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THE *IN-VITRO* EFFECT OF FUNGAL DEXTRANASE ON HUMAN DENTAL PLAQUE

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Summary—Suspensions of dental plaque from eighteen children with high caries activity were hydrolyzed *in vitro* by a partially purified fungal dextranase isolated from *Penicillium funiculosum* NRRL 1768. The dextranase preparation appeared to lack 1,4-glucanhydrolase and sucrase activity. The release of reducing substances from the plaque was measured by use of a 3,5-dinitrosalicylate reagent. Hydrolysis occurred at pH 5·1 but not at pH 7·0. Approximately 20 per cent of the total carbohydrate of plaque was consumed by the dextranase (2–3 per cent of the plaque dry weight). No isomaltose was found when the hydrolysates were analysed by a thin layer chromatography procedure which could detect as little as 0·1 μg of isomaltose. The findings suggest that the quantity of dextran in these plaque samples is low.

INTRODUCTION

CERTAIN strains of streptococci have been implicated as the causative agent of dental caries in hamsters (FITZGERALD and KEYES, 1960; KRASSE, 1966) and in rats (GIBBONS *et al.*, 1966). These organisms appear to be pathogenic by virtue of their ability to synthesize extracellular polysaccharides from sucrose. One type of polysaccharide, dextran (α-1, 6 glucan), appears to be important in plaque formation (WOOD and CRITCHLEY, 1966; GIBBONS and BANGHART, 1967; GUGGENHEIM and SCHROEDER, 1967). The importance of dextran as a determinant of caries production in these model systems has been demonstrated by the fact that attempts to prevent dextran accumulation by addition of dextranase to the drinking water (FITZGERALD *et al.*, 1968) or high molecular weight dextran formation by addition of low molecular weight dextrans to the diet (GIBBONS and KEYES, 1969) have been successful in inhibiting caries in hamsters.

The significance of the role of dextran in the formation of human carious lesions is not clear. Its concentration in human plaque has been estimated to be 2 per cent of the dry weight by immunologic methods (GIBBONS and BANGHART, 1967) or 10 per cent of the dry weight by indirect chemical methods (WOOD and CRITCHLEY, 1967, 1969). Attempts to prevent or minimize human plaque formation by dextranase mouthwashes have been equivocal (CALDWELL *et al.*, 1971; KEYES *et al.*, 1971; LOBENE, 1971).

In the present study, an attempt has been made to demonstrate and quantitate dextran in plaque obtained from tooth surfaces of children with high levels of caries activity by the direct method of measuring the breakdown products of plaque derived from exposure to dextranase.

MATERIALS AND METHODS

Source of dextranase

Dextranase 110 was isolated from spent cultures of *Penicillium funiculosum* NRRL 1768 grown in medium containing 1 per cent Pharmacia Dextran 110, molecular weight 110,000 (w/v), and 1 per cent Corn Products Steepwater. The initial pH was adjusted to pH 6 (TSUCHIYA *et al.*, 1952). Incubation was for 5 days at 30°C in a water-bath provided with a shaker oscillating at 60 times per minute.

Dextranase 110 was isolated by a modification of the method proposed by BAILEY and CLARKE (1959). The fungus was removed from the spent cultures by filtration through Whatman No. 1 filter paper. Fractional precipitation of the dextranase was achieved by adding 10g increments of $(NH_4)_2 SO_4$ to 100 ml of culture filtrate and then assaying each precipitate for dextranase. Most of the dextranase was found in the 35–55 per cent $(NH_4)_2 SO_4$ fraction. The 35–55 per cent $(NH_4)_2 SO_4$ fraction from each 1500 ml of spent medium was dissolved in 10 ml of 0·01 M sodium citrate buffer, pH 6, and dialysed at 40°C against 1 litre of the same buffer. The buffer was changed 3 times during the 48-hr period. The retentate was lyophilized, and the dried powder was stored *in vacuo* over $CaCl_2$ at 4°C.

Assay for dextranase activity

The lyophilized dextranase was dissolved in water to give 5 per cent w/v solutions. Aliquots (0·05 ml) containing from 50 to 250 activity-units of dextranase were mixed with 0·1 ml of a 1·67 per cent solution of Dextran 7840, molecular weight 200,000 (Nutritional Biochemicals Corporation), in 0·1 M potassium acetate buffer, pH 5·1, in 13 × 100 mm Pyrex tubes. The tubes were stoppered and incubated at 40°C in a water-bath for varying periods of time. Following the incubation, 0·15 ml of 3,5-dinitrosalicylate reagent (BOREL, HOSTETTLER and DEUREL, 1962) which gives a colour reaction with reducing groups was added to each tube. After mixing, the tubes were heated for 5 min in a boiling water bath. The contents were cooled, diluted with 2·7 ml of distilled water, and the absorbance determined at 530 nm. Standard solutions of isomaltose and inactivated preparations of the enzyme in the presence of the appropriate substrate were manipulated in like manner. One activity unit of dextranase was defined as the amount of dextranase necessary to liberate 1 mg of isomaltose from Dextran 7840, in 1 hr at 40°C (TSUCHIYA *et al.*, 1952).

Starch and sucrose as substrates for dextranase

The presence of α-1,4-glucanhydrolase and of sucrase has been noted in preparations of fungal dextranase (GOLDSTEIN, personal communication; GOLD, PRESTON and BLECHMAN, 1970). These enzyme activities were sought in the dextranase 110 preparation. To determine the activity of these enzymes, 250 units of dextranase 110 were added to tubes containing solutions of either 1·67 per cent corn starch, 1·67 per cent soluble starch, 1·67 per cent sucrose or 1·67 per cent Dextran 7840 in 0·1 M potassium acetate buffer, pH 5·1. After incubation for 15 min at 40°C, the extent of hydrolysis was determined by the use of 3,5-dinitrosalicylate reagent.

Collection of dental plaque

Dental plaque was collected from 18 children, 5–8 yr old, who were either untreated patients in the Pedodontic Clinic of the School of Dentistry, The University of Michigan, or kindergarten pupils at the Perry Elementary School, Ypsilanti, Michigan. Each of the children had at least 8 untreated carious teeth, as determined by radiographic and/or visual examination. The dental plaque was supragingival material removed from the buccal, lingual and approximal surfaces of all teeth in each patient by means of a sterile periodontal scaler. The plaque from each child was homogenized in 2 ml of 0·1 M potassium acetate buffer, pH 5·1, by the use of a Pyrex glass homogenizer. The resulting plaque suspension was immediately immersed in boiling water for 20 min. If this were not done, endogenous plaque catabolism gave rise to reducing substances which obscured the assay results. Heating a solution of Dextran 7840 for 20 min at 100°C did not increase its ability to react with 3,5-dinitrosalicylate reagent.

Incubation of dextranase with dental plaque

The 18 dispersed samples of dental plaque were incubated with varying concentrations of dextranase 110 for 1 hr at 37°C, pH 5·1, and the extent of hydrolysis was estimated with the 3,5-dinitrosalicylate reagent. In other experiments, the homogenates of plaque were incubated with 250 units

of dextranase 110 for 1, 5 and 15 min. The "total" concentration of carbohydrates in 8 of the samples was estimated by the use of the anthrone reagent (NEUFELD and GINSBERG, 1966), with glucose as the reference standard.

Reaction products occurring in suspensions of dental plaque after incubation with 250 units of dextranase 110 for 15 min at 37°C were examined by thin layer chromatography. One to 5 μg aliquots were placed on Gelman ITLC-SG support and were developed with solvents comprised of either chloroform, methanol and acetic acid (90:10:15) (HAER, 1967) or ethyl acetate, pyridine and water (8:2:1) (HAER, 1967). The carbohydrate reaction products were detected with an anisaldehyde-sulphuric acid spray (STAHL, 1965). With this reagent, as little as 0·1 μg of isomaltose could be visualized.

Since the pH of saliva is approximately 7·0, it was of interest to assess the ability of dextranase 110 to catalyse the hydrolysis of the dextran in the plaque samples at pH 7·0 as well as at pH 5·1. Plaque specimens from 8 of the children were collected in 0·1 M sodium phosphate buffer, pH 7·0. Suspensions of the plaque samples were then incubated with 50, 100, and 250 units of dextranase 110 for 1 hr at 37°C.

The activity of dextranase 110 on plaque was compared to the activity of a commercial preparation of dextranase (Nutritional Biochemicals Corporation). The N.B.C. dextranase (250 activity units) was incubated with homogenized plaque from 5 of the children for 15 min at pH 5·1.

RESULTS

Partial characterization of dextranase 110

Fungal dextranase has a pH optimum at 5·1 (TSUCHIYA *et al.*, 1952). Dextranase 110 appeared to have a similar pH activity as its ability to release reducing substances

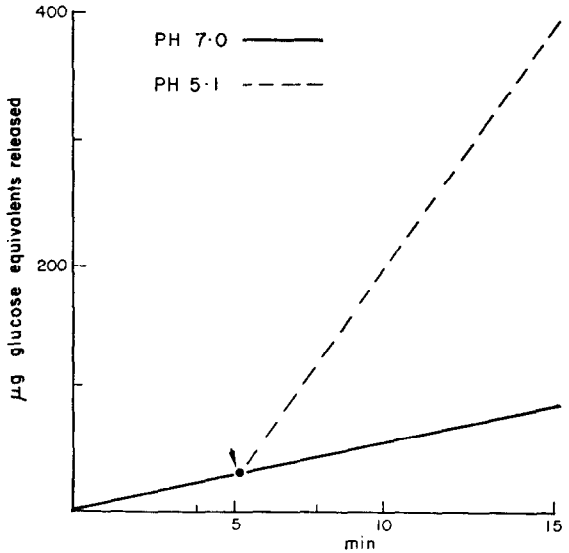

FIG. 1. The effect of pH on dextranase activity. To a series of tubes, each containing 0·1 ml of a 1·67 per cent solution of Dextran 7840 in 0·1 M sodium phosphate buffer, pH 7·0 0·05 ml of a solution of dextranase 110 (250) was added. ← After incubation for 5 min at 37°C, 0·1 ml of 0·06 M hydrochloric acid was added to some of the tubes (– – – –) to lower the pH to 5·1. To the remaining tubes 0·1 ml of 0·06 M sodium chloride solution was added so as to adjust the volume and the ionic concentration of these tubes. Thereafter incubation was continued for an additional 10 min at 37°C. ← The extent of hydrolysis (glucose equivalents liberated) was determined by the use of the 3,5-dinitrosalicylate reagent.

from dextran and/or plaque was markedly improved by a pH shift from 7·0 to 5·1 (Fig. 1).

Dextranase 110 was relatively specific for dextran, as Dextran 110, Dextran 7840 and Dextran 2000 (molecular weight 2,000,000) were degraded to give a colour reaction with 3,5-dinitrosalicylate reagent. When soluble starch, corn starch or sucrose served as substrates, only small amounts of reducing compounds were released (Fig. 2). A more specific measure of dextran degradation would be the detection of isomaltose as a digestion product. Isomaltose was found by thin-layer chromatography methods as a breakdown product when 250 units of dextranase 110 were incubated with 2 per cent solutions of Dextran 110, Dextran 7840 and Dextran 2000. No isomaltose or other breakdown products could be detected by thin-layer chromatography when dextranase 110 was incubated with sugar or soluble starch. If smaller amounts of dextran served as substrate i.e. 0·1 per cent Dextran 7840, no isomaltose could be detected.

Incubation of dextranase 110 *with dental plaque*

Hydrolysis of plaque carbohydrate as evidenced by a release of reducing substances occurred in all 18 of the plaque samples after exposure to dextranase 110 for 1 hr at 37°C and pH 5·1 (Table 1). The extent of hydrolysis was roughly proportional to the concentration of the enzyme. When plaque was incubated with 50 activity units (AU), only 7 per cent of the total plaque carbohydrate was hydrolysed; whereas,

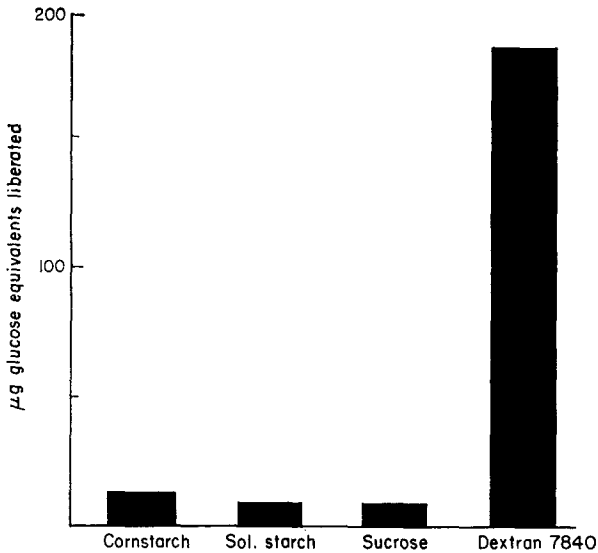

FIG. 2. The effect of dextranase on cornstarch, soluble starch, sucrose, and Dextran 7840. To a series of tubes, each containing 0·1 ml of a 1·67 per cent solution of either corn starch, soluble starch, sucrose, or Dextran 7840 in 0·1 M potassium acetate buffer of pH 5·1, 0·05 ml of a solution of dextranase 110 (250 units) was added. After incubation for 15 min at 40°C, the extent of hydrolysis (glucose equivalents liberated) was determined by the use of the 3,5-dinitrosalicylate reagent.

TABLE 1. THE EFFECT OF DEXTRANASE ON DENTAL PLAQUE*

Enzyme conc. (AU)	Glucose equiv. released	Percentage of plaque carbohydrate
50	2·4 ± 1·8 μg†	7‡
100	4·9 ± 2·6	14·3
250	7·0 ± 1·6	20·4

* 60 min incubation, pH 5·1, 37°C.
† Average of 18 plaque samples.
‡ Total plaque carbohydrate: 34·3 μg; range: 19–65 μg.

TABLE 2. HYDROLYSIS OF DENTAL PLAQUE BY DEXTRANASE (250 A.U.)*

Incubation time (min)	Glucose equiv. released	Range
1	2·6 μg	0–7·5
5	3·3	0–9·0
15	7·5	1·5–11·0

* Eighteen plaque samples.

when 250 AU were added, approximately 20 per cent of the plaque carbohydrate was hydrolysed (Table 1). In the other experiments, 250 AU of dextranase 110 were incubated for varying periods of time with the plaque samples. The hydrolysis of plaque was found to be a function of time with maximum release of reducing substances occurring after 15 min incubation (Table 2). Further incubation did not result in increased hydrolysis. If one assumes that the reducing equivalents released upon incubation with 250 AU of dextranase 110 reflected complete hydrolysis of plaque dextrans, then dextran accounted for 10–47 per cent of plaque carbohydrate with an average value of 20 per cent. When the same samples were spotted on thin-layer plates, no isomaltose could be detected.

When plaque samples from 8 children were collected in 0·1 M sodium phosphate buffer, pH 7·0, and incubated with 50, 100 and 250 AU of dextranase 110 for 1 hr at 37°C, none of the samples was hydrolysed.

Plaque samples from 5 children were exposed to either 250 AU of dextranase 110 or 250 AU of N.B.C. dextranase in 0·1 M potassium acetate buffer, pH 5·1, for 15 min at 37°C. Hydrolysis occurred only in samples containing dextranase 110. The commercial preparation was active in that it hydrolysed Dextran 7840.

DISCUSSION

The results of the present investigation showed that all 18 plaque samples gave rise to reducing compounds when exposed to dextranase 110. Since dextranase 110 appeared to be specific for dextran, these reducing compounds were assumed to be derived from dextran. As such, dextran accounted for about 20 per cent of the plaque carbohydrate. As the carbohydrate content of plaque is approximately 10–17 per cent of the dry weight (KREMBEL, FRAN and DELUZARCHE, 1969; LOESCHE *et al.*, 1971), the dextran in these plaque samples would account for about 2–3 per cent of the dry weight. This result, however, should be interpreted with some caution as no isomaltose could be detected in plaque suspensions that had been hydrolysed by exposure to dextranase 110. GOLD, PRESTON and BLECHMAN (1970) could not detect isomaltose release from plaque samples in similar experiments. The failure to detect isomaltose could mean (1) that dextranase 110 was attacking other plaque carbohydrates giving rise to reducing sugars, (2) that there was no dextran in the plaque samples, (3) that the dextranase 110 was not active against the dextrans in plaque, (4) that the amount of dextran present in plaque was too little for degradation products to be detected by thin-layer chromatography. The last cited possibility can be supported by results of this investigation. A suspension of 0·1 per cent Dextran 7840 which was hydrolysed by dextranase 110 failed to yield any isomaltose by thin-layer chromatography. Thus, for isomaltose to be detected in digested plaque, more than 1 mg of dextran per ml of plaque suspension would be needed. The dry weight of the plaque samples collected in the present study ranged from 5 to 20 mg. If 10 per cent of the dry weight was due to dextran (WOOD and CRITCHLEY, 1969), then there would be about 0·5–2 mg of dextran in the entire plaque suspension. If the plaque dextran was completely degraded by dextranase 110, then isomaltose would be barely detectable by thin layer chromatography. However, if dextran comprised only 2 per cent of the plaque dry weight (GIBBONS and BANGHART, 1967), it would be unlikely that any isomaltose could be detected. This latter value would be supported by the results of the colorimetric assay which showed that reducing substances released by dextranase accounted for approximately 2–3 per cent of the plaque dry weight.

The dextranase assay must for another reason be interpreted with some caution. If the preparation contained an α-1,4-glucanhydrolase or other glucocidases, then reducing groups could be released from plaque carbohydrates other than dextrans, giving erroneously high values. The dextranase 110 preparation employed was inactive against soluble starch, corn starch and sucrose. Endogenous catabolism of α-1,4-glucans was prevented by heat-inactivation of the plaque samples. Conversely, if the dextranase 110 preparation was not capable of degrading branched dextrans, then erroneously low values would be recorded. This possibility needs further investigation. In this regard, the dextranase 110 preparation was superior to the N.B.C. dextranase, which suggested that it contained a more complete enzyme system against plaque dextrans than did the commercial preparation.

In the human studies where a dextranase mouthwash was tested, dental plaque was exposed to highly concentrated solutions of dextranase for several intermittent periods of from 2 to 5 min each. In our *in vitro* system, dispersed plaque samples

required 15 min exposure to dextranase for 2–3 per cent of the dry weight to be degraded. This would suggest that in the *in vivo* studies where plaque is not dispersed, longer treatment periods might be necessary. In our *in vitro* study, a pH of 5·1 was necessary to show activity of dextranase 110. When a pH 7·0 dextranase preparation was used in an *in-vivo* study, no reduction in plaque was found (CALDWELL *et al.*, 1971).

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Résumé—Des suspensions de plaque dentaire de dix huit enfants à activité carieuse élevée sont hydrolysées *in vitro* par une dextranase fungique partiellement purifiée, isolée à partir de *Penicillium funiculosum* NRRL 1768. La préparation de dextranase ne semble pas présenter d'activité en 1,4-glucanehydrolase et sucrase. La libération de substances réductrices de la plaque est mesurée en utilisant un réactif 3,5-dinitrosalicylate. L'hydrolyse se fait à pH 5,1, mais non pas à pH 7,0. Environ 20 pour cent de la totalité des hydrates de carbone de la plaque est éliminé par la dextranase (2–3 pour cent du total en poids sec de la plaque). On ne trouve pas d'isomaltose par analyse des hydrolysats en chromatographie en couche mince, pouvant détector jusqu'à 0,1 μg d'isomaltose. Ces résultats indiquent que le contenu en dextrane des plaquesétudiées est faible.

Zusammenfassung—Plaque-Suspensionen von 18 hochgradig kariesaktiven Kindern wurden *in vitro* mit Hilfe einer teilweise einer gereinigten Pilz-Dextranase aus *Penicillium funiculosum* NRRL 1768 hydrolysiert. Der Dextranase schien 1,4-Glukanhydrolase und Sukraseaktivität zu fehlen. Die Freisetzung reduzierender Substanzen aus der Plaque wurde mit Hilfe eines 3,5-Dinitrosalicylat-Reagenz bestimmt. Die Hydrolyse trat bei pH 5,1, nicht jedoch bei pH 7,0 auf. Ungefähr 20 Prozent des gesamten Plaque-Kohlenhydrats (2–3 Prozent des Plaque-Trockengewichts) wurden von der Dextranase gespalten. Wenn die Hydrolysate dünnschichtchromatographisch bis zur Empfindlichkeitsgrenze von 0,1 μg untersucht wurden, ließ sich Isomaltose nicht auffinden. Die Ergenbnisse legen es nahe, daß die Menge Dextran in diesen Plaqueproben gering ist.

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