Journal of Solid-Phase Biochemistry, Vol. 1, No. 3, 1976

PROPERTIES OF HUMAN PAROTID AMYLASE IMMOBILIZED BY COVALENT BINDING TO GLASS OR SEPHAROSE OR BY REACTION WITH IMMOBILIZED ANTIBODY[1]

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Accepted August 17, 1976

Human parotid amylase was immobilized by covalent binding to CNBr-activated Sepharose, to Corning GAO-3940 silica glass biomaterial support by the diazonium reaction or reaction with glutaraldehyde, or as a result of the antigen–antibody reaction between rabbit anti-human parotid amylase IgG that was covalently bonded to GAO glass and soluble amylase. The amylase directly bonded to the supports showed constant activity at flow rates of 3–15 ml/min through a 1.76-cm^3 (8-mm diameter) support bed, did not lose enzyme into a circulating starch solution, retained its activity in the presence of soluble antiamylase IgG, was optimally active at 35°–40°C, and lost activity at 40°–45°C. When the enzyme was bound by interaction with immobilized antibody, full activity was expressed, but some enzyme was solubilized by a circulating starch solution. Immobilization of either amylase or antiamylase IgG makes dissolution of the antigen–antibody bond difficult.

INTRODUCTION

Proteins, including enzymes and antibodies, have been immobilized by a variety of methods (1, 2). Among these are adsorption on glass and covalent attachment to various substrates. Immobilized antigens and antibodies have been used for the isolation of antibodies and antigens, respectively, and immobilized enzymes have been used as models for membrane-bound enzymes and for a variety of experimental and preparative purposes. In this paper we report the tenacious retention, in the face of procedures designed to break the antigen–antibody bond, of the enzymatic activity of human parotid amylase reacted with antiamylase IgG that is covalently bound to Sepharose or glass beads and of amylase covalently bound to the supports

[1] This is publication No. 75-26 of the Department of Medical Genetics, Indiana University School of Medicine, and was supported in part by the Indiana University Human Genetics Center PHS GM 21054 and research grant PHS GM 19178. A preliminary report of this work was presented to the First Chemical Congress of the North American Continent, Mexico City, Mexico, November 30–December 5, 1975.

and then reacted with the antiamylase antibodies. The activity of these support-enzyme:antibody and support-antibody:enzyme columns is compared with that of support-enzyme columns, and attempts are described to use the latter as an affinity column for purification of antiamylase IgG.

MATERIALS

Purified human parotid amylase was obtained from Robert C. Karn of this department. The rabbit antihuman parotid amylase IgG columns have been described before (3). The silica glass was a gift from H. Weetall or was purchased from Corning. GAO-3940 silica glass biomaterial support, which has a nominal pore diameter of 55 nm, is 96% silica glass with reactive primary amine groups. The particle size range is 177–840 μm. Sepharose 6B was purchased from Pharmacia. Columns were prepared in 2-ml glass syringes (Becton, Dickinson Co.) of 8-mm diameter, bed height 35 mm.

METHODS

GAO glass was activated by the diazo method or with glutaraldehyde, (3) or as described in the Corning Biological Products Group pamphlet "Biomaterial Supports." Sepharose 6B was activated with cyanogen bromide according to Porath et al. (4). Amylase activity was determined by the method of Bernfeld (5). To determine the hydrolytic activity of the immobilized amylase, a starch solution of the appropriate concentration and temperature was pumped through the thermostated column of immobilized enzyme at the desired rate. Fractions were then collected after equilibration of the column with the starch. From five to eleven fractions were assayed and the amylase activity calculated as follows: Units = mg maltose/ml × 3 × flow rate (ml/min) × 1/dilution of starch × 1/void volume (ml).

RESULTS

Activity and Stability of Amylase Immobilized Directly on Glass or Agarose

The activity and stability were determined using amylase bound to GAO glass by the diazo reaction, as a Schiff base, or bound to CNBr-activated Sepharose. In each case preparations exhibiting amylase activity were obtained. The calculated activity of the columns was always less than that of the enzyme apparently bound to the column. The fraction of activity applied that was expressed varied from 16–29% for glass to 0.2% for the Sepharose column.

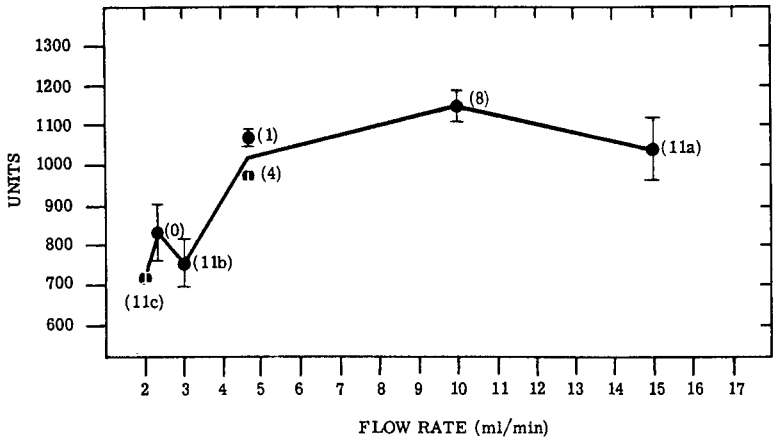

FIG. 1. Effect of flow rate on the activity of GAO-glutaraldehyde-amylase. Glass weight, 0.5 g; column diameter, 8 mm, height, 35 mm; void volume, 0.25 ml; amylase bound, 4,570 units; temperature, 30°C. Starch (20%) was pumped through the column and activity determined from the maltose equivalents produced. Each point represents the mean of six determinations. The vertical lines represent ± 1 *SD*. The figures in parentheses represent the days after the first experiment (2.3 ml/min) was performed.

The variation of activity with flow rate for amylase linked to GAO glass by glutaraldehyde is shown in Fig. 1. The activity is constant for flow rates above 3–4.7 ml/min. There is no indication that the age of the column, at least up to 11 days, affected the results. In other experiments a column of GAO-glutaraldehyde-amylase[2] glass and one of GAO-diazo-amylase glass showed some loss of activity after 13 days and 1 day, respectively. The data on stability are shown in Fig. 2. Inspection of the data obtained by analyzing the starch digests immediately after collection (clear bars) shows some variation in activity, which may be up or down. However, when the starch eluted from the column was permitted to incubate for 4.5–24 h before assay for maltose (shaded areas), there was almost always some increase in apparent activity. This did not occur when the starch was pumped through a GAO-diazo-lysine glass column, indicating that the starch solution was

[2] Abbreviations used are: GAO-glutaraldehyde-amylase and GAO-diazoamylase, amylase immobilized on GAO glass biomaterial supports by reaction with glutaraldehyde or the diazonium reaction, respectively; GAO-diazo IgG:amylase, amylase bound to IgG that was immobilized on GAO glass by the diazonium reaction. GAO-diazo-lysine, lysine immobilized on GAO glass by the diazonium reaction; Sepharose-amylase, amylase immobilized on CNBr-activated Sepharose; Sepharose-amylase:IgG, IgG bound to amylase that was immobilized on CNBr-activated Sepharose.

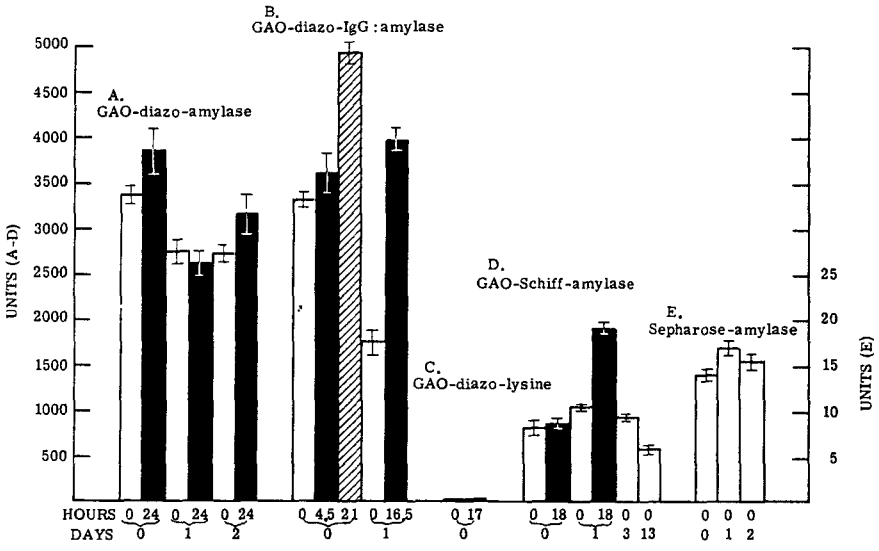

FIG. 2. Stability of immobilized ligands. Activity of the columns against starch was determined on day 0 and from 1 to 13 days later. The data are grouped by preparation and day of determination. Clear bars, activity determined on freshly collected eluate on day indicated; shaded bars, apparent activity determined on eluate held at 30°C for a number of hours indicated (increase due to amylase eluted from the column); vertical lines, $\pm 1SD$.

stable for 24 h and that some activity was eluted from the substrate-amylase columns.

Effect of Temperature on Activity

The temperature effect was determined on the column used for the flow-rate experiments. The bulk of the experiments consumed 5 days and an additional experiment at 30°C was performed on day 26. The activity increased slightly (19.8%) from 30° to 40°C and fell abruptly (36.8%) between 40° and 45°C, then remained constant to 50°C. The column apparently suffered some irreversible change as the lower level of activity reached at 50°C was retained even when the enzyme was reassayed at 30°C. Three weeks after the initial experiment, the activity of the column had fallen from the initial value of 636 ± 38 units to 462 ± 21 units after the run at 50°C to a final 175 ± 6.7 units. This drop (72.5%) is considerably more than that (about 24.5%, from Fig. 1) occasioned by the change in flow rate from about 5 ml/min of the initial experiments to the 2 ml/min of the last experiment.

Effect of Antiamylase IgG on Amylase Activity

In the previous paper (3) we described how difficult it is to elute amylase from an antiamylase IgG affinity column and stated that a GAO-diazo-IgG : amylase glass column retains enzymatic activity. Prior to attempts to quantitate the activity of such a column, equal portions of a single batch of GAO glass were covalently linked to antihuman parotid amylase IgG, human parotid amylase, or lysine by the diazo reaction. Amylase (1,080 units) was circulated through the IgG column overnight before circulation of the starch and assay. The activity and stability of these columns are shown in Fig. 2A through C. The GAO-diazo-IgG : amylase glass column was remarkably stable, although starch eluted more amylase from it than from the GAO-diazo-amylase glass column.

The effect of IgG on immobilized amylase was tested with columns of glutaraldehyde-amylase glass and Sepharose-amylase. The results obtained with these columns are summarized in Table 1. The activity of the Sepharose-amylase column was assayed in the usual manner. Following the assay IgG obtained from a rabbit that had been immunized against a virus (control IgG) was passed through the column. No change in amylase activity was noted. Antiamylase IgG was circulated through the column and no

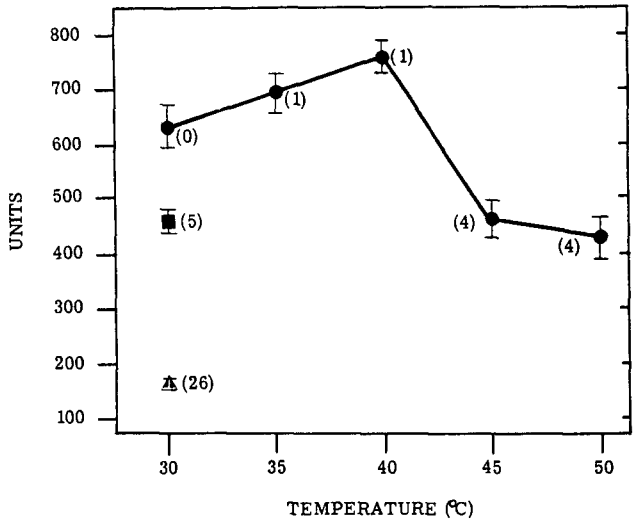

FIG. 3. Effect of temperature on the activity of GAO-glutaraldehyde-amylase glass. For technique and column description, see legend to Fig. 1. Flow rate, 5 ml/min; starch concentration, 20%. Day 0 (figure in parentheses) corresponds to day 11 of Fig. 1.

TABLE 1. **The Effect of Antiamylase IgG on Amylase Immobilized on Sepharose**

Treatment	Activity (units)
None	14.1
Control IgG (16 h)	15.6
Antiamylase IgG (16 h)	16.5
Elution of IgG	17.3
Antiamylase IgG (16 h)	15.2

attempt made to elute the antibody. This specific IgG also had no inhibitory effect on the column. The experiment with antiamylase IgG was repeated three times.

After each application of IgG the column was washed with 0.04 M phosphate buffer, pH 8.0, and eluted with 0.1 M acetic acid. The eluate was assayed for antiamylase IgG by double immunodiffusion against amylase. Only the eluate from the first experiment was positive for the specific IgG. For the GAO glass experiments, equal amounts of the immobilized enzyme were placed in two columns and 20% starch pumped through at 5.5 ml/min. Antiamylase IgG was circulated through one column overnight. The columns were rinsed with buffer, and then assayed for amylase activity 5 days later. Both columns had identical activities (880 units). Treatment with 0.1 M acetic acid failed to elute immunologically active IgG from the column treated with antibody. Both columns lost activity with time at approximately the same rate.

Acid Dissociation of Amylase:Antiamylase

Because it was conceivable that the amylase:antiamylase bond is unusually stable even when the reaction takes place between both components in solution, an attempt was made to dissociate precipitated amylase from its antibody. Amylase was precipitated from solution with antibody. The precipitate was then removed by centrifugation, washed in borate-buffered saline (6), and incubated for 3 h in 0.05 M glycine-HCl buffer, pH 2.7, before application to a 1.5- × 100-cm column of Sephadex G-200. The column was eluted with the same buffer at a flow rate of 0.12 ml/min. Six 2-ml fractions were collected, followed by eighty-six 1-ml fractions. The A_{280} and amylase activities were determined on representative fractions. The material absorbing at 280 nm eluted in two peaks. The first peak approximated the void volume and contained amylase activity but relatively

little of the material absorbing at 280 nm, whereas the second peak contained the bulk of the protein but no amylase activity.

DISCUSSION

The data presented above show that human parotid amylase can be immobilized on glass supports or Sepharose and still retain its enzymatic activity. The enzyme may be immobilized by direct covalent linkage to the solid support but perhaps more interestingly by means of the reactions between the enzyme in solution acting as an antigen and the antienzyme IgG bound covalently to the solid support. In the latter case, a remarkably stable bond is formed and the enzyme is removed with difficulty even by 0.1 M acetic acid (3). The bond is not as stable as that formed covalently between the solid support and the enzyme and it can be severed, at least in part, by starch, which is the substrate for the enzyme. Epton and Thomas (7) reported the partial dissociation of immobilized α-amylase by starch. The preparation of active, immobilized amylases of other than human origin has been reported before. Flemming et al. (8) immobilized gluco-amylase of unknown origin on glass. Bernfeld and Wan (9) immobilized hog pancreas α-amylase by entrapping it in polyacrylamide. An active β-amylase preparation was similarly obtained. β-amylase was bound to CNBr-activated agarose by Schell et al. (10). Axen and Porath (11) prepared the isothiocyanate derivative of β-amylase and Sephadex G-25 and G-200, but neither of these preparations was active.

The properties of enzymes are sometimes altered by immobilization. The action pattern of *B. subtilis* α-amylase was altered by immobilization on cellulose derivatives (12), and the heat stability of the enzyme (13) was increased by immobilization in acrylamide. It was not the purpose of this study to characterize the immobilized amylase extensively, but it would appear that the resistance of the rather heat-labile parotid amylase (14) is not extensively changed by immobilization.

It is of interest that the antigen–antibody bond between immobilized amylase and its antibody is so strong. Immobilized antigens or antibodies have been used to prepare the corresponding antibody or antigen. In general the antigen–antibody bond has been readily broken by changes in pH. The unusual stability of the bond between antihuman amylase IgG immobilized on glass and amylase was first noted by Hodes and Glier (3). Because it was conceivable that this unusual stability was also a characteristic of soluble amylase and antiamylase, attempts were made to precipitate from solution the antigen–antibody complex and then to separate its components. This was rather readily done, although the recoveries of the components, which

were easily solubilized by buffer at pH 2.8, indicated that both the antigen and antibody were denatured.

The results of the reverse experiment, in which a column of solid amylase was used to chromatograph antiamylase IgG, were not entirely satisfying. Circulation of antiamylase IgG through the column of immobilized amylase did not result in inhibition of enzymatic activity. There are two possible explanations: (1) amylase–antiamylase is enzymatically active or (2) antiamylase IgG does not bind to immobilized amylase. In favor of the former explanation are the experiments cited above with immobilized antibody and the demonstration of activity against starch in situ following immunoelectrophoretic separation or immunodiffusion of amylase. The fact that very little of the circulated IgG was retained by the amylase columns (data not shown) argues for the second explanation. However, tenacious binding of purified antibody to viral hepatitis virus on columns of Sepharose covalently linked to viral hepatitis antigens was reported by Cullen (15) and remarked on by others in discussion of that paper.

It is interesting to note the difference in activity between the amylase insolubilized on Sepharose and that bound to glass. No spacer was used with the Sepharose, whereas in the case of the glass supports the *p*-aminophenyl group was used to extend the propylsiloxy side chain of the glass. The failure of Axen and Porath (11) to obtain an active derivative of β-amylase and Sephadex may have been due to the inability of the substrate, soluble starch, to approach the enzyme bound by a short tether.

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