EFFECT OF PHOSPHATE IONS AND DIMETHYL SULFOXIDE ON THE AMYLOLYTIC ACTIVITY OF SELECTED α-, β-, AND GLUCOAMYLASES

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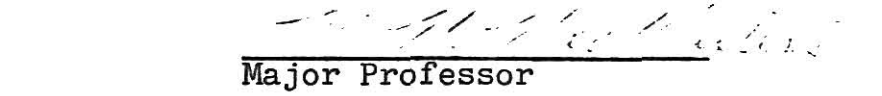

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INTRODUCTION

Diets supplemented with various phosphates were found to prevent dental caries in rats. Sodium trimetaphosphate has the highest cariostatic activity of the several phosphates tested and is currently the preferred compound for control of dental caries in animals (1).

Since all the phosphates are absorbed into the system in the form of orthophosphate, sodium trimetaphosphate may perform its cariostatic function in the oral cavity. One way it could do this would be by enhancing the activity of enzymes present in the saliva. Thus food materials would be very quickly and efficiently removed from the dental surfaces and the crevices of the teeth. It was of interest to investigate the effect of sodium trimetaphosphate on the activity of selected α-, β-, and glucoamylases to determine if any effect on enzyme activity occurred.

The effect of varying concentrations of sodium trimetaphosphate, dibasic sodium phosphate, monobasic sodium phosphate, sodium fluoride, and dimethyl sulfoxide on the activity of porcine pancreatic α-amylase, sweet potato β-amylase, and glucoamylases from Aspergillus niger have been investigated in this research.

The effect of dimethyl sulfoxide on enzyme activity was of interest due to the fact that it is used as a solvent in the analytical and experimental extraction of starch from natural sources (2).

LITERATURE REVIEW

Diastatic enzymes hydrolyze starch, yielding maltose as the principal end product. Under favorable conditions some starches yield as high as 70 to 80 % maltose (3).

There are three mechanisms for the endwise synthesis and degradation of a polymer molecule: the single chain mechanism in which the enzyme completes the degradation of a single polymer molecule before transferring its action to the next; the multichain mechanism, in which the enzyme adds or removes single units in completely random fashion from all available polymer ends; and the multiple attack mechanism in which multiple enzyme attacks on the same substrate molecule are possible before the complex dissociates (4).

α-Amylases hydrolyze interior α-D-(1,4)- linkages of starch and are therefore classified as endo-enzymes (5). In the early stages of the reaction only dextrins are formed, but after hydrolysis has proceeded to about 25% maltose-equivalent maltose appears. The concentration of maltose increases with continued conversion while the concentration of dextrins decreases. Glucose is formed in the later stages of the reaction (5). Robyt and French have shown the multiple attack nature of porcine pancreatic, human salivary, and Aspergillus oryzae α-amylases (5). With porcine pancreatic α-amylase under optimal conditions, pH 6.9, the degree of multiple attack was 6 for action on amylose, but at pH 10.5 this dropped to zero, which corresponded to a multichain mechanism. Under optimum conditions, human salivary α-amylase gave a degree of multiple attack of only three (5).

β-Amylases act only on the non-reducing end of the starch chain and split off maltose units stepwise down the chain. Only maltose and the unreacted portion of the starch molecule are present at any time. The enzyme is classified as an exo-enzyme. The reaction is complete at about 50% conversion to maltose since the enzyme cannot hydrolyze the α-D-(1,6)- linkages at branch points (6). Hopkins and Jelinek (7) have shown a multiple attack pattern for β-amylase action on amylose. The Michaelis constants of soya bean β-amylase acting on potato amylose or on the short chain fission products were of the same order. Thus, the enzyme showed no decided preference for either long or short chains. The early disappearance of the short chains from a mixture of long and short-chain polysaccharides was also demonstrated and must be attributed to a multichain mode of attack by the enzyme; the hydrolysis of short chains being soon completed because they are short. The single chain and the multichain theories of β-amylase action have been demonstrated by Hopkins and Jelinek (7). Bird and Hopkins (8) showed the multichain action of β-amylase on amylose fission products. Paper chromatographic analysis of the fission products of maltohexaose by crystalline β-amylase indicated that the intermediate product, maltotetraose, accumulated until the hexasaccharide disappeared; maltotriose slowly disappeared. Diminution in chain length of the residue and the presence of short-chain fission products during hydrolysis of amylose by β-amylase were observed, indicating a multichain mechanism (8).

Bailey and French (4) observed a multiple attack mechanism for β-amylase. Using a ^{14}C-labelled linear polysaccharide of known chain length, sweet potato β-amylase was found to give four enzymic attacks per effective encounter with the substrate molecule.

Bailey and Whelan (9) studied the action pattern of β-amylase. The action pattern was studied under a variety of conditions using two maltodextrins and a synthetic amylose of average chain length of 49 units. The action pattern was always intermediate between single- and multi-chain. The enzyme removes several maltose units during each encounter with a substrate molecule (9) which is a multiple attack action pattern.

The glucoamylase from Aspergillus niger is capable of hydrolyzing α-D-(1- 4) linkages and α-D-(1- 6) linkages in a variety of glucosyl oligosaccharides (10). The initial hydrolysis of a substrate by glucoamylase occurs at the non-reducing end and proceeds at a faster rate if the terminal unit is linked by an α-D-(1- 4) linkage rather than an α-D-(1- 6) linkage to the remainder of the oligosaccharide molecule (10). α-D-(1- 4)-Linkages are hydrolyzed approximately forty times more rapidly than the α-D-(1- 6) linkages. Glucoamylase was found to produce glucose at the same rate, in weight per unit time, in equimolar solutions of amylose differing widely in original chain length and glucose was the only sugar found by paper chromatography (11).

The glucoamylase from Aspergillus niger has been isolated and purified by use of DEAE-cellulose ion-exchange chromatography (13, 14). The enzyme obtained was in a highly purified state and

was observed to consist of two forms. The glucoamylase from Rhizopus delemar was purified by chromatography on Sephadex gel and ion-exchange resins (12). The action pattern of the purified glucoamylase from Rhizopus delemar on starch and malto-oligosaccharides is similar to that of glucoamylase from A. niger.

The effect of varying concentrations of sodium phosphate (0.005-0.05 M) on the activity of porcine pancreatic α-amylase has been investigated (15). Hydrogen ion activities were kept constant by adjustment with equimolar solutions of mono- and disodium phosphate. The activity of pancreatic amylase was found to be independent of the concentrations of sodium phosphate under favorable conditions. In the presence of 0.004 M sodium citrate, the enzyme was found to have optimal activity in solutions of pH 7.0 to 7.2. The activity of the enzyme was almost the same in the presence of citrate ion when compared to phosphate ions. The activity of the enzyme showed no change in the presence of sodium acetate and it was difficult to control the hydrogen ion activities as is desirable. Sodium borate in concentrations of 0.001-0.01 M did not influence enzyme activity. The activity was found to be slightly lower than the controls, but the difference was within experimental limits of error. As long as the hydrogen ion activities were maintained constant there was no significant effect of phosphate (0.0005 to 0.05 M) or borate (0.001 to 0.01 M) salts. In the presence of 0.05 M sodium chloride, the activity of the enzyme was found to be independent of the concentrations of phosphate (0.0005 to 0.05 M) and of borate (0.001 to 0.01 M) as long as the hydrogen ion activities were maintained optimal for enzyme activity (15).

The effect of various neutral salts such as sodium chloride, potassium chloride, lithium chloride, sodium sulfocyanate, sodium fluoride, sodium sulfate, and sodium phosphate on the activity of pancreatic α-amylase have been studied (16). Sodium chloride was found to have the most favorable influence upon enzymic activity. Lithium chloride (0.005, 0.02, and 0.05 M) was found to give 80 to 90% of the optimal activity of pancreatic α-amylase in the presence of sodium chloride. Potassium or sodium chlorides activated pancreatic α-amylase to the same extent but the concentration of potassium chloride required was slightly greater than that of sodium chloride. Of all the anions studied, chloride ion was the most effective and the salts were placed in the following order according to their influence on the hydrolysis of starch by pancreatic amylase: "sodium chloride > potassium chloride > lithium chloride > sodium bromide > sodium nitrate > sodium sulfocyanate > and sodium fluoride" (16).

The influence of more than one salt at a time on the activity of pancreatic α-amylase has been investigated (16). Enzyme activity was measured in the presence of 0.02 M sodium chloride with additions of sodium nitrate (0.01 - 0.02 M) or sodium sulfate (0.01 - 0.10 M). Enzyme activity decreased with increasing concentrations of the second salt. Anions having a favorable effect upon the hydrolysis of starch by pancreatic α-amylase were arranged in the following order: "chloride > bromide > nitrate > chlorate sulfocyanate > and fluoride" (16). The concentration of the salts required for optimal enzymic activity was found to be: 0.01 M for sodium chloride, potassium chloride, and sodium bromide;

0.05 M for sodium nitrate or sodium chlorate; 0.10 M for sodium sulfocyanate; and 0.20 M for sodium fluoride. As the salt concentration decreased below these concentrations the enzyme was found to be most active in increasingly more acid solutions (17).

The concentration of various neutral salts yielding optimum enzyme activity were reported to be 0.01 - 0.005 M for sodium chloride, 0.03 to 0.05 M for potassium chloride, 0.03 to 0.20 M for sodium bromide, and 0.10 to 0.20 M for sodium nitrate (18).

Muus et al. (19) studied the effect of various ions on the rate of inactivation of salivary α-amylase and the stabilizing effect of chloride ion. Heavy metals such as copper and silver were found to inactivate the enzyme. Copper sulfate (3×10^{-6} M) gave a rate of inactivation similar to that of commercial phosphate buffer. If it is assumed that copper in the reagent is responsible for the difference in rate between the commercial phosphate buffer and the recrystallized barbital buffer, it would mean that the salt contained approximately 0.002% copper in the anhydrous salt. The effect of copper and silver ions was found to be reversed by cyanide ion, this was due to cyanide competing with binding of heavy metals. Versenate, which is a strong complexing agent for metal ions, did not decrease the rate of inactivation, but, on the contrary, it increased the rate of inactivation. Salyrgan, a -SH reagent, was found to have no effect on the enzyme activity at 5×10^{-5} M, but with mercuric chloride at the same concen-

tration it reduced the rate of enzymic reaction to one-fifth of the rate without the inhibitor. Bromide was found to activate salivary α-amylase and its activating effect was about 80% that of chloride ion. As a stabilizer, it was found to act in a similar manner to that of chloride ion. At lower salt concentration, the effect was greater, and maximum activation was obtained with a chloride concentration of 0.01 M. Even a chloride concentration of 10^{-6} M was found to give slight activation of highly purified salt-free amylase (19).

Beryllium and aluminum salts in concentrations from 10^{-2} to 10^{-5} M were found to inhibit α-amylases from man, hog, rat, and dog to varying degrees (20). Magnesium, barium, and strontium salts were found not to inhibit these enzymes. At 10^{-2} M concentration of beryllium sulfate, the inactivation was not instantaneous but required about 30 minutes, at 10^{-3} to 10^{-4} M concentration inactivation required about 60 minutes. In the presence of certain proteins, such as bovine plasma albumin and gelatin, the enzyme was found to be protected against inhibition by beryllium. The effect of sodium oxalate and the sodium salt of phytic acid on hog pancreatic and human salivary α-amylases have been studied (20). Although both oxalate and phytate are known to precipitate calcium, it was found that, at 10^{-3} M concentration, oxalate had no effect and phytate caused only 20% inhibition. Citrate at 10^{-3} M concentration had no effect on either amylase.

The requirement for calcium by α-amylases was studied by Hsiu *et al.* (21) using α-amylases from *Bacillus subtilis* and

human saliva. The calcium bound to these enzymes was removed by chelation or electro-dialysis and the removal of calcium was accompanied by a loss of enzymic activity. This requirement for calcium was interpreted in the following manner: "By forming a tight metal chelate structure, the metal produces intramolecular cross-links similar in function to disulfide bridges, which confer to the α-amylase molecule the structural rigidity required for effective catalytic activity" (21).

A wide range of salts such as sodium hippurate (0.1 M), sodium succinate (0.2 M), and sodium DL-malate (0.10 M) were found to increase the activity of α-amylase when estimated by a standard graph method (22). Positive effects were observed only with ionized compounds in which the charges could separate in solution; zwitterions such as glycine and alanine, unlike sodium glutamate, had no stimulating effects. The stimulations were not due to either specific cation or anion effects since no significant differences were found when the chlorides (40 mM), and sulfates (20 mM) of sodium and potassium were tested at equal metal-ion concentrations. Under optimum conditions the activity of the mixture of α- and β-amylases fell below the sum of activities of the enzymes acting separately. When potassium chloride was added to the mixture of amylases, the breakdown of starch was found to be accelerated and the activity of the enzyme equalled or even slightly surpassed the sum of activities of the enzymes measured separately. Potassium sulfate (0.03 M) was found to have no effect on the activity of α-amylase, but was found to

accelerate slightly the activity of a mixture of α- and β-amylases. With 0.50 M potassium chloride, the activity of β-amylase was found to be slightly accelerated (22).

Warren et al. (23) showed that, when the enzyme β-amylase utilizes uncharged substrates, neutral salts inhibit the activity in an order of increasing effectiveness for anions: "acetate < chloride < bromide ≅ nitrate < iodite < perchlorate ≅ thiocyanate." Neutral salts, at concentrations which only partly inhibit the activity of β-amylase, increase the rate of reaction of its sulfhydryl groups with 5,5'-dithiobis-(2-nitrobenzoic acid) in an order of increasing effectiveness for anions: "acetate < chloride < perchlorate < thiocyanate," which is similar to the order of inhibition (23).

Urea (5 to 9%) was found to be without inhibitory effect on salivary amylase at temperature of 37-39° C and pH 7.0, in the presence of C.01 M sodium chloride. In the absence of sodium chloride and at temperatures above 50° C, amylase underwent a spontaneous progressive inhibition. The inhibitory action of urea was counteracted by a 0.10% mixture of dibasic and monobasic ammonium phosphate of pH 7.0. Application of pressure to the reaction mixture increased amylase activity by 30% in the absence of urea and 12% in the presence of 5% urea (24).

The presence of sodium chloride was found to be necessary for the liquefaction of starch paste by pancreatic α-amylase. For malt and fungal amylases sodium chloride was not necessary for liquefaction but sodium chloride greatly accelerated the

rate (25).

Addition of 0.02 to 2.0 mg of cobalt chloride to a solution of human saliva was found to increase the amylolytic activity two- to three-fold. Cobalt nitrate in doses of 1.0 mg or less showed no significant effect on salivary amylase, while higher doses (2-20 mg) inhibited enzyme activity. Cobalt chloride markedly increased the activity of pancreatic amylase. Cobalt nitrate also increased the activity of pancreatic amylase but low doses of cobalt sulfate showed no significant effect (26).

Manganese chloride was found to increase the activity of pancreatic and salivary amylases. Manganese sulfate in doses of 2-10 mg increased pancreatic amylase activity two-fold but showed a marked inhibitory effect on salivary amylase (27).

No reports of work concerning the effect of various ions on the activity of glucoamylases has been found by this author.

MATERIALS AND METHODS

Materials:

Porcine pancreatic α-amylase (twice crystallized, DFP-treated) and sweet potato β-amylase (crystallized) were purchased from Worthington Biochemical Corp., Freehold, N. J. Sodium trimetaphosphate was supplied by Monsanto Co., St. Louis, Mo. All other chemicals were reagent grade unless otherwise specified.

Purification of Glucoamylase I and II:

Glucoamylase I and II were isolated and purified by ion-exchange chromatography on DEAE-cellulose (13). DEAE-cellulose was washed with acid and alkali in the manner described by Lineback et al. DEAE-cellulose was wetted by standing overnight in water. The supernatant fluid and fine particles were removed through a glass tube with suction. This procedure was repeated approximately ten times. The resin was then filtered and washed successively in a Buchner funnel with 0.5 M hydrochloric acid, distilled water, 0.5 M sodium hydroxide, and water. The resin was filtered after each washing and fine particles removed when necessary. The DEAE-cellulose was filtered and the resulting pad suspended in 0.025 M citrate-phosphate buffer of the desired pH. The mixture was equilibrated by stirring and then adjusted to the desired starting pH with 1.0 M citric acid or potassium hydroxide. Columns (3.5 x 45 cm) were packed with a slurry of the resin adjusted to pH 8.0 in 0.025 M citrate-phosphate buffer. The columns were washed with about 300 ml of 0.025 M citrate-phosphate buffer, pH 8.0, and were equilibrated overnight at 4° C. prior to use.

A commercial glucoamylase preparation (Diazyme concentrate, Miles Chemical Co., Elkhart, Indiana) was used as the source of the crude enzyme. The crude enzyme was supplied as a tan powder. Glucoamylase I and II were isolated and purified using the gradient elution system reported by Lineback et al. (13). The flow rate of the column was maintained at one ml per minute by means of a peristaltic pump and fractions of 15 ml were collected automatically. Protein components were located by measuring their absorbance at 280 nm. Three protein components were obtained during the purification. The first protein component was transglucosidase and was discarded. The second protein component was glucoamylase II and the third protein component was glucoamylase I. Fractions containing glucoamylase I and II were collected separately and further purified by rechromatography on DEAE-cellulose ion-exchange columns using a gradient elution system (13).

The protein concentration of glucoamylases I and II was determined by the Lowry method (28). An aliquot (1.0 ml) of the suitably diluted enzyme was treated for 10 minutes at room temperature with 5.0 ml of the alkaline copper reagent. Folin-Ciocalteau reagent (0.5 ml) was then added with stirring and the absorbance was determined at 660 nm after the color had developed for 30 minutes at room temperature. A standard curve was constructed with bovine serum albumin.

Glucoamylase Assay:

Glucoamylases I and II were diluted with 0.05 M acetate buffer, pH 4.8, to yield solutions containing 80 and 60 ug protein per ml, respectively. The activity of the enzyme was determined

by measuring the amount of glucose liberated by the enzyme from a starch substrate (13). The enzyme (0.5 ml) was incubated with 1.5 ml of 4% starch (Lintner soluble) in 0.05 M acetate buffer, pH 4.8, for one hour at 30° C. The reaction was terminated by adding 5.0 ml of absolute ethanol. Glucose was determined in an aliquot (1.0 ml) of the supernatant solution, diluted five-fold, using a glucose oxidase reagent (Glucostat, Worthington Biochemical Corp., Freehold, N. J.). An aliquot (0.5 ml) of the diluted supernatant solution was incubated with 5.0 ml of the enzyme-chromophore solution for 30 minutes at 30° C. The reaction was terminated by adding two drops of 4 M HCl, and the absorbance was measured at 400 nm. A standard curve was prepared using 20-100 ug glucose. Specific activity was expressed as mg of glucose produced per hour per mg of protein, determined by the Lowry method (28).

Effect of various salts and dimethyl sulfoxide on glucoamylase activity:

Solutions of sodium trimetaphosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M), dibasic sodium phosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M), monobasic sodium phosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M), sodium fluoride (0.0005 M, 0.005 M, 0.05 M, 0.10 M), and dimethyl sulfoxide (0.00045 M, 0.0045 M, 0.045 M, 0.095 M, 1.0 M, 5.0 M, 10.0 M) were prepared in 0.05 M acetate buffer, pH 4.8. Glucoamylases I and II were dialyzed against each of these solutions for twelve to fifteen hours at 4° C. The solutions were replaced with fresh solvent at 5-hour intervals. Aliquots (0.5 ml) of the dialyzed enzyme solutions were then

incubated with 4% starch (1.5 ml) in 0.05 M acetate buffer, pH 4.8, for one hour at 30° C. The glucose formed was determined as previously described (13). A control was run without the salts or DMSO.

Effect of various salts and dimethyl sulfoxide on α-amylase activity:

Salt and dimethyl sulfoxide solutions of the concentrations previously listed for glucoamylase were prepared in 0.02 M sodium glycerophosphate hydrochloride buffer, pH 6.9. Porcine pancreatic α-amylase (twice crystallized, DFP-treated), previously diluted to a concentration of 20 Units per ml, was dialyzed against these solutions for 12 to 15 hours at 4° C and finally diluted to 0.5-1.5 Units per ml. The enzyme had 10 mM of chloride and 3 mM Ca ions. α-Amylase activity was determined by the Nelson colorimetric modification of the Somogyi copper procedure (30). An aliquot (1.0 ml) of the dialyzed and properly diluted enzyme solutions was incubated with a 1% starch solution (1.0 ml) in 0.02 M sodium glycerophophate hydrochloride buffer, pH 6.9, for 10 minutes at 25° C. After 10 minutes, 0.5 ml of the digest was added to one ml of the Nelson copper reagent and diluted to two ml with distilled water. The solution was placed in a boiling water-bath for 20 minutes, cooled in running tap water for 5 minutes and one ml of the arsenomolybdate reagent was added. The mixture was shaken until the evolution of carbon dioxide ceased, allowed to stand at room temperature for 10 minutes and diluted to 25 ml with distilled water. The absorbance of the resulting solution was measured at 520 nm. A standard curve was prepared using

50-500 µg of maltose (31). A control was run without the above mentioned salts or DMSO.

Effect of various salts and dimethyl sulfoxide on β-amylase activity:

Salts and dimethyl sulfoxide (DMSO) solutions of the concentrations previously tested for the glucoamylases and α-amylase were prepared in acetate buffer, pH 4.8, containing 0.5 mM reduced glutathione and 0.05% serum albumin. Sweet potato β-amylase was diluted to about 20 Units per ml and dialyzed against the salts and DMSO as done previously for the glucoamylases and α-amylase. The dialyzed solutions were diluted to 1.5 Units per ml with acetate buffer, pH 4.8, containing 0.5 mM reduced glutathione and 0.05% serum albumin, so that the enzyme concentration would liberate 10 - 20 mg of maltose in 30 minutes from 28 ml of 0.6% starch in acetate buffer, pH 4.8 (32, 33). An aliquot (2.0 ml) of the properly diluted β-amylase solution was incubated with 28 ml of starch solution (0.6% starch + 3 ml of 0.2 M acetate buffer, pH 4.8) containing the desired concentration of salt or DMSO for 30 minutes at 35° C. Maltose produced in the digest was determined by the Nelson colorimetric modification of the Somogyi copper procedure (30). A standard curve was prepared using 50-500 ug maltose (32). A control was without the salts or DMSO.

All starch substrates contained the desired salts or DMSO concentration in the suitable buffer and pH for the particular enzyme.

RESULTS AND DISCUSSION

Isolation and purification of glucoamylase from Aspergillus niger:

Two forms of glucoamylase of *Aspergillus niger* were isolated by chromatography on DEAE-cellulose ion-exchange resins. Three major protein components (Fig. 1) were obtained from elution of the proteins with a solvent gradient of decreasing pH. The pattern obtained was very similar to that reported (13) for purification of the same enzyme under identical conditions and the components were assumed to correspond in each pattern. The first protein component was assumed to be a glucosyl transferase and was discarded. The second protein component was glucoamylase II and the third component was glucoamylase I. Pazur and Ando (14) also purified glucoamylase from *A. niger* by chromatography on DEAE-cellulose ion-exchange columns using a stepwise elution system and obtained two forms of the enzyme (glucoamylase I and II). The activities of the purified enzymes were determined by measuring the amount of glucose produced from a starch substrate. Values obtained for glucoamylase I agreed with those reported (13) while the values for glucoamylase II were found to be slightly higher.

Effect of phosphate ions on glucoamylase activity:

The effect of several concentrations of different phosphate anions on the activity of purified glucoamylases was studied by dialyzing the purified glucoamylase against a buffered solution of the salt and then measuring the amount of glucose produced by the enzyme from a starch substrate. Varying concentrations of sodium trimetaphosphate (0.0005 *M*, 0.005 *M*, 0.05 *M*, 0.10 *M*) were found to have no effect on the enzymatic activity of glucoamylase I (Table I).

Fig. I. Chromatography of crude glucoamylase from A. niger on DEAE-cellulose. The fraction labelled I and II refer to glucoamylase I and II, respectively.

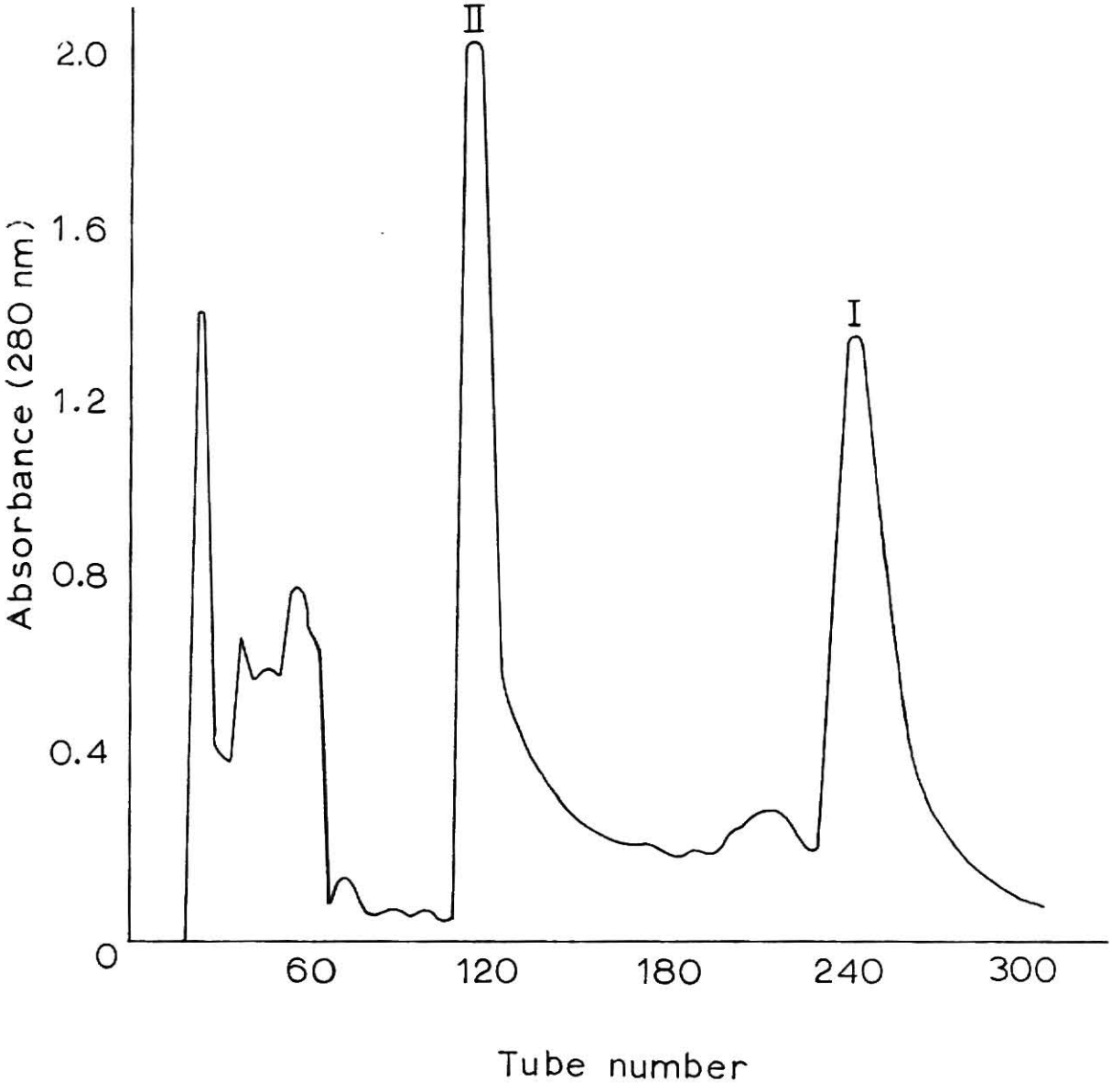
II
I
2.0
1.6
1.2
0.8
0.4
0
Absorbance (280 nm)
60
120
180
240
300
Tube number

TABLE I. EFFECT OF SODIUM TRIMETAPHOSPHATE ON GLUCOAMYLASE I ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	150	168	145	156	155
0.0005	138	158	145	147	147
0.005	160	160	150	150	155
0.05	149	158	146	150	151
0.10	152	152	143	146	148

* Activity given in mg of glucose produced per mg of protein.

TABLE II. EFFECT OF SODIUM TRIMETAPHOSPHATE ON GLUCOAMYLASE II ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	177	191	177	182	182
0.0005	177	205	175	173	183
0.005	159	173	170	170	168
0.05	175	175	159	167	169
0.10	168	170	166	166	168

* Activity given in mg glucose produced per mg protein.

Slightly decreased values were obtained for glucoamylase II (Table II) but the values were within the range of experimental error and, thus, there was no significant effect on the activity of the enzyme.

The activities of glucoamylase I and II were studied in the presence of dibasic and monobasic sodium phosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M; Tables III, IV, V, VI). Slightly decreased values were obtained for enzymatic activity of glucoamylase I (Table III) in the presence of 0.10 M and 0.05 M dibasic sodium phosphate, but this may be due to a small variation in the hydrogen ion concentration. In this investigation the initial pH was kept constant (± 0.1 Unit) by addition of 1 M potassium hydroxide or citric acid. However, the values for enzyme activity were within the experimental error (less than three standard deviations from the mean of the control). The standard deviation for all control values was 12.8 for glucoamylase I and 11.1 for glucoamylase II. The activities of glucoamylase I and II were not significantly affected in the presence of either monobasic or dibasic sodium phosphates.

Effect of phosphate ions on the activity of α-amylase:

α-Amylase was dialyzed against several concentrations of buffered salt solutions. The activity of the enzyme was then determined by measuring the amount of maltose produced from a starch substrate.

Sodium trimetaphosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M) was found to have no significant effect on enzyme activity (Table VII). The activity of the enzyme appeared to be slightly

TABLE III. EFFECT OF DIBASIC SODIUM PHOSPHATE ON GLUCOAMYLASE I ACTIVITY.

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	172	166	170	170	169
0.0005	147	152	149	150	150
0.005	144	156	151	150	150
0.05	144	151	146	147	147
0.10	138	147	143	144	143

*Activity given in mg glucose produced per mg protein.

TABLE IV. EFFECT OF DIBASIC SODIUM PHOSPHATE ON GLUCOAMYLASE II ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	172	177	175	174	174
0.0005	182	179	178	182	180
0.005	177	182	180	178	179
0.05	182	168	175	176	175
0.10	161	168	165	164	164

*Activity given in mg glucose produced per mg protein.

TABLE V. EFFECT OF MONOBASIC SODIUM PHOSPHATE ON GLUCOAMYLASE I ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	162	162	146	146	154
0.0005	166	169	140	146	155
0.005	164	162	142	144	152
0.05	156	156	142	142	149
0.10	156	156	138	152	150

*Activity given in mg glucose produced per mg protein.

TABLE VI. EFFECT OF MONOBASIC SODIUM PHOSPHATE ON GLUCOAMYLASE II ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	173	168	156	166	166
0.0005	163	180	170	159	168
0.005	168	154	154	161	159
0.05	159	156	147	156	154
0.10	166	159	152	168	161

*Activity given in mg glucose produced per mg protein.

TABLE VII. EFFECT OF SODIUM TRIMETAPHOSPHATE ON α-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	68	71	70	75	71
0.0005	74	71	71	75	73
0.005	75	73	75	73	74
0.05	75	71	77	75	74.5
0.10	66	65	69	65	66

*Activity given in μg maltose formed per ml digest per minute.

TABLE VIII. EFFECT OF DIBASIC SODIUM PHOSPHATE ON α-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	71	71	75	77	73.5
0.0005	62	62	68	75	67
0.005	69	75	77	75	74
0.05	71	75	75	75	74
0.10	74	81	75	77	77

*Activity given in μg maltose formed per ml digest per minute.

reduced in the presence of 0.10 M sodium trimetaphosphate but the extent was not significant.

Varying concentrations of dibasic (Table VIII) and monobasic (Table IX) sodium phosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M) were without significant effect on the activity of α-amylase. Sherman et al. (15) studied the effect of sodium phosphate on the activity of pancreatic α-amylase by varying the total concentration of sodium phosphate in the solution from 0.0005 M to 0.05 M while the hydrogen ion activities were maintained constant by addition of suitable proportions of equimolar solutions of mono- and dibasic sodium phosphate. The activity of pancreatic α-amylase was found to be independent of these concentrations of phosphate provided the hydrogen ion activities of the solutions were suitably adjusted. In the presence of 10 mM chloride ion, the activity of the enzyme in this study appeared to be independent of the concentrations of phosphate from 0.0005 M to 0.10 M, provided the optimum hydrogen ion activity was maintained (pH 6.9). This is in agreement with the results of Sherman et al. (15).

Effect of phosphate ions on the activity of β-amylase:

The effect of several concentrations of different anions on the activity of β-amylase was studied by dialyzing the enzyme against a buffered solution of the salt and then measuring the amount of maltose produced by the enzyme from a starch substrate.

Sodium trimetaphosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M) was found to have no effect on enzyme activity (Table X). The lowest two concentrations appeared to decrease the activity slightly but the extent was not significant.

TABLE IX. EFFECT OF MONOBASIC SODIUM PHOSPHATE ON α-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	80	81	71	79	78
0.0005	80	81	72	81	78.5
0.005	73	79	79	77	77
0.05	77	83	81	79	80
0.10	79	81	78	85	80.8

*Activity given in μg maltose formed per ml digest per minute.

TABLE X. EFFECT OF SODIUM TRIMETAPHOSPHATE ON β-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0.	19.7	17.0	16.7	15.7	17.3
0.0005	19.0	15.7	14.0	14.0	15.7
0.005	17.7	14.7	15.0	15.0	15.6
0.05	18.3	17.3	17.0	16.7	17.3
0.10	18.0	17.0	17.7	16.7	17.4

*Activity given in μg maltose formed per ml digest per minute.

Dibasic (Table XI) and monobasic (Table XII) sodium phosphate (0.0005 M, 0.005 M, 0.05 M, 0.10 M) were found to have no significant effect on β-amylase activity. There appeared to be a slight increase in enzyme activity at 0.10 M, 0.05 M, 0.005 M concentrations of dibasic sodium phosphate (Table XI) and a slight decrease in enzyme activity at 0.0005 M. However, the values were within experimental error and were not considered significant. Monobasic sodium phosphate was found to be without any effect on the activity of β-amylase at all concentrations investigated.

The above results indicate that the phosphate ions tested had no significant influence on the activity of α-amylase, β-amylase, and glucoamylase I and II. Porcine pancreatic α-amylase was used in this investigation because it is very similar to salivary amylase (35). Since sodium trimetaphosphate has been found to have no influence on the enzymes investigated in this study, it seems unlikely that its cariostatic function (1) is due to any enhancement of enzymic activity in the oral cavity. The use of sodium trimetaphosphate in the production of corn syrups or dextrose is also not warranted since it has no beneficial effect on the amylolytic systems used.

Effect of sodium fluoride on the activity of glucoamylase I and II:

Varying concentrations of sodium fluoride (0.0005 M, 0.005 M 0.05 M, 0.10 M) were found to have no significant effect on the activity of glucoamylase I and II (Tables XIII and XIV), α-amylase (Table XV) or β-amylase (Table XVI). Sherman et al. (16) studied the influence of sodium fluoride on the activity of pancreatic

TABLE XI. EFFECT OF DIBASIC SODIUM PHOSPHATE ON β-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity*				
	1	2	3	4	Average
0	18.0	18.0	16.3	17.7	17.5
0.0005	14.3	14.0	15.7	14.7	14.7
0.005	19.3	17.7	19.3	18.7	18.8
0.05	18.0	19.0	19.0	18.3	18.6
0.10	18.7	17.7	19.3	18.3	18.5

*Activity given in μg maltose formed per ml digest per minute.

TABLE XII. EFFECT OF MONOBASIC SODIUM PHOSPHATE ON β-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity*				
	1	2	3	4	Average
0	18.0	15.9	17.1	17.1	17.0
0.0005	17.7	16.7	16.3	17.0	16.9
0.005	17.0	14.6	17.1	16.2	16.2
0.05	17.7	17.7	15.7	17.0	17.0
0.10	18.3	16.7	18.3	17.7	17.8

*Activity given in μg maltose formed per ml digest per minute.

α-amylase and reported that 0.2 M to 0.3 M concentration gave some activation of the enzyme.

A slight increase in the activity of β-amylase was observed in this study between concentrations of 0.005 M and 0.05 M sodium fluoride and the enzyme was found to have optimal activity in the presence of 0.005 M sodium fluoride. However, the increases were not significant.

Effect of dimethyl sulfoxide on glucoamylase activity:

The activities of glucoamylase I (Table XVII) and II (Table XVIII) were studied in the presence of varying concentrations of dimethyl sulfoxide (0.00045 M, 0.0045 M, 0.045 M, 0.095 M, 1.0 M, 5.0 M, 10.0 M). Dimethyl sulfoxide concentrations less than 1.0 M did not effect enzymatic activity. At 1.0 M dimethyl sulfoxide there was a 20% decrease in the activity of glucoamylase I. This decrease was nearly three standard deviations from the control value and hence lay on the borderline of significance. Similar results were obtained for glucoamylase II, with the exception that the activity of glucoamylase II was still uneffected in 1.0 M dimethyl sulfoxide. As the concentration of the dimethyl sulfoxide increased, the activity of glucoamylase I and II continued to decrease. There was a significant decrease in the activity of both glucoamylase I and II at dimethyl sulfoxide concentrations of 5.0 M and 10.0 M.

Libby (2) reported that a glucoamylase (Rhizopus species) solution containing 18% dimethyl sulfoxide showed no change in enzyme activity during four-months use. The specific activity of the glucoamylase was not determined at 18% dimethyl sulfoxide, but

TABLE XIII. EFFECT OF SODIUM FLUORIDE ON GLUCOAMYLASE I ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	150	140	146	142	144
0.0005	162	170	146	132	152
0.005	160	182	140	110	148
0.05	162	144	136	164	148
0.10	164	162	144	136	151

*Activity given in mg glucose produced per mg protein.

TABLE XIV. EFFECT OF SODIUM FLUORIDE ON GLUCOAMYLASE II ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	189	166	163	168	171
0.0005	173	166	176	156	165
0.005	180	161	168	175	171
0.05	182	154	173	166	169
0.10	182	154	175	170	170

*Activity given in mg glucose produced per mg protein.

TABLE XV. EFFECT OF SODIUM FLUORIDE ON α-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	81	71	73	79	76
0.0005	79	80	74	74	77
0.005	73	72	73	73	73
0.05	79	75	71	71	74
0.10	77	77	68	74	74

*Activity given in μg maltose formed per ml digest per minute.

TABLE XVI. EFFECT OF SODIUM FLUORIDE ON β-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	18.0	17.7	19.1	19.7	18.6
0.0005	16.7	20.2	18.0	20.3	18.8
0.005	20.7	23.0	20.0	20.7	21.1
0.05	20.7	20.0	20.3	20.3	20.3
0.10	18.0	19.8	18.3	17.3	18.4

*Activity given in μg maltose formed per ml digest per minute.

the hydrolysis of starch was reported to be very fast (complete in ten minutes) under the conditions used.

Effect of dimethyl sulfoxide on α-amylase activity:

The effect of varying concentrations of dimethyl sulfoxide on the activity of α-amylase was investigated. At concentrations of less than 1.0 M dimethyl sulfoxide, no effect was observed on the activity of α-amylase (Table XIX). Concentrations above 1.0 M were found to inhibit the enzyme activity significantly. The decrease may be due to a solvation effect of dimethyl sulfoxide on the enzyme in which the protein conformation is changed.

Effect of dimethyl sulfoxide on the activity of β-amylase:

The effect of varying concentrations of dimethyl sulfoxide on the activity of sweet potato β-amylase was studied by measuring the amount of maltose produced by the enzyme from a starch substrate. Dimethyl sulfoxide at concentrations of 1.0 M or less was found to have no effect on the activity of β-amylase (Table XX). There was a significant decrease in enzymic activity at 5.0 M and 10.0 M concentrations. The activity of β-amylase was completely inhibited in 10.0 M dimethyl sulfoxide.

Farkas and Gobel have reported that addition of dimethyl sulfoxide (2-30%) increased the activity of barley glucose-6-phosphate dehydrogenase several hundred percent as measured by the increase in NADP reduction with this enzyme. The V_{max} was observed to be increased following addition of dimethyl sulfoxide while the K_{max} for both NADP and glucose-6-phosphate was lowered. Under similar conditions, low concentrations of dimethyl sulfoxide had no stimulatory effect on yeast glucose-6-phosphate dehydroge-

TABLE XVII. EFFECT OF DIMETHYL SULFOXIDE ON GLUCOAMYLASE I ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	182	174	158	164	169
0.00045	174	176	160	164	168
0.0045	176	180	164	160	170
0.045	190	184	158	170	175
0.095	170	166	164	164	166
1.0	134	134	130	138	134
5.0	88	88	88	90	89
10.0	32	30	32	30	31

*Activity given in mg glucose produced per mg protein.

TABLE XVIII. EFFECT OF DIMETHYL SULFOXIDE ON GLUCOAMYLASE II ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	177	193	177	185	183
0.00045	170	177	177	173	174
0.0045	182	184	175	159	175
0.045	203	173	161	173	178
0.095	175	173	168	163	170
1.0	170	154	162	160	164
5.0	93	100	96	98	97
10.0	33	33	34	35	34

*Activity given in mg glucose produced per mg protein.

TABLE XIX. EFFECT OF DIMETHYL SULFOXIDE ON α-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	68	68	70	71	69
0.00045	56	58	59	57	58
0.0045	57	58	60	59	59
0.045	59	59	59	56	58
0.095	59	59	61	61	60
1.0	73	72	73	75	73
5.0	39	40	39	40	40
10.0	13	13	13	13	13

*Activity given in μg maltose formed per ml digest per minute.

nase and high concentrations inhibited the enzyme. The enzyme activities in this investigation were also unaffected by low concentrations of dimethyl sulfoxide but were inhibited by concentrations of dimethyl sulfoxide greater than 1.0 M. Thus, the results indicate that, when dimethyl sulfoxide is used to extract starch from natural sources, the starch may be determined by digestion with glucoamylase, or amylolysis limits may be measured in the normal manner, without effect on the enzymic activity when the concentration of DMSO is less than 1.0 M.

TABLE XX. EFFECT OF DIMETHYL SULFOXIDE ON β-AMYLASE ACTIVITY

Concentration (M)	Enzyme Activity *				
	1	2	3	4	Average
0	19.0	17.2	18.0	17.3	17.9
0.00045	16.7	17.0	17.0	17.0	17.0
0.0045	15.0	17.0	18.8	17.7	17.1
0.045	17.2	16.0	17.7	17.3	17.0
0.095	18.0	16.7	17.3	18.0	17.5
1.0	17.7	17.7	15.6	17.2	17.0
5.0	6.7	6.6	6.6	6.6	6.6
10.0	0	0	0	0	0

*Activity given in μg maltose formed per ml digest per minute.

SUMMARY

Sodium trimetaphosphate has been reported to have the highest cariostatic activity of several phosphates tested and is currently the preferred compound for control of dental caries in animals (1). It was of interest to investigate the effect of sodium trimetaphosphate on the activity of α-, β-, and glucoamylases to determine if any effect on enzyme activity occurred.

The effect of various phosphate ions, fluoride ions, and dimethyl sulfoxide on the activity of glucoamylase I and II from Aspergillus niger, porcine pancreatic α-amylase, and sweet potato β-amylase was studied by measuring the amount of glucose and maltose produced from a starch substrate. Phosphate and fluoride ions (0.0005 M to 0.10 M) were found to have no significant effect on the activity of the enzymes studied.

Dimethyl sulfoxide at 5.0 M and 10.0 M concentrations were found to inhibit the enzyme activity significantly in all cases. At 1.0 M or less dimethyl sulfoxide had no effect on the enzymic activity, but as the concentration increased the enzymic activity was found to decrease. Dimethyl sulfoxide at 10 M concentration was found to completely suppress β-amylase activity.

Since sodium trimetaphosphate has been found to have no influence on the enzymes investigated in this study, it seems unlikely that its valuable cariostatic function is due to an enhancement of enzyme activity in the oral cavity. The use of sodium trimetaphosphate in the production of corn syrups is also not warranted since it has no beneficial effect on the amylolytic systems used. When dimethyl sulfoxide is used to extract starch

from natural sources, the starch may be determined by digestion with glucoamylase or amylosis limits may be measured in the normal manner without effect on the enzymic activity when the concentration of dimethyl sulfoxide is less than 1 M.

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EFFECT OF PHOSPHATE IONS AND DIMETHYL SULFOXIDE ON THE AMYLOLYTIC ACTIVITY OF SELECTED α-, β-, AND GLUCOAMYLASES

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The primary objective of the study was to investigate the effect of various phosphate ions and dimethyl sulfoxide on the activity of selected α-, β-, and gluco-amylases. The activity of the amylases was determined by measuring the maltose produced by the enzyme from a starch substrate. Glucoamylase activity was assayed by measuring the glucose produced by the enzyme from starch.

The effects of varying concentrations of sodium trimetaphosphate, dibasic sodium phosphate, monobasic sodium phosphate, sodium fluoride, and dimethyl sulfoxide on the activity of porcine pancreatic α-amylase, sweet potato β-amylase, and glucoamylase I and II from Aspergillus niger were investigated.

Sodium trimetaphosphate (0.0005 M to 0.10 M), dibasic sodium phosphate (0.0005 M to 0.10 M), monobasic sodium phosphate (0.0005 M to 0.10 M), and sodium fluoride (0.0005 M to 0.10 M) were found to have no significant effect on the amylolytic activity of α-, β-, and gluco-amylases. Dimethyl sulfoxide at 5.0 M and 10.0 M concentrations was found to have a significant inhibitory effect on the activity of α-, β-, and gluco-amylases. The activities were unchanged at dimethyl sulfoxide concentrations of 1.0 M or less. As the concentration of dimethyl sulfoxide increased, enzyme activities were decreased. Dimethyl sulfoxide (10 M) completely suppressed the activity of β-amylase.