Carbohydrate Research, 36 (1974) 23–33

PREPARATION AND PROPERTIES OF A CONJUGATE CONTAINING DEXTRANASE AND CONCANAVALIN A

SIDNEY A. BARKER, ALAN G. GIBLIN, CHARLES J. GRAY,
Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (Great Britain)

AND WILLIAM H. BOWEN
Royal College of Surgeons Research Establishment, Downe, Kent (Great Britain)

(Received November 27th, 1973; accepted for publication, January 29th, 1974)

ABSTRACT

Conditions for the reaction of concanavalin A and dextranase with glutaraldehyde have been established to give a soluble, intermolecularly cross-linked conjugate possessing both dextranase and concanavalin activities. Evidence is presented that the dextranase and concanavalin molecules are linked to each other in the conjugate. The conjugate gives a different pattern of hydrolysis products on incubation with dextran than does dextranase.

INTRODUCTION

In recent years, much research work has been directed towards reduction of the incidence of dental caries. The reported approaches include studies with fluoride[1], glycerophosphates[2], and dextranases[3]. It is this last approach which has led to the present work.

In 1966, Gibbons *et al.*[4] examined the plaque produced on the surface of teeth and found that significantly more polysaccharide was produced by cariogenic than non-cariogenic bacteria. The polysaccharides present in plaque were later identified as being mainly dextrans, *i.e.*, (1→6)-linked glucans.

It is probable that layers of this type of polysaccharide on the surface of teeth contribute to the onset of caries by entrapping certain micro-organisms and providing a suitable environment for their activity. Consequently, it seemed likely that degradation of the polysaccharide coating would prevent the development of this environment, and might be beneficial in reducing the incidence of caries.

Attempts to achieve this degradation by using dextranase enzymes (EC 3.2.1.11), which hydrolyse α-D-(1→6) linkages, have been reported[3,5], but the results were not conclusive. Whereas the inclusion of dextranase in the diet of hamsters was reported[6] to completely inhibit development of caries, other workers[5] have indicated that no beneficial result was obtained when dextranase was used for children or adults. In almost all cases, however, a major problem was the introduction of the enzyme into

the mouth so that it remained in contact with the teeth in high enough concentration for a significant length of time. The natural flow of saliva normally removes soluble material very rapidly.

Concanavalin A is an agglutinin, obtained from the jack bean, which binds firmly to certain polysaccharides, including dextrans. The aim of this work was to prepare a conjugate of dextranase and concanavalin A, such that the strong affinity of concanavalin A for the dextran in plaque would result in the retention of the dextranase in the vicinity of the material on which it was required to act.

The principle of attaching a protein having a desirable biological activity to another species that is able to control the concentration of that activity at a particular site is, of course, a general one, and it is probable that it can be applied in many spheres of therapeutic treatment.

EXPERIMENTAL

General. — Buffers were made up according to the methods described by Dawson *et al.*[7]. The pH of each buffer was checked on an E.I.L. model 23A pH meter. Absorbances were determined on a Pye Unicam SP-500 spectrophotometer, using microcells with a path length of 1 cm.

Paper chromatograms were developed by downward irrigation on Whatman No. 1 paper (46 × 57 cm) with 1-butanol–pyridine–water (6:4:3), and detection was effected with ammoniacal silver nitrate at 100° for 15 min.

All mixing and stirring was carried out with a Griffin vortex stirrer, unless otherwise stated. All columns were prepared and packed in such a way as to ensure maximum reproducibility of results.

Assay for dextranase activity. — Dextranase activity was calculated by determining the reducing sugar produced on incubation of the enzyme with a 0.5% aqueous solution of B-512 dextran (Sigma Ltd.) at pH 5.0 and 37°, using 3,5-dinitrosalicylic acid[8]. The enzyme, which was isolated from a Penicillium species and had a specific activity of 202 i.u./mg, contained some α-D-galactosidase activity but was eluted as a single peak from Biogel P-200. In certain experiments, reducing sugar was determined by the phenol–sulphuric acid method[9]. Protein concentrations were determined by the method of Lowry *et al.*[10].

Determination of concanavalin A binding-power. — Concanavalin A binding-power was determined by the turbidimetric method[11], using glycogen.

Investigation of the reaction of glutaraldehyde with dextranase and concanavalin A. — (*a*) Solutions (1 mg/ml in 1 ml of 0.2M citrate buffer, pH 5.0) were severally treated with 25% aqueous glutaraldehyde (0, 20, 50, 100, 200, and 500 μl). The solutions were stirred and stored at 20° for 1 h. After centrifugation, the supernatants were then assayed for binding power. Corresponding samples of dextranase solution (1 mg/ml in 1 ml of distilled water) were also treated with glutaraldehyde, as described above, and then assayed for activity.

(*b*) The above experiment was repeated, using solutions containing 0.5 mg/ml of concanavalin A and dextranase. The results are shown in Table I.

Preparation of a dextranase–concanavalin A conjugate, using glutaraldehyde at pH 5.0. — A solution of dextranase (0.5 mg/ml in 0.1M citrate buffer, pH 5.0; 400 ml) was mixed with a solution of concanavalin A (0.5 mg/ml in 0.1M citrate buffer, pH 5.0; 400 ml). 25% Aqueous glutaraldehyde (10 ml) was added, and the mixture was stirred and incubated at 21° for 1 h.

A portion (10 ml) of the reaction mixture was then applied to a column (30 × 1.5 cm) of Biogel P-300 and eluted with distilled water. Fractions (1 ml) were collected, scanned at 280 nm, and assayed for dextranase and concanavalin A activity. The column had previously been calibrated with dextranase and concanavalin A. The results are shown in Fig. 1. The remainder of the reaction mixture was then dialysed against distilled water (6 × 2 l) to remove excess glutaraldehyde.

Precipitation of the conjugate with glycogen. — A solution (1 ml) of conjugate (0.7 mg/ml) was mixed with glycogen (1 mg/ml in 0.1M phosphate buffer, pH 7.2; 0.4 ml) and incubated at 37° for 4 h.

A saturated solution (0.5 ml) of sodium chloride in distilled water was then added, the mixture was stored for 16 h and then centrifuged, and the supernatant was assayed for dextranase activity. A 23% retention of activity was observed; 100% activity was retained when the conjugate was replaced by dextranase or a dextranase–concanavalin A mixture.

Incubation of dextranase with various concentrations of substrate. — Aliquots (2.5 ml) of 0.2% solutions of B-512 and B-742 dextrans in 0.2M citrate buffer (pH 5.0) were incubated with dextranase solution (0.5 mg/ml in distilled water; 0.20 ml) at 37°. Aliquots (0.5 ml) were removed after 0, 10, 20, and 30 min, and the liberated reducing-sugar was determined. The results are shown in Figs. 2 and 3.

Effect of the conjugate on various concentrations of substrate. — A solution (0.2 ml) of conjugate (0.7 mg/ml) was incubated at 37° with 2.5 ml of B-512 dextran solutions of various concentrations in 0.2M citrate buffer (pH 5.0). Similar experiments were carried out using 0.2-ml portions of a dextranase–concanavalin A mixture of identical protein concentration, but which had not been treated with cross-linking reagent. The solutions were assayed for reducing sugar after 0, 10, 20, 30, and 120 min. The results are shown in Fig. 4.

The reaction profile of the action of conjugate on B-512 dextran after treatment of the conjugate with isomaltohexaose. — An aliquot (0.4 ml) of a solution of isomaltohexaose (1 mg/ml in distilled water) was incubated with 1 ml of conjugate solution at 25° for 4 h. An aliquot (0.2 ml) of this solution was then incubated at 37° with 2.5 ml of a 0.5% dextran solution in 0.2M citrate buffer (pH 5.0). A similar experiment was carried out using 1 ml of conjugate solution which had been treated with 0.4 ml of distilled water in place of the isomaltohexaose. The solutions were then assayed for reducing sugar after 0, 10, 20, 30, and 60 min. The results are illustrated in Fig. 5.

Action of dextranase and conjugate on dextran (B-512). — Solutions (0.4 ml) of conjugate (0.7 mg/ml) and dextran (4% in distilled water; 2.5 ml) were incubated. Aliquots (0.5 ml) taken at 0, 30, and 240 min were heated at 95° for 10 min to stop

the reaction. Similarly, 0.4 ml of a dextranase solution (0.5 mg/ml in distilled water) was incubated with dextran. The products from each reaction were examined by p.c. (40-h irrigation), using D-glucose, isomaltose, maltose, isomaltotriose, isomaltotetraose, isomaltopentaose, and isomaltohexaose as reference compounds. The results are shown in Table II.

Action of dextranase, conjugate, and dextranase–concanavalin A mixture on isomaltotriose and isomaltotetraose. — Aliquots (0.4 ml) of conjugate, dextranase (0.5 mg/ml in distilled water), and 1:1 dextranase–concanavalin A mixture (protein concentration identical to that of the conjugate) were separately incubated with 1 ml of solutions of isomaltotriose and isomaltotetraose (4% in distilled water). Samples were removed at 0 and 240 min and, after the enzyme had been heat-inactivated, examined by p.c. (40-h irrigation), using D-glucose, isomaltose, isomaltotriose, and isomaltotetraose as reference substances. The results are shown in Table III.

Hydrolysis of dextran by modified dextranase. — Aliquots (1 ml) of dextranase (1 mg/ml in 0.2M acetate buffer, pH 5.0) were separately incubated with acetaldehyde (0.1 ml), 25% aqueous glutaraldehyde (0.1 ml), and distilled water (0.1 ml) for 1 h at 20°. The solutions were then passed down columns (80 × 5 mm) of Biogel P-10 and eluted with distilled water. Fractions (1 ml) were collected and scanned at 280 nm. The fractions of high molecular weight were combined and assayed for dextranase activity. An aliquot (0.2 ml) of each fraction was then incubated with 2.5 ml of a 4% aqueous solution of dextran. Aliquots (0.5 ml) were removed after 0, 30, and 240 min, placed in a boiling water bath for 10 min to stop the reaction, and then examined by p.c.

RESULTS AND DISCUSSION

Glutaraldehyde (pentane-1,5-dial) is a well-known reagent for cross-linking protein molecules[12]. Among the advantages of this reagent are the mild conditions of temperature and pH under which it is effective, and also its relatively low toxicity[13]. Glutaraldehyde reacts with exposed free-amino groups, especially the ε-amino groups of lysyl residues[14]. The reaction is complicated[15], and the cross-linking is irreversible.

The reaction has mainly been used to produce insoluble derivatives of proteins by extensive cross-linking[12]. However, we required derivatives which, although cross-linked, were soluble, and in which the biological activities of the proteins were retained.

Donelly and Goldstein[16] showed that concanavalin A is insolubilized by treatment with glutaraldehyde at pH 7.0, with good retention of its binding properties. Solutions of concanavalin A at two different concentrations were therefore treated with various amounts of glutaraldehyde. Dextranase was also treated in a similar way. The results (Table I) indicated that concanavalin A, at 1.0 mg/ml, was insolubilized by all quantities of glutaraldehyde except the highest. This high proportion of glutaraldehyde, however, caused major losses of dextranase activity when the dextranase concentration was also 1 mg/ml.

TABLE I

EFFECT OF GLUTARALDEHYDE ON DEXTRANASE AND CONCANAVALIN A

Glutaraldehyde added (*μl*)	*Dextranase*		*Concanavalin A*	
	1 mg/ml Activity (%)	*0.5 mg/ml Activity* (%)	*1 mg/ml Binding* (%)	*0.5 mg/ml Binding* (%)
0	100	100	100	100
20	98	98	Insolubilized	87
50	99	97	Insolubilized	Insolubilized
100	102	98	Insolubilized	Insolubilized
200	86	97	Insolubilized	Insolubilized
500	14	79	83	72

The dextranase at 1 mg/ml could not be insolubilized, and its activity was virtually unaffected until 200 or more *μl* of glutaraldehyde solution were used. The very significant loss of activity of dextranase when 500 *μl* of glutaraldehyde solution were used is probably due to extensive cross-linking of the protein molecules, rather than to reaction with residues essential for enzymic activity. Extensive cross-linking of the dextranase molecules is likely to reduce activity, in that easy access of the large dextran molecule to the active sites of the enzyme molecules would be impeded.

When a lower (0.5 mg/ml) concentration of concanavalin A was used, no insolubilization occurred when 20 *μl* of glutaraldehyde solution was added. This quantity of glutaraldehyde produced essentially no loss of activity with dextranase (0.5 mg/ml) and, therefore, these conditions were used for the preparation of a batch of the conjugate.

The elution pattern (Fig. 1) of the conjugate on gel-filtration chromatography (Biogel P-300) revealed no material corresponding to unreacted dextranase or

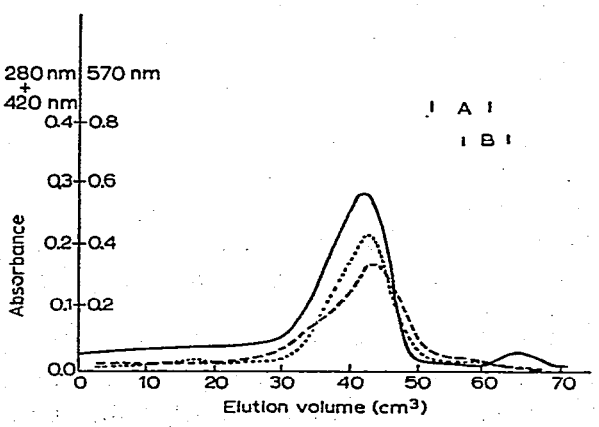

Fig. 1. Elution pattern of dextranase–concanavalin A conjugate: absorptions at 280 (——), 420 (——), and 570 nm (.....). Elution volumes: A, dextranase; B, concanavalin A.

concanavalin A. The major protein peak, which was eluted earlier than either of the two starting materials and was within the exclusion limit of the column, corresponded therefore to material of higher molecular weight. The material in this peak had concanavalin A and dextranase activities.

The appropriate assays showed that the conjugate contained 84% of the original protein used and had 43 units/mg of dextranase activity (*cf.* 202 for dextranase). For a conjugate containing equal weights of concanavalin and dextranase, the maximum activity should be 100 units/mg. Estimates of the dextranase activity of the conjugate based on initial rate measurements must be treated with some caution (see later). However, it is not surprising that dextranase conjugated with another protein molecule shows a decreased enzymic activity. The presence of another protein molecule is likely to impede seriously access of the dextran substrate-molecules to the enzyme. This is especially likely when the enzyme is conjugated with a protein which itself has an affinity for the substrate.

In what is essentially a random-coupling process, the possibility exists that not all of the dextranase molecules are linked to concanavalin A. Dextranase–dextranase species, for example, might also be produced. In order to determine the extent of conjugation of dextranase molecules to concanavalin A, the conjugate was treated in solution with glycogen. Glycogen, which forms an insoluble complex with concanavalin A, is not a substrate of dextranase. When solutions of glycogen and dextranase are mixed, precipitation does not occur. If the dextranase is covalently bound to concanavalin A, however, precipitation of the complex with glycogen should also remove the dextranase from solution. After precipitation of the conjugate with glycogen, it was found that the supernatant lost 77% of the original dextranase activity.

Since concanavalin A is not totally precipitated from solution, and the concanavalin A activity of the conjugate was only 87% of that of concanavalin (assuming a 1:1 ratio in the conjugate), it is reasonable to conclude that most, if not all, of the dextranase in the conjugate is linked to concanavalin A molecules.

The elution pattern (Fig. 1) indicates that any dextranase not attached to concanavalin A is in a form of high molecular weight and must therefore be part of a dextranase–dextranase conjugate.

Conjugation of concanavalin A to dextranase profoundly affects the enzymic activity, with regard to both time course and hydrolysis products.

For dextranase, if the substrate concentration is not high enough to cause saturation of the enzyme, the initial rate of formation of reducing sugar is proportional to the substrate concentration. Typical results are shown for dextrans B-512 and B-742 in Figs. 2 and 3, respectively.

The time course of the reaction (Fig. 4) of the conjugate with dextran B-512 shows slow production of reducing sugar at the beginning and a later increase; there is a time lag before the maximum rate is achieved. This effect is probably due to the interaction of the concanavalin A molecules in the conjugate with the molecules of the dextran substrate. The binding between the dextran and concanavalin A moieties

could impede the reaction of the dextranase species with the large dextran molecules, both by steric hindrance and also by decreasing the mobility of the conjugate. After a short time-lag, during which a slow hydrolysis of dextran to smaller molecules takes place, the rate of production of reducing sugar increases, probably for two reasons.

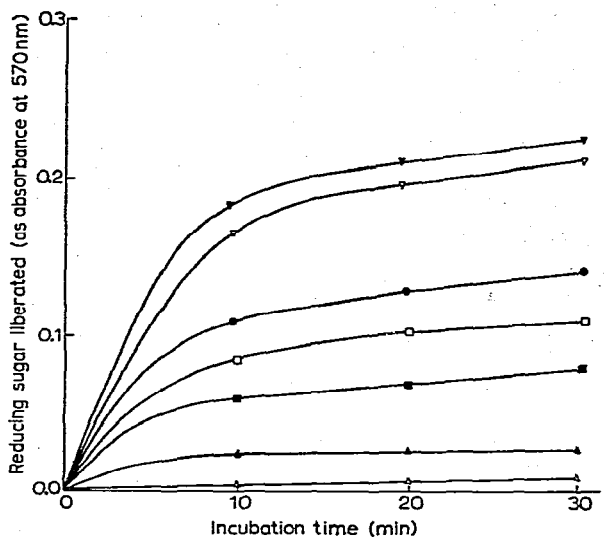

Fig. 2. Production of reducing sugar on incubation of dextranase with various concentrations of B-742 dextran, namely 0.1 (△), 0.2 (▲), 0.4 (■), 0.5 (□), 0.6 (●), 0.8 (▽), and 1% (▼).

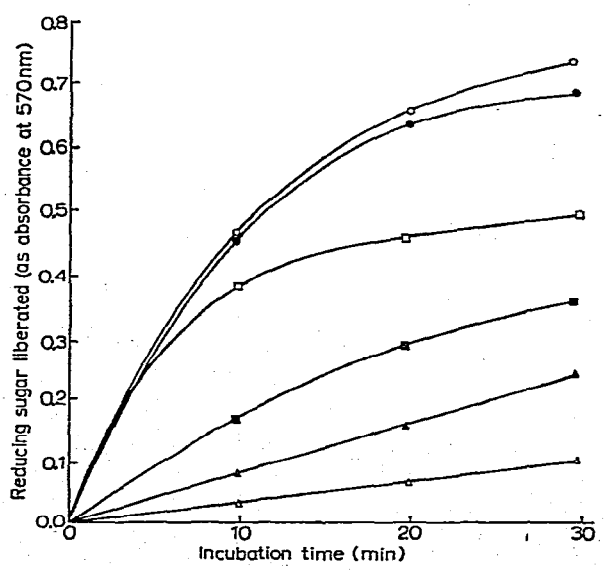

Fig. 3. Production of reducing sugar on incubation of dextranase with various concentrations of B-512 dextran, namely 0.1 (△), 0.2 (▲), 0.4 (■), 0.6 (□), 0.8 (●), and 1% (○).

Firstly, because the smaller molecules compete with the dextran for access to the binding sites of concanavalin A, and thus reduce the steric hindrance and increase the mobility of the conjugate. Secondly, the smaller molecules compete effectively with the larger dextran molecules for access to the active sites of the dextranase. This hypothesis predicts that addition of small substrate-molecules should cause the abolition

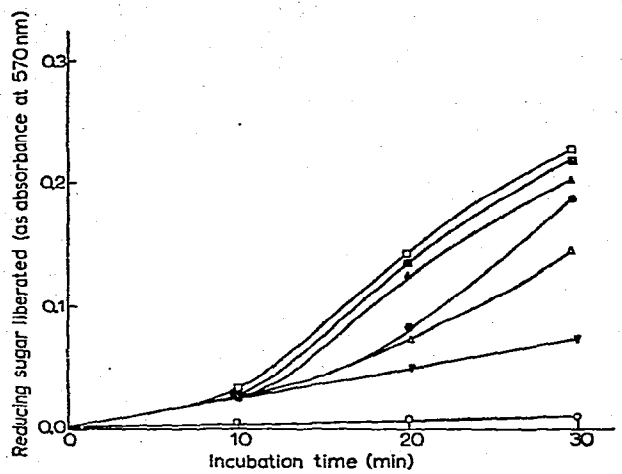

Fig. 4. Production of reducing sugar on incubation of dextranase–concanavalin A conjugate with various concentrations of B-512 dextran, namely, 0.1 (○), 0.2 (▼), 0.4 (△), 0.5 (◒), 0.6 (▲), 0.8 (■), and 1% (□).

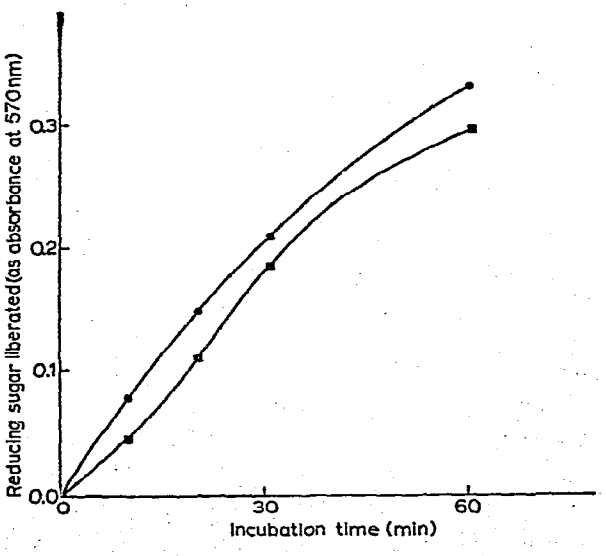

Fig. 5. Incubation of dextranase–concanavalin A conjugate with B-512 dextran before (■) and after (●) incubation of conjugate with isomaltohexaose.

of the time lag. Fig. 5 shows clearly that, in the presence of isomaltohexaose, the concave, upper portion of the initial curve was abolished.

The product pattern obtained when the conjugate was incubated with dextran (B-512) was different from that obtained when the dextran was treated with the native dextranase (Table II). Dextranase ultimately degrades dextran to D-glucose and isomaltose, and the production of D-glucose in the later stages is accompanied by a decrease in the quantity of isomaltotriose. Dextranase degrades isomaltotriose to D-glucose and isomaltose, and isomaltotetraose to isomaltose (Table III). However,

TABLE II

PRODUCTS FORMED ON HYDROLYSIS OF B-512 DEXTRAN BY DEXTRANASE–CONCANAVALIN A CONJUGATES, AS OBSERVED ON PAPER CHROMATOGRAPHY

Product	*Incubation*					
	Dextranase	*Conjugate*	*Dextranase*	*Conjugate*	*Dextranase*	*Conjugate*
Dextran	+++	+++	++	++	−	−
Isomaltohexaose	−	−	−	++	+	−
Isomaltopentaose	−	−	−	+	−	−
Isomaltotetraose	−	−	−	−	−	−
Isomaltotriose	−	−	+++	+++	−	+++
Isomaltose	−	−	+	−	+++	+++
D-Glucose	−	−	+	−	+++	−

Key: −, absent; +, present; ++, strong; +++, intense.

TABLE III

PRODUCTS FORMED ON HYDROLYSIS OF ISOMALTOTRIOSE AND ISOMALTOTETRAOSE BY DEXTRAN AND DEXTRANASE–CONCANAVALIN A CONJUGATES, AS OBSERVED ON PAPER CHROMATOGRAPHY

	Isomaltotriose			*Isomaltotetraose*		
	Dextranase	*Conjugate*	*Mixture*	*Dextranase*	*Conjugate*	*Mixture*
Isomaltotetraose	−	−	−	+	+	+
Isomaltotriose	+	+++	+	−	−	−
Isomaltose	+++	−	+++	+++	+++	+++
D-Glucose	+++	−	+++	−	−	−

Key: −, absent; +, present; ++, strong; +++, intense.

no D-glucose was produced when the dextran was incubated with the conjugate (Table II), suggesting an inability to hydrolyse isomaltotriose. This view was confirmed when the conjugate was shown to cleave isomaltotetraose to give isomaltose, but had no effect on isomaltotriose (Table II). A mixture of dextranase and concanavalin showed normal dextranase activity.

Two possible explanations can be suggested for these observations. Firstly, that there are two dextranases present in the preparation, one of which is an exo-glycosidase which cleaves the terminal D-glucose residues from oligo- and poly-saccharide substrates, and is inactivated by the treatment with glutaraldehyde. If such an enzyme were present, the formation of D-glucose immediately on incubation with dextran or isomaltotetraose would be expected. This is not the case. Moreover, a significant loss in activity on treatment of the dextranase with the dialdehyde would be expected, and Table I shows that this is not the case.

The alternative explanation is that the preparation contains a single dextranase which requires at least two residues between the non-reducing end and the point of cleavage. The change in product pattern must then be accounted for either by the chemical modification of the protein at the lysine residues, or the incorporation of the dextranase in the conjugate with some consequent conformational or steric change. The effect of glutaraldehyde and acetaldehyde on dextranase was therefore examined.

Treatment of dextranase with acetaldehyde (which modifies amino groups in proteins[18]) had no effect on the subsequent product-pattern. However, treatment with glutaraldehyde changed the product pattern, in that the production of D-glucose was no longer observed. These results suggest that simple modification of the amino groups of the enzyme is not responsible for the inability of the enzyme to hydrolyse isomaltotriose. The cross-linking process, involving dextranase molecules, or dextranase and concanavalin A molecules, appears to be responsible for the change in product pattern.

It is unlikely that the cross-linking process, which produces a three-dimensional network, sterically prevents the approach of isomaltotriose but allows access to larger oligosaccharides. It is probable, therefore, that the process results in a conformational change in the dextranase molecules, such that the binding of isomaltotriose becomes so weakened that it is no longer an effective substrate. Isomaltotetraose, with its extra D-glucose residue, presumably still binds sufficiently strongly for the enzyme action to occur.

ACKNOWLEDGMENTS

The authors thank Professor B. Cohen for helpful advice and suggestions, and the Medical Research Council for financing this investigation.

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