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Characterization of an Extracellular Dextranase from *Fusarium moniliforme*

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An extracellular dextranase (EC 3.2.1.11) was purified approximately 75-fold from cell-free culture filtrates of *Fusarium moniliforme*. The purified dextranase was of the endo type, and isomaltose was identified as the primary end product of dextran hydrolysis. The molecular weight of the dextranase was determined to be 39,000 by gel permeation chromatography. The enzyme was most active at pH 5.5, and the temperature optimum was near 55 C. Activity was not inhibited by either ethylenediaminetetraacetic acid or iodoacetate. The K_m for dextran with an average molecular weight of 10,000 was estimated to be 1.1×10^{-4} M. The electrophoretic mobility of the dextranase was distinctly different from that of a *Penicillium*-derived commercial dextranase. The *F. moniliforme* dextranase was also found to differ from the commercial preparation by its greater relative activity against glucans isolated from *Streptococcus mutans*.

Studies have indicated that dextranase (EC 3.2.1.11, alpha-1,6-glucan 6-glucanohydrolase) preparations derived from *Penicillium* were only partially successful in reducing dental caries and periodontal disease in experimental animals and humans (4, 5, 11, 20). The limited therapeutic success of the *Penicillium*-derived dextranase may be related to the observation that the dextran-like glucans produced by different caries-inducing streptococci differ in their susceptibility to degradation by this enzyme (11). Therefore, the isolation of additional dextranases could reveal a more effective preparation with improved therapeutic properties. As a result of a survey for new sources of this enzyme (20a), we discovered dextranase activity by *Fusarium moniliforme*. The present report describes the subsequent isolation and characterization of the *F. moniliforme* dextranase.

MATERIALS AND METHODS

Culture methods. A dextranase-producing culture of *F. moniliforme* ISU-32 was used throughout this investigation. As a result of preliminary studies, an initial culture pH of 8 and an incubation period of 14 days were found to be optimal for maximum dextranase production by this organism. The fermentation was maintained under aeration (4 liters/min) at 30 C. Cells were grown in a synthetic dextran broth which contained per liter: dextran (clinical; Sigma Chemical Co.), 10 g; DL-asparagine, 0.1 g; NaCl, 3 g; $MgSO_4$, 0.2 g; $NH_4H_2PO_4$, 1 g; $K_2HPO_4 \cdot 3H_2O$, 1 g; KI, 100 μg; boric acid, 10 μg; ammonium molybdate, 10 μg; $MnSO_4$, 10 μg; $FeSO_4$, 50 μg; thiamine, 200 μg; riboflavine, 200 μg; pyridoxine, 200 μg; nicotinic acid, 200 μg; *p*-aminobenzoic acid, 200 μg; calcium pantothenate, 200 μg; inositol, 10 μg; biotin, 2 μg. The final pH of the medium was adjusted to 8.0 with 1 N NaOH. A 10-liter quantity of the medium was prepared and inoculated by mycelial transfer (1 cm^2) from a stock culture of *F. moniliforme*. Stock cultures were grown on solidified (1.5% Noble agar, Difco) synthetic dextran medium and stored at 4 C.

Dextranase assay. Enzyme preparations (1.0 ml) were diluted with substrate buffer so that a linear relation existed between activity and end product formation. The enzyme was added to 4.0 ml of dextran (20 mg/ml; dextran 10, Pharmacia) in 0.1 M sodium phosphate buffer, pH 6.0, and the mixture was incubated for 2 h at 37 C. All enzyme and substrate solutions were equilibrated to the assay temperature prior to mixing. A heat-inactivated (15 min at 80 C) enzyme preparation was used as the zero time control. The reaction was terminated by the addition of 1.0 ml of 3,5-dinitrosalicylate reagent (3) to 1.0 ml of the sample. The resulting mixture was heated in a boiling water bath for 5 min, cooled to room temperature, and mixed with 10.0 ml of deionized water. The absorbance of each solution was determined at 540 nm. The concentration of reducing sugar present in the samples was determined with a maltose standard curve in the range of 0 to 1 mg. A unit of dextranase activity was defined as that amount of enzyme required to release from the substrate (dextran) 1 mg of reducing sugar after 2 h of contact at 37 C in 0.1 M phosphate buffer, pH 6.0. The unit thus defined is similar to that described by Chaiet et al. (6).

Dextranase purification. The culture broth was clarified by Buchner filtration through Whatman no. 1 filter paper, followed by vacuum filtration with

0.45-μm membrane filters (Millipore Corp.). The resulting cell-free filtrate was brought to 30% saturation with solid ammonium sulfate and centrifuged at 18,000 $\times$ g after standing overnight. The precipitate was discarded, and the supernatant was brought to 90% saturation with solid ammonium sulfate. The resulting precipitate was re-dissolved in deionized water and dialyzed against 5 mM phosphate buffer, pH 6.0, overnight at 4 C with constant stirring.

The dialyzed ammonium sulfate fraction was applied onto a diethylaminoethyl-cellulose (Cellex-D, Bio-Rad Labs) anion exchange column. The enzyme was eluted from the column (4.5 by 42.5 cm) with a linear gradient of 0 to 1 M NaCl in 5 mM phosphate buffer, pH 6.0, at a flow rate of 1.6 ml/min. Ten-milliliter volumes were collected at 10 C. The absorbance at 280 nm was determined for the eluted fractions, and dextranase assays were performed to locate the range of peak activity. Peak fractions, 58–85, were combined, dialyzed, concentrated by freeze-drying, and stored at 4 C.

End product analysis. Ascending paper chromatography was performed on Whatman no. 3MM paper and developed with an ethyl acetate-pyridine-water (10:4:3, by volume) solvent system (18). Three ascents were found optimal for resolving the dextran hydrolytic end products. Reducing sugar spots were detected by an alkaline silver nitrate reagent (23). The samples included sugar standards at a concentration of 2 mg/ml, an enzymatic digest of dextran, and a heat-inactivated enzymatic control. The standards used were glucose, maltose, and isomaltose. The enzymatic digest was prepared by incubating 1.0 ml (164 units) of a partially purified *F. moniliforme* dextranase preparation in 4.0 ml of a 20 mg of dextran per ml-0.1 M phosphate buffer solution, pH 6.0, at 37 C for 3 h. The control preparation was identical to the active preparation with the exception that the enzyme was heat inactivated for 15 min at 80 C prior to incubation. Ten microliters of each sample was applied to the chromatogram.

Electrophoretic dextranase assay. The electrophoretic conditions were employed as described by Davis (8). A procedure for locating dextranase activity after protein separation by polyacrylamide gel electrophoresis was developed in this study. The gel column was longitudinally sliced after electrophoresis. One-half of the sliced gel was placed in contact with the surface of an assay plate containing dextran (10 mg/ml) and 1.5% agar in 0.2 M sodium acetate buffer, pH 5.5. After 3 h at 37 C the plate was then flooded with 95% ethanol, and dextranase activity, indicated by a zone of clearing, could be observed within 24 h. The second half of the gel slice was stained for proteins with Coomassie brilliant blue R250 (7). Identification of the stained protein band as dextranase was accomplished by repositioning the stained gel on the assay plate and noting the position of corresponding activity, if present. The technique allows the direct visualization of dextranase activity without the necessity of eluting the enzyme from the gel column.

Gel permeation. A column (1.5 by 85 cm) of Bio Gel P-150 (Bio-Rad) and an elution buffer of 0.05 M sodium phosphate and 0.1 M NaCl, pH 6.0, were used to estimate the molecular weight of dextranase. A 0.5-ml sample was chromatographed at a flow rate of from 0.25 to 0.30 ml/min. Blue dextran (Pharmacia) was used to determine the void volume, and fractions of 3.0 ml were collected at 4 C. The column was calibrated (1) with protein standards having the following molecular weights: bovine serum albumin (Armour), 67,000; ovalbumin (Miles), 45,000; pepsin (Mann), 35,000; and ribonuclease A (Mann), 13,700.

Preparation of streptococcal glucans. Extracellular water-soluble and -insoluble glucans were prepared from *Streptococcus mutans* SL-1 by modification of earlier procedures (17). Overnight seed cultures grown in Todd-Hewitt broth (Difco) with 0.5% glucose were inoculated (10 ml) into 350-ml volumes of Todd-Hewitt broth plus 5% sucrose. The cultures were routinely incubated at 37 C in an atmosphere of 95% N and 5% CO_2 for 24 h. The culture-soluble glucan was isolated by removing the cells (11,000 $\times$ g) and fractionation of the supernatant with 95% ethanol. The 35 to 50% precipitate was washed with 50% ethanol, re-dissolved, and reprecipitated.

Water-insoluble glucan was separated from cells that had been washed twice with 0.85% NaCl by extraction with 1 N KOH (17). After centrifuging at 11,000 $\times$ g to remove cells, the clear supernatant was neutralized with acetic acid and the 35 to 50% ethanol precipitate was collected. The precipitate was treated with Pronase (2 mg/ml in 0.2 M borate buffer, pH 7.6) for 2 h at 37 C (Calbiochem), and the water-insoluble polysaccharide was dialyzed against deionized water, reprecipitated, washed with 50% ethanol, and freeze dried to a coarse, white, fluffy product.

RESULTS

The relative change in specific activity at the various steps of purification is shown in Table 1. Although separation within this range was not sharp, ammonium sulfate concentrations greater than 30% saturation but less than 90% precipitated about 95% of the dextranase activity from the cell-free culture supernatant. The precipitate was dialyzed and further purified by

TABLE 1. *Purification of F. moniliforme dextranase*

Procedure	Activity (U/ml)	Protein (mg/ml)	Sp act (U/mg)	Total activity (Units)	Purification (fold)
Culture filtrate	43.5	1.175	37	369,750	1
Ammonium sulfate and dialysis	230.0	0.225	1022	348,304	27
Diethylaminoethyl-cellulose	100.0	0.036	2778	16,908	75

diethylaminoethyl-cellulose chromatography. A single peak of dextranase activity was eluted from the column. Concentration of the fractions containing dextranase activity gave a 75-fold increase in specific activity.

Dextran derived from *Leuconostoc mesenteroides* NRRL-B-512 has been reported to be a 95% alpha-1,6-linked glucan (17). The reducing sugar end products liberated from this substrate (Dextran-10, Pharmacia) by the *F. moniliforme* dextranase were studied by paper chromatography. The principal reducing sugar end product of dextran hydrolysis was found to be isomaltose (Fig. 1). No maltose was released in the 3-h incubation period. A trace of glucose was also detected as an end product as well as intermediate higher-molecular-weight oligomers. The heat-inactivated enzyme preparation remained free of any reducing sugar end products.

Effect of pH and temperature. The pH optimum of *F. moniliforme* dextranase was studied at 37 C. A substrate solution for each pH value was prepared by adding dextran (10 mg/ml; average molecular weight, 10,000; Pharmacia) to 0.2 M acetate buffer over a pH range of 2.8 to 6.5. Quantitative dextranase assays were performed for each pH value (Fig. 2A), and the greatest activity was found to occur at pH 5.5. A sharp drop in activity was observed between pH 6.0 and 6.5.

The temperature optimum of *F. moniliforme* dextranase was also studied. The assay conditions were the same as those described in the dextranase assay except for incubation temperature. The highest activity occurred at 55 C, and a sharp decrease in activity was observed between 55 and 65 C (Fig. 2B). Dextranase solutions were stable at 4 C for at least 6 months within a pH range of 5.5 to 7.5.

Molecular weight. A linear correlation of the logarithm of the molecular weight of globular proteins and their elution pattern in gel chromatography has been observed (1). The molecular weight of the *F. moniliforme* dextranase was determined from a series of gel permeation experiments by comparing the elution behavior of the enzyme with proteins having pub-

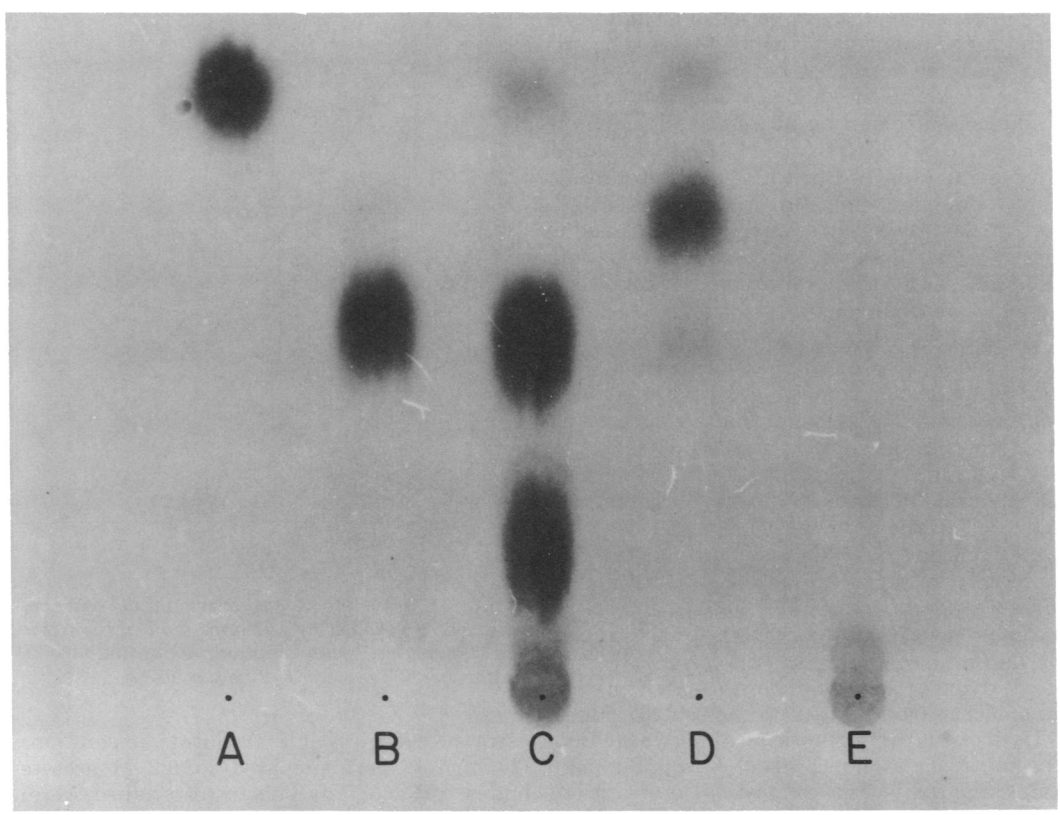

FIG. 1. *Reducing sugar end products resulting from dextran hydrolysis by the F. moniliforme dextranase. The samples applied were: (A) glucose; (B) isomaltose; (C) enzymatic digest; (D) maltose; (E) heat-inactivated control.*

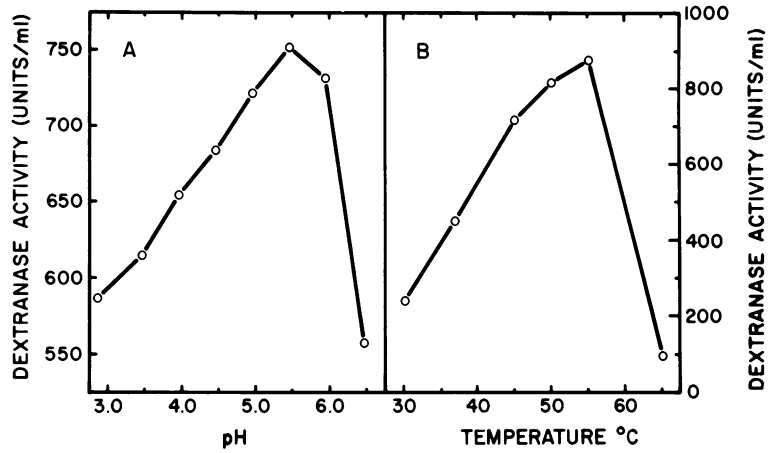

FIG. 2. *Effect of pH (A) and temperature (B) on activity of dextranase from F. moniliforme.*

lished molecular weights (Fig. 3). A molecular weight of 39,000 was assigned by this method.

Kinetic properties. The initial velocities of the dextranase activity on substrate concentrations (Dextran-10, Pharmacia) ranging from 2.0 to 0.01 mM and an assay period of 30 min were determined under standard conditions. The initial velocities were directly proportional to substrate concentration, and the saturation kinetics appeared to be typically Michaelis-Menten in form. A K_m of 1.1×10^{-4} M was obtained from a Lineweaver-Burk plot of the resulting data. However, since a uniform-molecular-weight dextran substrate does not exist and a single reducing sugar end product is not initially released, the K_m value can only represent an approximation for this enzyme.

Effect of reagents. The effects of iodoacetic acid and ethylenediaminetetraacetic acid (EDTA) on *F. moniliforme* dextranase activity were studied. The reagent additive concentrations were prepared in 0.2 M acetate buffer, pH 5.5, containing 20 mg of dextran-10 (Pharmacia) per ml and brought to 37 C. Either an active dextranase or a heat-inactivated enzyme, heated to 37 C, was mixed with the buffered substrate solutions. The reaction mixtures were incubated at 37 C for 2 h, and activity was measured by the assay procedure. EDTA and iodoaceate had little or no effect on the dextranase activity under the specified conditions (Table 2), relative to the control value.

Hydrolysis of streptococcal polyglucans. The purified dextranase from *F. moniliforme* was compared with a commercial preparation from *Penicillium* (Worthington) with respect to relative activity on streptococcal glucans. The relative activities of the dextranases were determined by modifying certain conditions of the assay procedure (Table 3). Substrate solutions (20 mg/ml) were prepared in 0.2 M acetate buffer, pH 5.5. The substrates studied were: dextran-10 (Pharmacia) and both water-soluble and -insoluble glucans purified from *S. mutans* SL-1.

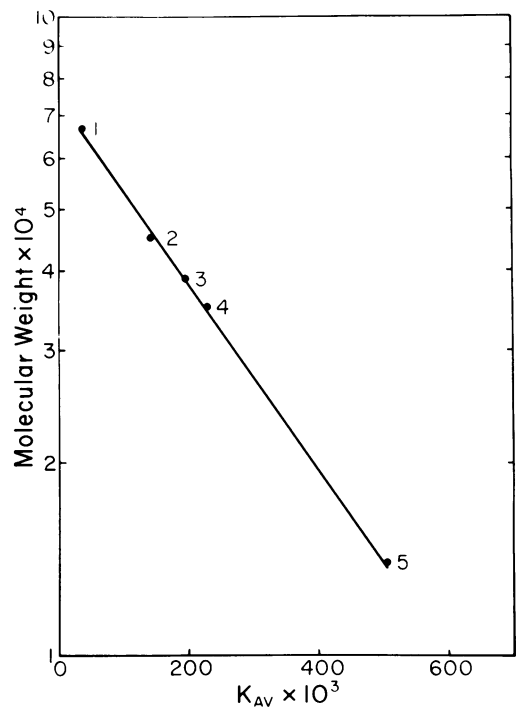

FIG. 3. *Molecular weight determination of F. moniliforme dextranase by gel permeation on Bio Gel P-150. (1) Bovine serum albumin; (2) ovalbumin; (3) dextranase; (4) pepsin; (5) ribonuclease A.*

Electrophoretic assay. An electrophoretic

TABLE 2. *Effect of EDTA and iodoacetate on dextranase activity*

Additive	Concn (mM)	Molar ratio[a]	Activity (U/ml)
None			115
EDTA	10.0		100
Iodoacetate	0.05	10,000	126
	0.005	1,000	103

[a] Iodoacetate-dextranase, based on a dextranase molecular weight of 40,000.

TABLE 3. *Relative activity of dextranase from Fusarium and Penicillium on different substrates*

Dextranase source	Substrate	Relative activity (%)
Penicillium sp.[a]	Dextran-10[b]	100.0
	Water-soluble glucan[c]	1.0
	Water-insoluble glucan[c]	0.8
Fusarium moniliforme	Dextran-10	100.0
	Water-soluble glucan	19.8
	Water-insoluble glucan	5.8

[a] Worthington Biochemical Corp.
[b] Pharmacia.
[c] Isolated from *S. mutans*.

assay was also used to compare the two dextranase preparations. Dextranase activity was identified after polyacrylamide gel electrophoresis. A zone of hydrolysis on agar plates containing dextran indicates the location of electrophoretically separated dextranase in the stained protein pattern (Fig. 4). The *F. moniliforme* dextranase was found to have a lesser mobility relative to the *Penicillium*-derived dextranase, and the two activities were readily separated and distinct under the electrophoretic conditions employed in this investigation. Each dextranase was observed to have its same relative mobility whether run in separate gel columns or mixed. Two widely separated sites of dextranase activity from the same gel column were only observed if a sample solution containing both enzyme preparations was applied to the column.

DISCUSSION

The *F. moniliforme* dextranase was purified approximately 75-fold in specific activity relative to the original cell-free culture filtrate by ammonium sulfate fractionation, dialysis, and diethylaminoethyl-cellulose column chromatog-

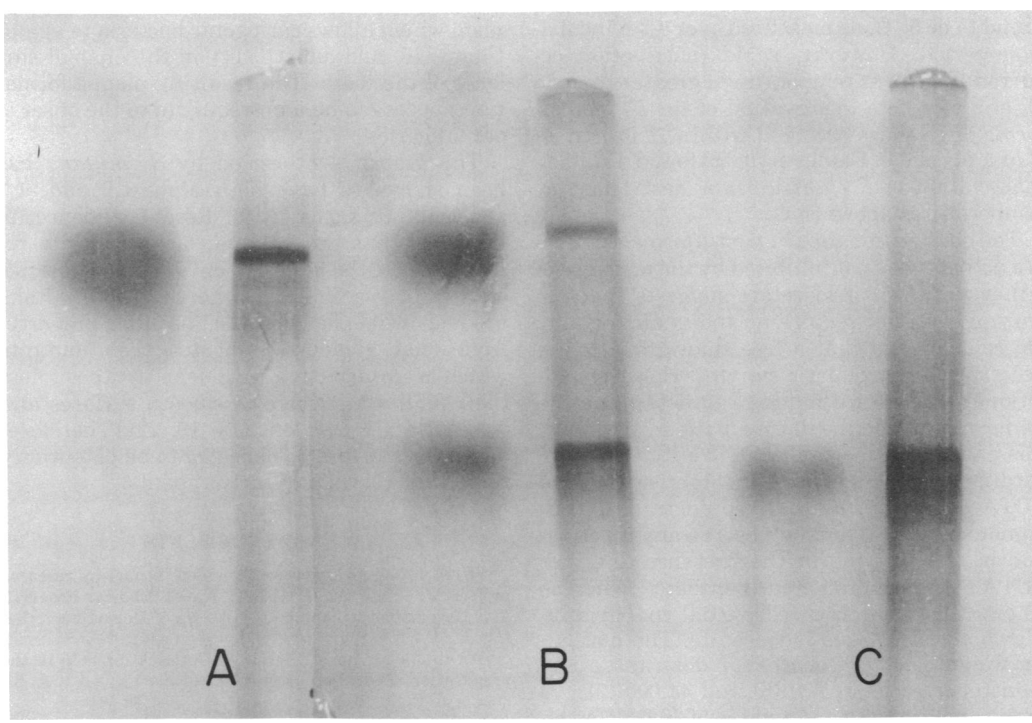

FIG. 4. *Polyacrylamide gel electrophoresis of dextranase preparations showing patterns of protein staining and sites of enzyme activity. About 40 μg of protein per dextranase preparation was applied to each gel column. (A) Purified dextranase from F. moniliforme; (B) a mixture of samples A and C; (C) a commercial dextranase derived from Penicillium. The specific sites of enzymatic dextran hydrolsis appear as dark areas in the photograph. Anode at bottom.*

raphy. A single area of dextranase activity was observed in the electrophoretic assay technique and a single peak of activity was eluted from both ion exchange and gel permeation column chromatography. The use of a defined synthetic medium eliminated the necessity of introducing extraneous proteins which could interfere with the purification of the enzyme.

Some properties of the *F. moniliforme* dextranase were similar to those reported for other fungal dextranases (13, 21, 24). The finding that isomaltose is the primary hydrolytic end product of *F. moniliforme* dextranase activity in the present investigation (Fig. 1) has also been observed for dextranase derived from *Penicillium* (24). The isolation of trace amounts of glucose and some intermediate high-molecular-weight oligomers along with isomaltose after dextran hydrolysis indicates a random rather than a stepwise hydrolysis of the polysaccharide. Therefore, it was concluded that the enzyme represents a dextranase of the endo type.

The pH optimum for the purified *F. moniliforme* dextranase was found to be about 5.5. A pH optimum of 6.0 was reported for *P. funiculosum* dextranase (21). The temperature optimum of the *F. moniliforme* dextranase was found to be 55 C, as measured over a 2-h incubation period. However, rapid inactivation occurred (Fig. 2) at temperatures greater than 55 C, and complete inactivation of the *F. moniliforme* dextranase occurred within 15 min at 80 C at a pH of 6.0. Chaiet et al. (6) found a similar inactivation of *P. funiculosum* dextranase at temperatures above 55 C.

The observation that *F. moniliforme* dextranase activity was not inhibited by the presence of either EDTA or iodoacetate suggests that the enzyme does not require an inorganic metallic ion cofactor and that a free thiol group is not necessary for enzymatic activity. However, additional studies are required to determine the conformation and the nature of the active site of this enzyme. Some conflicting results exist regarding the effects of specific thiol reagents and EDTA on *Penicillium*-derived dextranase. Fukumoto et al. (13) did not observe any inactivation of this enzyme in the presence of either EDTA or *p*-chloromercuribenzoate, whereas others (21) have observed partial inactivation with both EDTA and iodoacetate. The molecular weight of *P. funiculosum* dextranase has been reported as 41,000 (6) and 44,000 (21). A similar molecular weight value of 39,000 for the *F. monoliforme* dextranase was determined in the present investigation.

Although many similarities between the *F. moniliforme* dextranase and *Penicillium*-derived enzyme were observed, some important molecular and enzymatic differences were also found. When the two dextranases were compared by the electrophoretic assay procedure, a major difference in their relative mobilities was observed. A potentially more important difference was the greater relative activity that the *F. moniliforme* dextranase had toward the extracellular glucans isolated from *S. mutans*. Approximately a 20- and a 7-fold greater relative activity was observed on the water-soluble and -insoluble glucans, respectively, compared to the *Penicillium*-derived dextranase. However, both enzymes were similar in that each had its lowest activity on the water-insoluble glucan and its highest activity on the commercial dextran.

As pointed out earlier, dextranase may be an important therapeutic agent for the prevention or control of dental caries and periodontal disease. A possible protective mechanism of dextranase action could involve the interference with bacterial plaque formation. *S. mutans* and other extracellular glucan-producing streptococci have been isolated from dental plaque (14, 25), and dextran has been identified as a component of human dental plaque (15). The synthesis of this polysaccharide may serve as a mechanism which allows cariogenic bacteria to agglutinate (16) and adhere (14) on the enamel surfaces of the teeth. The resulting plaque formation appears to be a prerequisite to the onset of dental caries.

The glucan synthesized by *S. mutans* has been shown to have both alpha-1,6 and -1,3 glycosidic linkages (2, 9). Recent evidence (9) indicates that the 1,6-links are primarily responsible for the adhesive or "sticky" properties of this polysaccharide, whereas the 1,3-links are responsible for its water-insoluble property. Additional evidence indicates that mutants which were defective in glucan synthesis lost their ability to adhere to smooth surfaces and were also less virulent (12, 19, 22). Therefore, dextranases could be expected to be potentially important caries preventive agents.

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