Human Pancreatic α-Amylase
II. Effects of pH, Substrate and Ions
on the Activity of the Enzyme

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ABSTRACT

Purified human pancreatic α-amylase (α-1,4-glucan 4-glucano-hydrolase, EC 3.2.1.1) was found to be stable over a wide range of pH values (5.0 to 10.5) with an optimal pH for the enzymatic activity of 7.0. The Michaelis constant of the enzyme at optimal pH and assay conditions was found to be 2.51 mg per ml for soluble starch. Halide ions were required for the activity of the enzyme whereas sulfate and nitrate were not. The order of effectiveness of activation was found to be: Cl->Br->I->F-. Calcium and magnesium were activators at concentrations of 0.001M and 0.005M, respectively, but exhibited inhibitory effects at concentrations higher than 0.005M. At 0.01M ethylenediamine tetraacetic acid (EDTA) concentration the enzymatic activity upon seven min incubation, was inhibited up to 96 percent. The inhibition of EDTA and calcium could be reversed upon addition of calcium and EDTA, respectively.

Introduction

Human pancreatic α-amylase (HPA)* has been obtained in a highly purified form in this laboratory by using ammonium sulfate fractionation, Sephadex G-100 and DEAE-Sephadex A-50 column chromatography.12 Its hydrodynamic properties and amino acid composition have been determined and compared with amylases from various other sources.12 Some physical and chemical properties of this enzyme were also reported by other investigators.1,4,11 This subsequent paper reports some studies of the effects of pH, starch substrate, and various ions on the activity of the enzyme.

Materials

HPA was purified using the procedure as previously described.12 Soluble starch was the product of Merck and Co., Inc., N. J. Maltose and other chemicals were reagent grade.

Methods

AMYLASE ASSAY

Amylase activity was determined by saccharifying method described by Bernfeld,3 in which the reducing groups...
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liberated from starch were measured by the reduction of 3,5-dinitrosalicylic acid. A unit of HPA activity was that which liberated one micromole of reducing group, calculated as maltose per minute at 20°. The enzymatic specific activity was expressed as units per milligram of protein.

PROTEIN ASSAY

Protein was determined by the method of Lowry et al10 using crystalline bovine serum albumin as a standard. The following buffers were used throughout the studies to determine the effects of pH on the activity of HPA: HCl-KCl (pH 2.0), glycine-HCl (pH 2.5–3.5), acetate (pH 4.0–5.5), phosphate (pH 6.0–8.0), Tris-HCl (pH 8.5–9.0), and glycine-NaOH (pH 9.5–11.0). All the buffers were 0.02M and contained 0.01M NaCl. Unless otherwise noted, the substrate used was 0.5 ml of 2 percent soluble starch solution in water.

The purified enzyme used in the studies of the effects of ions on the enzyme activity was dialyzed against one liter of 0.1M EDTA for seven days at 4° and subsequently dialyzed against three liters of deionized water at 4° for three days. The enzyme solution was diluted with deionized water to an approximate concentration of 0.9 μg of protein per 0.05 ml per assay.

Results

EFFECTS OF PH

1. On the activity of HPA: The effect of pH on the enzymatic activity was determined in the various buffers ranging in pH from 2 to 11. The results of the experiments are shown in figure 1. The enzyme is active in the reaction mixtures ranging from pH 4.5 to 10. The optimal pH of the enzyme under these assay conditions is 7.0.

2. On the stability of HPA: The experiments were carried out in the buffers ranging in pH from 2 to 11. The enzymatic activity was measured at pH 7.0 at 20°. All the activities were compared to the amylase activity at pH 7.0 under standard assay conditions. The results are shown in figure 2. The enzyme is stable over a wide range of pH values. Over a period of 30 min, the enzyme is stable from pH 5.0 to 10.5. Even after 45 hr, the enzyme is still fully active from pH 7.0 to 10.5. However, at pH 5.0 and 11.0, the activity decreases rapidly in the case of 1.5, 24, and 45 hr incubation. When the enzyme is incubated for only 30 min in the buffers, it still retains from 40 to 60 percent of its activity at pH 3.0 and 11.0, respectively.

The Km value of the enzyme was determined in 0.02M sodium phosphate buffer containing 0.01M NaCl, pH 7.0 and under standard assay conditions. The relationship between substrate concentration and reaction velocity of the enzyme is shown in figure 3. A Lineweaver-Burk plot of the enzymatic activity is also shown in figure 4. The slope of the line as calculated by the method of least squares is 0.1424. This resulted in a calculated value of Km for a soluble starch substrate of 2.51 mg per ml.

EFFECTS OF IONS ON THE ACTIVITY OF HPA

1. Anions: Six anions (chloride, bromide, iodide, fluoride, sulfate and nitrate, as sodium salts) were used in this study. Substrate was reacted with the purified enzyme preparation in 0.02M sodium phosphate buffer, pH 7.0. Sodium phosphate buffer was used in the reaction mixtures because sodium and phosphate ions do not influence the enzymatic activity of the amylase. Chloride in the reaction mixtures was the most effective activator for the amylase reaction, whereas fluoride was the least. Sulfate and nitrate had no
activating effect on the enzyme. In the absence of halide ions, no activity could be detected. The activities of the enzyme, plotted against concentrations of various active anions, are given in figure 5. A concentration of 10 mM of chloride is sufficient to give maximal activity. The order of effectiveness is $\text{Cl}^- > \text{Br}^- > \text{I}^- > \text{F}^-$. 

2. Cations: Only calcium nitrate and magnesium nitrate were investigated in detail. Other cations, such as manganese, copper, cobalt, zinc, and barium ions, were found to react directly with 3,5-dinitrosalicylic acid (color reagent) used in the assay system to form a precipitate or color which interfered with the colorimetric determination. The effect of calcium and magnesium on the amylase activity is shown in figure 6.

At a cation concentration of 0.001M, enzymatic activity was slightly increased in the case of calcium, while it was nearly equal to the control in the case of magnesium. Calcium and magnesium exhibited an inhibitory effect on amylase activity at concentrations higher than 0.005M. The enzymatic activity was completely inhibited at 0.20M of magnesium and 0.50M of calcium.

The inhibitory effect of calcium on the amylase activity, as a function of time, was also studied and the data presented in figure 7. At a calcium concentration of 0.02M, enzymatic activity was diminished by approximately 40 percent within one min after the initiation of incubation. This level of inhibition was observed over a period of 19 min of incubation. Addition of 0.01M EDTA resulted in an immediate restoration of the activity of the enzyme, demonstrating a reversibility of the inactivation of $\alpha$-amylase by calcium. However, restoration of only 90 percent of the total enzymatic activity was observed.

3. EDTA: The effect of EDTA on the amylase activity was carried out in 0.05M Tris-HCl-0.01M NaCl buffer, pH 7.5, and in the absence of divalent cations. In this series of experiments, the concentration of EDTA was varied from 0.001M to 0.080M. The effect of EDTA on the enzymatic activity is shown in figure 8. EDTA exhibited inhibitory effect on the amylase activity. The inhibition increased as the concentration of EDTA was increased. The enzymatic activity was com-

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**Figure 1.** Effect of pH on the activity of purified HPA. The experiments were carried out in buffers with pH ranging from 2.0 to 11.0. Enzyme activity was determined by standard assay procedure. The amount of enzyme used was 0.8 $\mu$g of protein assay.

**Figure 2.** Effect of pH on the stability of purified HPA. The enzyme, 0.9 $\mu$g per assay, was incubated with buffers, pH ranging from 3.0 to 11.0 at 37° for the following periods of time: (a) 0.5 hr; (b) 1.5 hr; (c) 24 hr; and (d) 45 hr. Enzymatic activity was measured in 0.05M phosphate—0.01 M NaCl buffer, pH 7.0, by standard assay procedure. All the activities determined are compared to the amylase activity at pH 7.0.
pletely abolished when the concentration of EDTA reached 0.080M.

The inhibitory effect of EDTA (0.01M) on the amylase activity as a function of time was also studied. When the enzyme was incubated with 0.01M EDTA, about 60 percent of amylase activity was lost within the first min of incubation, as shown in figure 9. After seven min of incubation, only 4 percent of the activity remained. This level of activity was observed over a period of 23 min of incubation. After 23 min, 0.02M calcium nitrate was added to the incubation mixture. A 60 percent reactivation of the amylase activity was observed within 2 min and about 80 percent of enzymic activity was recovered after 12 min.

Discussion

Bernfeld et al found that human pancreatic α-amylase was active over a range of pH 4.0 to 9.5 and had an optimal activity pH of 6.9. The enzyme was stable from pH 4 to 11 for 30 hr at 20°. The results of the present work are similar to these findings except that the enzyme was stable over a range of pH 7.0 to 10.5 when incubated at 37° for 45 hr, and its optimal pH was 7.0.

The Michaelis constant of HPA for soluble starch is 2.51 mg per ml. No Km value of this enzyme has been reported. The Km values of hog pancreatic α-amylase were found to be 0.18 mg per ml for amylase at 20° and 2.29 × 10^3 moles of glucosidic bond/liter for waxy maize starch at 40°. Halide ions were found to be necessary for the catalytic reaction of the HPA obtained in this laboratory. The order of activation by halide ions on the activity of α-amylases from human pancreas and saliva was similar. Chloride ions were also required for the activity of hog pancreatic and Bacillus subtilis α-amylases. Barley malt α-amylase was found to require calcium ions for its activity while β-amylases from barley malt and sweet potato required neither chloride nor calcium for
their activities. In the case of hog pancreatic α-amylase, calcium cannot replace chloride ions in the activation of the enzyme.

It had been shown that calcium-free, bacterial, hog pancreatic and human salivary α-amylases were essentially inactive and that the uptake of a single atom of calcium per molecule of protein restored full enzymatic activity. Addition of calcium to bacterial and fungal α-amylases reduced the rate of denaturation by heat, acid, and urea. Calcium also protected the amylase molecule from proteolytic digestion. It was proposed that calcium performed the functions of maintaining the protein in the proper configuration for biological activity and of stabilizing the secondary and tertiary structure of the enzyme.

It has been suggested that α-amylases contain a small number of sites to which calcium is firmly bound specifically with apparent association constants of $10^{12}$ to $10^{15}$. These high association constants enable the enzymes to collect traces of calcium from the surrounding environments such as buffers, glasswares, and chemicals especially starch substrates which commonly contain about 0.01 to 0.1 percent of calcium. These amounts of calcium in the starch are at least a thousand-fold in molar excess of the amounts of enzyme necessary to assay the system. This phenome-

**Figure 5.** Effect of anions on the activity of purified HPA. Sodium salts of these anions were used since sodium ions had no effect on the enzymatic activity. The reactions were carried out in 0.02 M sodium phosphate buffer, pH 7.0. Enzyme concentration of 0.9 μg of protein per assay was used and its activity was measured by standard assay procedure. One reaction mixture containing no anion, except phosphate ion, was used as control. Phosphate ions had no effect on the activity of the enzyme.

**Figure 6.** Effect of cations on the activity of purified HPA. Calcium nitrate and magnesium nitrate were used in this experiment. The enzyme used was dialyzed against 0.1 M EDTA at 4°C for seven days, and then dialyzed against deionized water at 4°C for three days. Appropriate concentration of enzyme (0.9 μg of protein per assay) was used in all reactions. Enzymatic activity was measured in 0.05 M Tris-HCl-0.01 M NaCl buffer, pH 7.5, by the standard assay procedure. One reaction mixture containing no cation was used as control.
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Figure 7. Reversible inactivation of purified HPA by calcium. Concentration of calcium: 18 μg of protein per ml, concentration of calcium nitrate: 0.02 M in 0.05 M Tris-HCl-0.01 M NaCl buffer, pH 7.5, 37°. Reactivation was obtained upon addition of 0.01 M EDTA to partially inactivated amylase solution. Standard assay procedure was used to determine the enzymatic activity.

Non also occurred in the present work in the HPA system because of the minute amounts of the enzyme (0.9 μg per assay) used in the system compared to 10 mg of starch used per assay. The EDTA-treated enzyme is reactivated by traces of calcium in the starch substrate during the incubation and gave full activity. This explains why the reaction mixture containing "no" calcium (control) still showed amylase activity and only slightly increased activity by the addition of calcium.

The activating effect of calcium on the HPA activity may be observed at a calcium concentration of 0.001M (figure 6). This concentration of calcium was sufficient to maintain and to stabilize the conformation of the amylase used in the assayed system, such that a maximal activity is obtained. Calcium concentrations of 0.005M or higher inhibit the amylase activity. This inhibition may be due to the excessive non-specific binding of calcium to the enzyme. If this were the case, the enzymatic activity should be restored when the calcium ions were removed from the enzyme molecules. Incubation of EDTA with the calcium-treated amylase does reverse the inactivation of the enzyme by calcium as shown in figure 7. The mechanism of reactions of these phenomena may be shown as follows:

\[
[\text{EMe}] + \text{Me} \rightarrow [\text{EMe}]\text{Me} \quad (1)
\]

active \hspace{1cm} inactive

\[
[\text{EMe}]\text{Me} + n\text{I} \rightarrow [\text{EMe}] + \text{MeI}_n \quad (2)
\]

Where [EMe] is the enzyme with endogeneous calcium, Me the calcium, I the EDTA, and n the number of moles of EDTA. The complex [EMe]Me is inactive because of the excessive non-specific binding calcium to the enzyme molecules.

The activity of purified HPA is also inhibited by EDTA as shown in figure 8. Chelating agents such as EDTA, or 1,10-phenanthroline have been postulated to reduce catalytic activity by forming coordination complexes with the constituent metal through the following possible mechanism:

\[
[\text{EMe}] + n\text{I} \rightarrow [\text{EMe}]\text{I}_n \quad (3)
\]

active \hspace{1cm} inactive

\[
[\text{EMe}] + n\text{I} \rightarrow \text{E} + \text{MeI}_n \quad (4)
\]

active \hspace{1cm} inactive

The complex [EMe]I_n is inactive since the enzymatically essential metal atom is blocked. The enzyme in equation 4 is inactivated by the removal of the metal. Once [EMe]I_n complex has formed, an
excess of metal ions should reverse the inhibition by competing with the enzyme for the chelating agent bound to its metal as shown in equation 5

\[ [\text{EMe}]_n + \text{Me} \rightarrow [\text{EMe}] + \text{MeI}_n \] (5)

[\text{EMe}]_n is reactivated by its dissociation owing to the addition of metal ions. In equation 4, the reaction may or may not be reversible, depending on whether the metal and protein can reassociate to restore the native state. EDTA used in the present investigation may have inhibited the enzyme reaction by either forming [\text{EMe}]EDTA complex (equation 3) or by removing metal from the enzyme molecule as shown in equation 4.

When the enzyme was dialyzed against EDTA, which was subsequently removed by dialyzing against deionized water, the enzyme should have been practically free from any metal. The ability of the HPA to concentrate calcium from surrounding media and restore the enzyme activity indicated that the inhibition of the enzyme by EDTA followed equation 4.

The inhibition of purified HPA activity by EDTA was reversed upon addition of excess calcium ions as shown in figure 9. These reactions indicate that [\text{EMe}]EDTA was formed (equation 3) and that recovery of the enzymatic activity followed equation 5.

References