*[b] *(μmol/min/unit)*	*Optimal pH*[c]
Native	0	0.93	0.250	6.5
	4	5.09	1.020	—
Proteolyzed	0	0.87	0.231	6.5
	4	5.15	1.080	—

[a]Dextran T-10 [b]Reactions were initiated by adding enzyme (20 μL, ~0 0516 unit of native, 0.0530 unit of proteolyzed) in 0.1% SDS to a mixture (180 μL) consisting of sucrose at concentrations varying from 0.25 to 500mM, 0.1M phosphate buffer, pH 6.1, and Triton X-100 (1.0%) in the presence or absence of Dextran T-10 (4mM). The enzyme was added at room temperature, the mixture rapidly heated to 37°, and maintained here for 10 min. The reactions were terminated by heating for 2 min at 100°, and aliquots (20 or 40 μL) were removed for analysis for D-fructose as described under Methods. The values for K_m and V_{max} were obtained from double-reciprocal plots of 1/rate *vs.* 1/sucrose concentration. [c]Enzyme (20 μL, ~0.05 unit) was reactivated by addition to 80 μL of 1% Triton X-100, and allowed to stand for 16 min at room temperature, at which time sucrose buffered with 0.2M sodium phosphate, 0.1M sodium acetate, at pH values ranging from 3.5 to 10.0, was added. After 20 min, the reaction mixtures were heated for 2 min at 100°, and aliquots (20 μL) were analyzed for D-fructose. Control reactions in the absence of enzyme were performed, and the values found were subtracted.

seen in Fig. 4 that the native and the proteolyzed forms have minor components with different isoelectric points. All of these appear to be isomeric forms of the proteolyzed protein, based upon SDS-gel electrophoretic analysis of these peaks. It should be pointed out that the native form was recovered only from the pI-5.14 peak. Thus, it appears that the native enzyme is an isoelectrically homogeneous entity.

Catalytic properties. The two enzyme forms isolated were compared with regard to their catalytic properties. Their pH optima, and such kinetic constants as K_m and V_{max}, are summarized in Table III. The kinetic constants were obtained from double-reciprocal plots, using sucrose as the variable substrate, in the presence and absence of Dextran T-10 (M_n ~9500) which served as a glucosyl acceptor. It may be seen that there is little or no difference between the two enzyme forms with regard to these constants. The pH optima were also compared; they appeared to be identical. Although the data are not shown, the pH–rate profiles for the two forms were virtually superposable.

It was also important to compare the products generated by the two forms of the enzyme. Although it was not readily possible to perform a detailed, structural analysis of the products, it was possible to obtain information concerning the ratio of α-D-(1→6) and α-D-(1→3) linkages. Previous work of Arnett and Mayer[22] had shown that this ratio can be established by ^{1}H-n.m.r. spectroscopy. Dextrans were

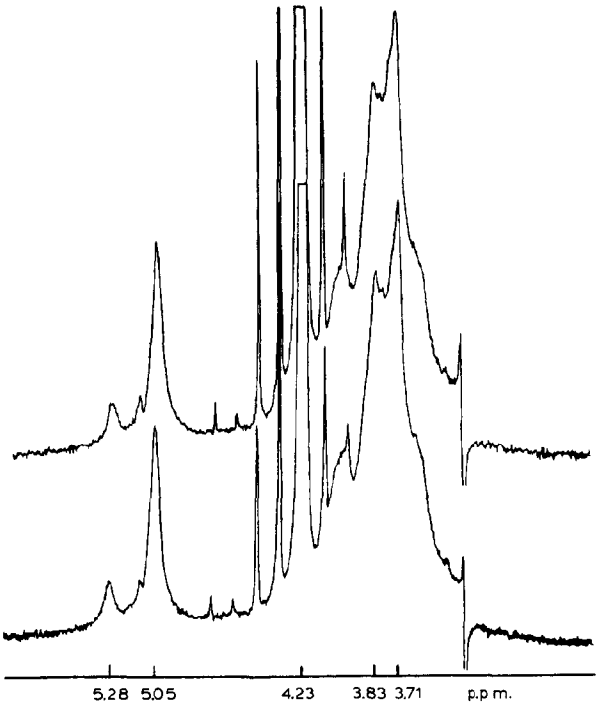

Fig. 5. ^{1}H-N.m.r. spectra of dextrans formed by native and proteolyzed enzymes. Dextran was synthesized in reactions of native (3.3 units) and proteolyzed (3.3 units) forms of dextransucrase, with sucrose (0.2M) in the presence of 0.1M sodium phosphate, pH 6.1, and 0.2% Triton X-100, in a total volume of 20 mL for 6 h. The polysaccharide was collected by precipitation with ethanol (2 vol.), and resuspended in water. The process was repeated twice, and then the product was prepared and subjected to ^{1}H-n.m.r. spectral analysis as described in the Experimental section. The upper tracing is the spectrum of the polymer formed by the native enzyme, and the lower is the product from the proteolyzed form.

produced enzymically in long-term reaction mixtures containing buffered sucrose and either the native or the proteolyzed form of dextransucrase. The products were isolated by repeated precipitation with ethanol, and then subjected to ^{1}H-n.m.r. spectral analysis as described in Fig. 5. The peaks at 5.05 and 5.28 p.p.m. are for the resonances due to α-D-(1→6) and α-D-(1→3) bonds, respectively. The spectra were almost identical in all regards, as may be seen by comparison at unassigned resonances, such as those at 3.71 and 3.83 p.p.m. The major peak (4.23 p.p.m.) corresponds to the HOD resonance, which is shifted upfield from its usual position by ~0.4 p.p.m.; this shift is caused by KSCN, which has the beneficial effect of lessening the interference of HOD with the anomeric-proton region. The ratio of α-D-(1→3) to α-D-(1→6) bonds was calculated from the areas of the peaks at 5.05 and 5.28 p.p.m. The native enzyme produced a polymer containing 17.4% of α-D-(1→3) bonds, whereas the proteolyzed form yielded a product with 17.3% of α-D-(1→3) bonds. It must, therefore, be concluded that the products are indistinguishable with regard to their linkage types as determined by ^{1}H-n.m.r. spectral analysis.

DISCUSSION

S. sanguis 10558 produces the enzyme dextransucrase (EC 2.4.1.5), which appears[14] to be a single polypeptide having a molecular weight of 174,000. The organism also produces a protease that catalyzes the conversion of the native form into a fully active, but smaller (mol. wt. 156,000) form[14]. The enzyme can exist as a large aggregate (mol. wt. $>5 \times 10^6$), which had been the cause of the low recoveries and poor reproducibility observed in previous purifications. The aggregates can be dissociated with[14] SDS, but high concentrations were found[15] to inactivate the enzyme completely. Nonionic detergents were found to be capable of stabilizing the protein[14] and relieving the inactivation[16,17] caused by SDS.

Taking advantage of this behavior with detergents, a purification procedure has been developed which provides purified enzyme (94% homogeneity) as an inactive SDS complex that can be fully reactivated with Triton X-100. This procedure has several advantages. First of all, proteolysis during the purification procedure appeared to be completely prevented; this was found to be a problem with other procedures, as the proteolyzed form had originally been observed in partially purified preparations of the enzyme[14,15]. The presence of SDS also permits the procedure to be carried out at room temperature. The yield of pure enzyme lies between 30 and 35%; however, the rest, which accounts for nearly all of the original enzyme, is recovered in fractions that are slightly less pure. The high yield of the enzyme attained at each step indicated that selective losses of unique forms were unlikely. The fact that the procedure is effective for the isolation of both native and proteolyzed forms is consistent with this idea. The highest specific activity for the native enzyme was 115 units per mg, although the pooled sample was 107 units per mg. Previous work in our laboratory[23] involved on the purification of the enzyme (in low yield) to a specific activity of ~200 units per mg of protein. The difference

between the specific activities in our earlier work and those found in the current preparation may be related to the fact that the initial preparation was in an aggregated state. It is possible that a true value for the protein could not be established under these conditions.

The properties of the purified enzyme forms were examined. The amino acid composition was found to be very similar to that reported by Shimamura *et al.*[24] for the GTF(S) isolated from *S. mutans*. Our analyses indicated that dextransucrase is rich in polar amino acids, especially the dicarboxylic amino acids and their amides. It is particularly noteworthy that the enzyme is devoid of cysteine, which means that its tertiary structure must be solely dependent on other types of secondary bonding, such as hydrogen bonding, and ionic and nonpolar interactions. In considering this observation, the behavior of the enzyme with detergents is even more remarkable. The classic model of SDS action on proteins[25] is that it disrupts nonpolar interactions and hydrogen bonding. In addition, high concentrations of sodium phosphate (employed in the presence of SDS for HA chromatography) would be expected to destabilize ionic bonding in the protein. The absence of cysteine eliminates the possibility that disulfide bonds are responsible for the retention of a sufficient amount of the tertiary structure to direct appropriate refolding. Thus, it would seem reasonable to conclude that SDS would cause a complete loss of the tertiary structure of the enzyme. If this is the case, then reactivation must involve refolding to an active form by a process that cannot take advantage of a partially retained, tertiary structure.

The generation of the proteolyzed form of the enzyme involves the loss of ~177 amino acid residues. A large proportion of these are polar, with a significant number being L-glutamic and L-aspartic acids. It is not known if the native form is degraded by a single cleavage at one end of the molecule, or whether there are multiple breaks. The fact that there are several proteolyzed forms that differ in their isoelectric points, but not molecular weights, might suggest that multiple cleavages occur and yield a series of closely related products.

The comparison of the enzymic properties of the native and proteolyzed forms of dextransucrase indicated that there is little or no difference between them. The specific activity of the proteolyzed form is slightly higher, which probably reflects the fact that ~10% of the protein have been removed. All other parameters examined, such as the pH profile, K_m, and product structure, were found to be virtually identical. It must be concluded that *S. sanguis* produces one form of dextransucrase that is proteolytically modified subsequent to its secretion from the cell. Both forms of the enzyme exhibit about a four-fold increase in V_{max} on addition of dextran to the reaction mixtures. This is similar to the results of earlier work from our laboratory[24], in which we observed in a kinetic analysis that the addition of Dextran T-10 caused a significant increase in V_{max} at concentrations >1.6mM. Because double-reciprocal plots showed a substantial, downward inflection at these concentrations, the data were interpreted as reflecting the activation of dextransucrase by the polysaccharide.

It appears that proteolytic modification neither alters the catalytic activity nor yields an enzyme having different product specificity. Because the analyses of the product formed by the purified, native enzyme showed that both α-D-(1→6) and α-D-(1→3) bonds are present, it must further be concluded that a single enzyme catalyzes the formation of both of these types of bonds. Previous work in our laboratory[22] had established that the dextran produced by *S. sanguis* grown in sucrose contained ~34% of D-glucopyranosyl residues linked α-D-(1→3). Half were in linear sequence (3-mono-*O*-substituted α-D-glucopyranosyl residues), while the other half were branch points (3,6-di-*O*-substituted α-D-glucopyranosyl residues). The polymers formed by the two forms of dextransucrase isolated in the present study had structural characteristics similar to each other, as seen by ^{1}H-n.m.r. spectroscopy. However, their proportion of a α-D-(1→3) bonding was about half that of polymer isolated from sucrose-grown cells. As ^{1}H-n.m.r. analysis is unable to discriminate between the linear and branching linkages, we were unable to determine whether the decrease in the proportion of α-D-(1→3) links is due to the loss of one specific type of bonding.

EXPERIMENTAL

Materials. — Lyophilized cultures of *S. sanguis* 10558 were obtained from the American Type Culture Collection. Brain Heart Infusion was purchased from Difco laboratories (Detroit, MI). Polyacrylamide, deuterium oxide (99.8% atom% of D), lauryl sulfate 95% (used in gel electrophoresis), and Sepharose CL-6B 200 were purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Gel HTP, Bio-lyte 4/6, and sodium dodecyl sulfate (99+%) were obtained from Bio-Rad Laboratories (Richmond, CA). All other reagents were obtained from common, commercial sources, and were of reagent quality.

Methods. — *Analytical procedures.* Enzyme activity was measured as previously described[13]. Samples (10 μL) that had been inactivated by means of 0.1% of SDS were reactivated by addition to a solution (90 μL) of Triton X-100 (1%) at room temperature. After 10 min, a substrate solution (100 μL) containing sucrose (20 μmol), Dextran T-10 (1 μmol), and sodium phosphate, pH 6.1 (10 μmol), was added. The rest of the procedure was identical to that previously described[13]. A unit of activity is defined as that amount of enzyme that will catalyze the release, from sucrose, of 1 μmol of D-fructose per min at 37°. Protein was estimated by its absorbance at 280 nm, or, quantitatively, by using the Lowry procedure[19]. Amino acid analyses were conducted on enzyme precipitated with trichloroacetic acid, followed by hydrolysis under vacuum in constant-boiling HCl (2.0 mL) containing 0.05% of 2-mercaptoethanol.

Hydrolyses were performed for 22 h, at which time the samples were dried *in vacuo* and redissolved in a solution (200 μL) of 0.2M lithium citrate, 1% thiodiglycol, and 0.1% of phenol at pH 2.2. A sample (50 μL) was analyzed with a Beckman Amino Acid Analyzer (Model 119CL). A spectrophotometric estimation

of tryptophan was made by a method similar to that of Goodwin and Morton[27]. Absorption spectra of the protein, tryptophan, and tyrosine in 0.1% SDS in 5 mM lithium phosphate, pH 6.5, were obtained. The assumption was made that the absorption spectrum of the enzyme was an additive function of the absorbances of tryptophan and tyrosine.

^{1}H-N.m.r. analyses on dextrans were conducted after three exchanges with deuterium oxide[22]. After the final resuspension in D_2O containing 4.36M KSCN, samples were dispersed by passage through a 22-gauge syringe-needle. Spectra were recorded with a Bruker WP-200 spectrometer at 24°, using 3-(trimethylsilyl)-1-propanesulfonic acid as the internal reference. Spectra were obtained in the F.t. mode, using a pulse width of 3 μs. A 16 K data set was used, for a spectral width of 2000.0 Hz. About 1000 scans were accumulated for each spectrum.

Gel electrophoresis was performed as previously described[14]. Glycerol-gradient isoelectric focusing was conducted in a U-tube (1.1 × 60 cm). The cathode electrolyte, which consisted of 0.2M NaOH in 60% (w/v) glycerol, was added to the tube. Aliquots (0.6 mL) of the reactivated, detergent-free enzyme (obtained by preparative ultracentrifugation) were individually mixed with appropriate ampholyte mixtures in glycerol; these were then sequentially layered into the U-tube, to produce a gradient of glycerol and ampholytes. The anode electrolyte, 0.03M H_3PO_4 (2 mL), was added, and electrofocusing was conducted at 400 V for 23 h at 0 to 4°. At the end of the experiment, glycerol (60%) was pumped into the dummy arm of the U-tube, to displace the formed gradient through the separation arm, and fractions were collected.

Preparation of crude enzyme. Cells were grown in Brain Heart Infusion (12 L) that had been subjected to ultrafiltration with two Amicon H1P10-20 hollow fiber cartridges. After sterilization, the low-molecular-weight filtrate was inoculated with an active culture (1 L), and the pH maintained at 6.5 by the addition of KOH. Additional D-glucose (90 g total) was introduced at appropriate times in order to maintain a concentration in the range of ~0.05 to 0.20%. Cells were removed by centrifugation prior to complete exhaustion of the D-glucose. The culture fluid was concentrated approximately thirty-fold, and the concentrate dialyzed by ultrafiltration with two Amicon H1P100-20 hollow fiber-cartridges. Dialysis was achieved after the concentration by continuous addition of distilled water until the conductivity of the effluent was <100 μmho/cm. The enzyme was recovered in >90% yield, and the S.A. (based upon protein analysis by the Lowry procedure) was increased by approximately 90-fold. The crude enzyme concentrate was clarified by centrifugation, rapidly frozen by dripping into liquid nitrogen, and the frozen pellets were stored at −70°.

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