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A PURIFICATION OF *Helix pomatia* ALPHA AMYLASE INVOLVING A NOVEL AFFINITY-BINDING PROCEDURE*

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ABSTRACT

Shellfish glycogen was cross-linked by treatment with cyanogen bromide followed by 1,6-diaminohexane. The resulting, insoluble product efficiently adsorbed *Helix pomatia* alpha amylase [(1 → 4)-α-D-glucan glucanohydrolase] from crude solutions of the enzyme at 0°, but only poorly at higher temperatures. A method was developed for the purification of *Helix pomatia* alpha amylase involving formation of an enzyme–adsorbent complex in the cold and recovery of the alpha amylase by suspending the washed complex in buffer at 37°. After chromatography of the desorbed alpha amylase on a column of Bio-Gel P-60, the enzyme was homogenous as judged by poly(acrylamide)-gel electrophoresis. An overall purification of 360-fold was achieved with a recovery of 35%.

INTRODUCTION

Alpha amylases may be adsorbed from solution onto insoluble starch granules[1–3]; this procedure has frequently been used for removal of alpha amylases from solutions of other amylolytic enzymes[4,5]. Affinity binding to starch granules has also been used for purification of alpha amylases, but the number of successful applications of this approach is rather small, possibly because of the difficulty of recovering the bound enzyme from the granules. Alpha amylases have hitherto been purified mainly by conventional protein-purification procedures[6,7].

We now describe the preparation of an insoluble, cross-linked glycogen that may be more useful than starch granules as an adsorbent for alpha amylases. The use of this material for purification of an alpha amylase from the juice of the snail, *Helix pomatia*, is described. Binding of alpha amylase to the adsorbent occurs readily

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*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

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in the cold; desorption of the enzyme is effected by suspending the amylase–adsorbent complex in a buffer at higher temperature (37°).

MATERIALS AND METHODS

Materials. — Oyster glycogen (type II, Sigma Chemical Company, St. Louis, MO) was dissolved in water before use and purified by precipitation of contaminating protein by addition of trichloroacetic acid (10%), followed by exhaustive dialysis of the supernatant solution and two precipitations of the polysaccharide by ethanol. Solutions of soluble starch (J. T. Baker Chemical Company, Phillipsburg, NJ) were dialyzed before use, against distilled water and centrifuged. Insoluble laminaran from *Laminaria digitata* was a gift from Dr. Eric T. Dewar; solutions were prepared by dissolving the material in water and the polysaccharide remained in solution during the course of enzymic digestion at 37°. Maltose was "extra pure" grade (British Drug Houses, Poole, Dorset, England).

Other materials used were D-glucose oxidase (type II, Sigma Chemical Company), horseradish peroxidase (grade II, Boehringer Mannheim Biochemicals, Indianapolis, IN), human serum albumin (Lister Institute, Elstree, Herts., England), Glusulase, a crude mixture of snail digestive-enzymes (Endo Laboratories, Garden City, NY), *o*-dianisidine dihydrochloride (Sigma), cyanogen bromide (Eastman Organic Chemicals, Rochester, NY), 1,6-diaminohexane (Aldrich Chemical Company, Milwaukee, WI), and Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, CA). Other chemicals were of reagent grade.

Analytical methods. — Protein concentrations were determined as by Lowry *et al.*[8], using bovine serum albumin as standard. In column fractions, protein was detected qualitatively by measurement of u.v. absorbance at 280 nm. Reducing sugars produced by enzyme action were determined by reduction of an alkaline copper reagent[9]. D-Glucose was determined specifically by using D-glucose oxidase[10].

Enzyme assays. — Alpha amylase and (1 → 3)-β-D-glucanase activities were determined from the rate of release of reducing sugars in digests (1.0 ml) containing substrate (soluble starch or laminaran, 5.0 mg), sodium acetate buffer (50mM, pH 5.0), calcium chloride (5mM), and a suitable amount of enzyme solution, incubated at 37°. Samples (200 μl) were removed from the enzyme digests at intervals for determination of reducing sugars. α-D-Glucosidase activities were determined by the rate of production of D-glucose in digests (0.5 ml) containing maltose (1.25 mg), sodium acetate buffer (50 mM, pH 5.0), calcium chloride (5mM), and a suitable amount of enzyme, incubated at 37°. Liberated D-glucose was determined after incubation for a suitable length of time, by using D-glucose oxidase reagent. One Unit (U) of enzyme activity is the amount that hydrolyses 1 μmol of glucosidic linkages per min under the specified conditions. Specific activities are the number of Units of activity per mg of protein.

Gel filtration. — Gel-filtration chromatography was performed at 2° on a column (1.5 × 85 cm) of Bio-Gel P-60 (100–200 mesh), eluted with acetate buffer

(50mM, pH 5.0) containing sodium chloride (1%). Fractions (5.0 ml) were collected automatically. Before use, the gel-filtration medium was prepared according to the manufacturer's recommendations[11].

Electrophoresis. — Electrophoresis of protein samples was performed in 7.5% poly(acrylamide) gels at pH 8.3 according to the method of Ornstein and Davis[12,13].

RESULTS

Synthesis of cross-linked glycogen adsorbent. — Glycogen (25 g) was dissolved in sodium carbonate solution (0.1M, pH 11.3, 1 liter). Solid cyanogen bromide (6.5 g) was added with vigorous stirring and the pH was maintained at 11.0 by addition of M sodium hydroxide solution. After 5 min, by which time the solution had become turbid, the pH was adjusted to 9.0 by addition of M hydrochloric acid solution and then 1,6-diaminohexane (4.0 g) added. The pH was then readjusted to 9.0 and stirring continued for 1 h. The precipitate that formed was collected by centrifugation (16,000 *g*, 10 min, 0°), washed by suspension in water (1.5 liters) and stirring for 1 h, and then recentrifuged. This washing procedure was repeated two more times. The product was then dried by washing on a Büchner funnel with abs. ethanol, and then with ether, followed by heating in a vacuum oven for 12 h at 30°. Prior to use, the cross-linked glycogen was made into the form of a fine suspension in water by stirring overnight, or by homogenization for 20 sec at low speed in a Potter–Elvejhem homogenizer.

Interaction of Helix pomatia *alpha amylase with cross-linked glycogen; binding characteristics and desorption conditions.* — The selective binding of *Helix pomatia* alpha amylase by cross-linked glycogen was demonstrated by addition of various amounts of the adsorbent (in the range 0–3.33 mg) to mixtures (1.8 ml) containing Glusulase (0.15 ml containing 22 Units of alpha amylase activity) in acetate buffer (37.5mM, pH 5.0) containing calcium chloride (7.5mM). After keeping for 15 min at 0° with occasional shaking, the mixtures were centrifuged (30,000*g*, 15 min, 0°) and the clear supernatant solutions assayed for activity towards starch, maltose, and laminaran. The results (Table I) showed that, of the activities measured, only alpha amylase interacted with the adsorbent; neither (1 → 3)-β-D-glucanase nor α-D-glucosidase were bound to a significant extent. The extent of binding of alpha amylase increased with the amount of adsorbent added. Under the conditions tested, almost complete (96%) binding of the enzyme was achieved with cross-linked glycogen in the amount of 3.33 mg/ml.

The effect of temperature on the binding of *Helix pomatia* alpha amylase to cross-linked glycogen was investigated using mixtures similar to the foregoing, containing 12.3 Units of alpha amylase activity/ml and 1.56 mg adsorbent/ml, incubated for 15 min at 0, 20, and 37°. The results, shown in Table II, demonstrate that a greater extent of binding of the enzyme to the adsorbent takes place at 0° than at higher temperatures.

Experiments were then performed to test the feasibility of isolating *Helix*

TABLE I

BINDING OF *Helix pomatia* ALPHA AMYLASE TO CROSS-LINKED GLYCOGEN AT 0°[a]

Cross-linked glycogen added (mg/ml)	*Alpha amylase activity in supernatant solution (U/ml)*	*Alpha amylase activity bound (%)*	*(1 → 3)-β-D-Glucanase activity in supernatant solution (U/ml)*	*α-D-Glucosidase activity in supernatant solution (U/ml)*
0	12.3	0	19.0	1.4
0.11	10.2	17	[b]	[b]
0.22	6.8	44	[b]	[b]
0.44	4.9	60	[b]	[b]
0.88	2.2	82	[b]	[b]
1.56	1.3	89	[b]	[b]
2.22	0.7	94	[b]	[b]
3.33	0.5	96	18.5	1.3

[a]Mixtures (1.8 ml) containing alpha amylase (12.3 U/ml) and the indicated amounts of cross-linked glycogen in acetate buffer (37.5mM, pH 5.0) containing calcium chloride (7.5mM) were kept for 15 min at 0°) with occasional shaking. After centrifugation, the amounts of alpha amylase activity remaining in the supernatant solutions were measured. [b]Not determined.

TABLE II

BINDING OF *Helix pomatia* ALPHA AMYLASE TO CROSS-LINKED GLYCOGEN AT DIFFERENT TEMPERATURES[a]

Temperature (°)	*Amount bound (%)*
0	89
20	29
37	6

[a]The starting amylase solution contained 12.3 U/ml of alpha amylase and was treated with 1.56 mg of cross-linked glycogen/ml as described in the text.

pomatia alpha amylase by binding the enzyme to cross-linked glycogen at 0° and recovering it by treatment of a suspension of the washed enzyme–adsorbent complex at a higher temperature (37°), and to determine the optimum conditions for this procedure. Mixtures (5–12 ml) were prepared containing Glusulase (1.0 ml, containing 110–150 Units of alpha amylase), sodium acetate buffer (40mM, pH 5.0), calcium chloride (8mM) and cross-linked glycogen (20–60 mg). After keeping for 20 min at 0° with occasional shaking, the mixtures were centrifuged (30,000g, 15 min, 0°) and the supernatant solutions removed. Each precipitate was then washed twice with 2–10 ml of acetate buffer (50mM, pH 5.0), containing calcium chloride (10mM) during 10 min at 0°. Desorption of the alpha amylase from the adsorbent was then effected by suspension of the washed precipitates in the same buffer (1–2.5 ml) and

incubation for 15 min at 37°. After centrifugation (30,000*g*, 15 min, 30°) the supernatant solutions were collected. In most cases, the desorption procedure was repeated and the two desorbed amylase solutions from each precipitate combined.

In all instances, 80–90% of the initial activity was bound to the cross-linked glycogen, this being somewhat less than expected on the basis of earlier results (Table I) but still an acceptable level. There was no evidence of desorption of alpha amylase from the adsorbent during the washing step, even when large amounts of buffer were used; indeed larger washes by buffer were preferable, as they served to remove more non-specifically bound protein, including (1 → 3)-β-D-glucanase. Treatment of the amylase–adsorbent complexes at 37° does indeed result in desorption of the amylase, although not as efficiently as might have been predicted from Table II. Thus, when alpha amylase (92 Units) adsorbed onto cross-linked glycogen (40 mg) was treated at 37° with 1.5 ml of buffer, 29.4 Units of activity were desorbed. A second treatment of the precipitate at 37° with 1.0 ml of buffer resulted in recovery of additional alpha amylase (10 Units). Binding of alpha amylase to cross-linked glycogen may therefore be inferred not to be a completely reversible process.

Use of quantities of adsorbent greatly in excess of that required for maximum binding resulted in lower specific activities and lower extents of purification of the desorbed alpha amylase than were obtained when smaller amounts of adsorbent were used. For example, the products recovered from 5.0-ml mixtures containing initially 150 Units of alpha amylase, by using 4, 8, and 12 mg of adsorbent/ml, had specific activities of 14.3, 10.9, and 8.4 Units/mg, corresponding to purification factors of 19.4-, 14.8-, and 11.4-fold, respectively, although the overall recoveries were not greatly different in all three instances. Use of excessive amounts of adsorbent clearly results in the contamination of the product with non-amylase protein that presumably interacts with the adsorbent by a mechanism other than biospecific adsorption; it is not removed during the washing step, but is desorbed during the recovery step.

Purification of Helix pomatia *alpha amylase.* — Based on the studies of the interaction of *Helix pomatia* alpha amylase with cross-linked glycogen and desorption of the amylase from the enzyme–adsorbent complex, the following procedure was chosen for isolation of the enzyme from the crude material by affinity binding. A mixture (12 ml) was prepared containing Glusulase (1.0 ml, containing 133 Units of alpha amylase) sodium acetate buffer (40mM, pH 5.0), calcium chloride (8mM), and cross-linked glycogen (40 mg). After keeping for 20 min at 0° with occasional shaking, the mixture was centrifuged (30,000*g*, 15 min, 0°) and the supernatant solution discarded. The precipitate was then washed twice; first with 10 ml, then with 6 ml of acetate buffer (50mM, pH 5.0), containing calcium chloride (10mM) for 10 min at 0°, and then desorption of the alpha amylase from the enzyme–adsorbent complex was effected by the suspension of the washed precipitate in the same buffer (2.0 ml) and incubation for 15 min at 37°. After centrifugation (30,000*g*, 15 min, 30°) the supernatant solution was collected and the desorption procedure repeated, this time with 1.0 ml of buffer. The two desorbed alpha amylase solutions were combined.

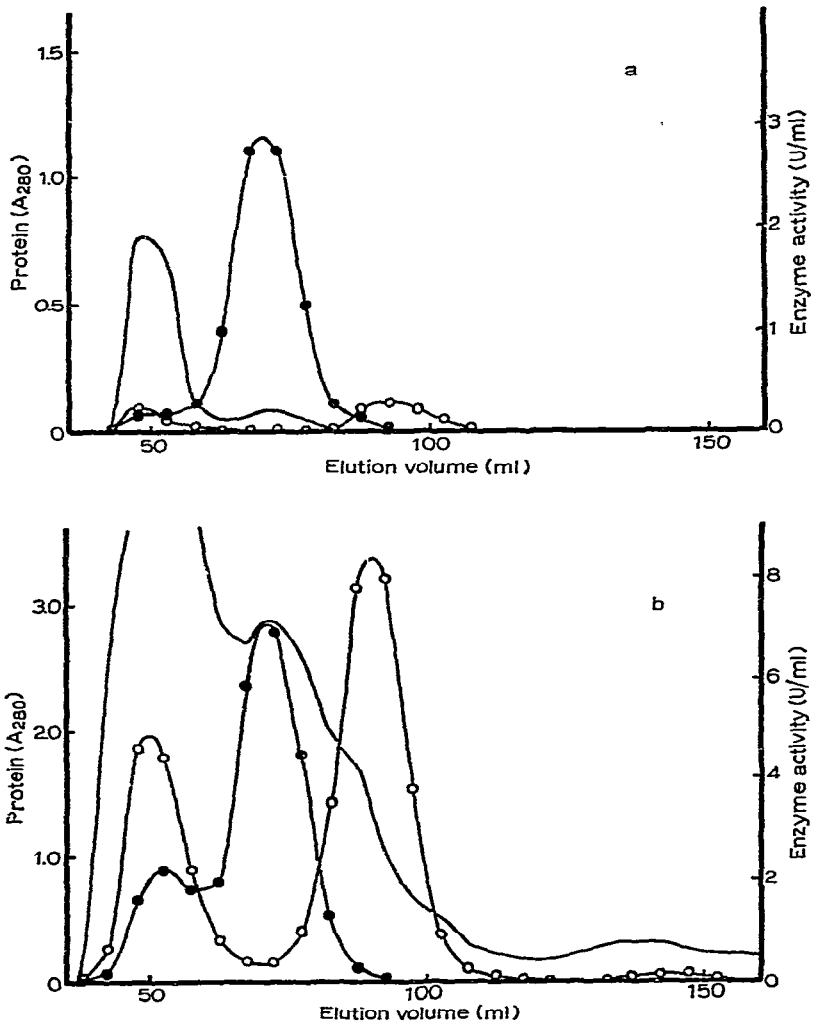

Fig. 1. (*a*) Chromatography of *Helix pomatia* alpha amylase preparation, after desorption from cross-linked glycogen, on a column (*1.5 × 85 cm*) of Bio-Gel P-60. ——, Distribution of protein; ●, alpha amylase activity; ○, (1 → 3)-β-D-glucanase activity. (*b*) Chromatography of crude Glusulase, corresponding in amount to that from which the alpha amylase in Fig. 1*a* was obtained, on the same column. The symbols are as in Fig. 1*a*, except that ● shows the activity of both α-D-glucosidase (45–60 ml) and alpha amylase (60–85 ml). For further experimental details, see the text.

A sample of the alpha amylase solution (2.0 ml, containing 30 Units of alpha amylase activity) obtained by desorption from cross-linked glycogen as just described was chromatographed on a column of Bio-Gel P-60. The results are shown in Fig. 1*a*. For comparison purposes, the results of chromatography of a sample of crude Glusulase under the same conditions are shown in Fig. 1*b*. Poly(acrylamide)-gel electrophoresis of the alpha amylase purified by affinity binding to cross-linked

glycogen, followed by chromatography on Bio-Gel P-60, showed the product to be homogeneous. Results of the overall purification are shown in Table III.

TABLE III

PURIFICATION OF *Helix pomatia* ALPHA AMYLASE

Step	*Total units (U)*	*Protein (mg)*	*Specific activity (U/mg)*	*Purification factor*	*Recovery (%)*
Crude snail juice	133	173	0.8	1	100
Adsorption on cross-linked glycogen[a]	110	[b]	—	—	83
Desorption from cross-linked glycogen	58	4.3	13.5	17	44
Chromatography on Bio-Gel P-60[c]	46	0.16	288	360	35

[a]Calculated by difference from measurements of the amounts of unbound alpha amylase in solution before and after treatment with cross-linked glycogen. [b]Not determined. [c]Performed with two-thirds of the material from the preceding step; however, the results were adjusted proportionately and are presented as if all the material had been subjected to chromatography on Bio-Gel P-60.

DISCUSSION

A number of affinity-binding procedures have been described for isolation of alpha amylases. One of the earliest methods is based on the interaction of alpha amylase with starch granules. A method for the preparation of crystalline, barley-malt alpha amylase depended on the use of a procedure of this type, the amylase being bound to wheat-starch granules in 40% ethanol and recovered by washing with a solution of calcium sulfate[14]. However, as different alpha amylases have different affinities for different types of starch granules[1-3] (this factor determining the efficiency of binding of an alpha amylase and the ease of recovering the amylase from the granules after adsorption), the success of the approach is likely to vary according to the affinity of the alpha amylase being purified for the particular starch being used as adsorbent. Although binding to starch granules has been used quite extensively for removal of alpha amylase from solutions of other amylolytic enzymes[4,5], it has not gained general acceptance as a procedure for purification of alpha amylases. Instead, a number of other affinity-binding procedures have been used, with varying degrees of success, for isolation of alpha amylases. An affinity procedure that depends on the ability of alpha amylases to bind to macrodextrins (partial degradation products of glycogen) in ethanolic solution has been used for isolation of several animal and plant alpha amylases[15-17]. Unfortunately, this method tends to give variable results because of the difficulty of determining the optimum conditions for production of the insoluble macrodextrin–amylase complexes. In two recent papers, the purification of alpha amylases by binding to starch cross-linked with epichlorohydrin or phosphorus oxychloride, and recovery by washing the enzyme–adsorbent complex with

starch or maltose has been described, although the purity of the products obtained was not rigorously investigated. However, in one example, it was demonstrated that the procedure served to separate alpha amylase and beta amylase[19]. Another biospecific adsorption procedure for alpha amylases has been described that is based on the use of immobilized cycloamylases[20]. None of these affinity-binding procedures has been fully evaluated in terms of their usefulness for the purification of alpha amylases from different sources.

Efforts to develop in our laboratories a universally useful adsorbent for removal of alpha amylase from solutions of other amylolytic enzymes have led to the synthesis of an insoluble derivative of glycogen. This is prepared by introducing reactive imidocarborate groupings into the polysaccharide by treatment with cyanogen bromide under alkaline conditions[21]. Addition of the α,ω-aliphatic diamine, 1,6-diaminohexane, results in inter- and, presumably, intra-molecular cross-linking of glycogen molecules with concomitant insolubilization. We have previously demonstrated the usefulness of this adsorbent for removal of alpha amylase from pancreatic extracts during the purification of hog-pancreatic α-D-glucosidases[22]. In other (unpublished) work, we have shown that the adsorbent has the ability to selectively bind alpha amylases from a wide variety of sources. In this publication, we describe the extension of these studies to the use of the cross-linked glycogen adsorbent for the purification of the alpha amylase from the juice of the snail, *Helix pomatia*.

A preliminary study of the characteristics of binding of *Helix pomatia* alpha amylase to cross-linked glycogen showed the binding at 0° to be essentially instantaneous. Increasing amounts of the adsorbent brought about the removal of increasing amounts of enzyme; thus, on a small scale, at 0° over 90% of the enzyme could be removed from a solution (containing initially 12.3 U/ml of alpha amylase) by amounts of cross-linked glycogen in excess of 1.6 mg/ml (Table I). A linear relationship was observed in a double logarithmic plot between the amount of enzyme bound (expressed as U/mg adsorbent) and the concentration of unbound enzyme. The data fit Freundlich's isotherm for surface adsorption in the form:

$$\log(\text{enzyme bound/mg adsorbent}) = 0.778 + 0.7 \log(\text{free enzyme concentration}).$$

The factor 0.7 is close to the theoretical value of 0.67 derived by Freundlich for adsorption of a solute onto a surface[23], and similar to that determined in the case of adsorption of pancreatic alpha amylase onto starch granules[3]. It may therefore be inferred that binding of *Helix pomatia* alpha amylase to cross-linked glycogen occurs by adsorption of the enzyme onto the surface of the adsorbent. There is deviation from the foregoing equation at concentrations of free enzyme in excess of 5 U/ml, suggesting saturation of the binding sites in the adsorbent under such conditions. However, none of the experiments performed permitted determination of the capacity of the cross-linked glycogen for alpha amylase.

The selectivity of the adsorbent was apparent from the lack of any significant binding of either (1 → 3)-β-D-glucanase, large amounts of which are present in snail juice[24-26], or the α-D-glucosidase that has previously been identified as a component of the amylolytic enzyme-system present in the juice[27]. The efficiency of binding was

found to be markedly dependent on the temperature of interaction, maximum binding being achieved at lower temperatures. Thus, at 0°, almost complete binding of alpha amylase was obtained, whereas at 37°, only 6% binding of the enzyme took place (Table II). No attempt was made to determine whether the poorer affinity-binding at higher temperatures was due to decreased affinity for the cross-linked glycogen or to hydrolysis of the adsorbent. However, the latter possibility seems unlikely as no free reducing sugars were detected in the supernatant solution after binding of the enzyme, although it should be recognized that hydrolysis of the cross-linked substrate without release of sugars into solution might conceivably have been taking place. The important practical aspect of the observation was, however, that it pointed immediately to a simple procedure for recovery of the adsorbed alpha amylase, namely by suspension of the amylase–adsorbent complex, formed at 0°, in buffer at higher temperature (37°) to bring about desorption.

A study was made of the adsorption of the enzyme under various conditions, the effect of washing the enzyme–adsorbent complex, and the recovery of the enzyme from the complex. A certain degree of irreproducibility was apparent, possibly because of the two-phase nature of the system under test. However, a number of important features of the binding and recovery procedure were evident. Under all conditions tested (different enzyme concentrations, different amounts of adsorbent) 80–90% of the initial activity was bound by the cross-linked glycogen. Although this amount was somewhat less than that expected on the basis of earlier experiments (Table I), probably because of less efficient interaction of enzyme and adsorbent in the larger incubation-mixtures, the extent of binding was still considered acceptable. Increasing the amount of cross-linked glycogen above a level of 4 mg/ml increased the extent of binding only slightly or not at all. For example, when 5-ml mixtures containing alpha amylase at a concentration of 30 U/ml were treated with adsorbent in amounts of 4, 8, and 12 mg/ml, the extents of binding were 76, 84, and 82%. It was found most convenient to use dilutions of the starting material giving an initial alpha amylase activity of 10–12 U/ml, this procedure helping to prevent non-specific binding, for example of (1 → 3)-β-D-glucanase. Use of more-concentrated alpha amylase solutions tended to result in more carry-over of non-specifically bound protein. On no occasion was any significant removal of bound alpha amylase observed during the washing of the enzyme–adsorbent complex so long as the washing was done at 0°. Fairly large volumes of buffer could therefore be used for washing the complex, thus decreasing the amount of nonspecifically bound material to a minimum. For preparative purposes, we have usually used two washes by buffer, each of about the same volume as the starting solution of amylase. Binding of alpha amylase to the cross-linked glycogen appeared not to be completely reversible by elevation of temperature, and a hysteresis effect appeared to be operative. This is evident from comparison of the results of binding at 37° [6% enzyme bound (Table II)] and desorption at 37° following binding at 0° [for example, 53% of bound enzyme recovered by two treatments at 37° (Table III)]. It is possible that the extent of recovery could be improved by incorporation of substrate during the desorption

step, but no attempt was made to do this as the yields of enzyme that could be recovered by desorption at 37° were considered acceptable. Use of excessive amounts of cross-linked glycogen for adsorption of alpha amylase had two deleterious effects. The greater the amount of cross-linked glycogen used, the greater the difficulty of recovering acceptable levels of the enzyme. Smaller amounts of cross-linked glycogen may bind slightly less alpha amylase, but the enzyme is released more readily during the recovery step. We have also found that increasing the amount of adsorbent decreases the specific activity and overall purification of the product obtained after desorption, pointing to the importance of avoiding the use of excessive amounts of cross-linked glycogen in the adsorption step.

Preparation of alpha amylase by affinity binding to cross-linked glycogen, using conditions for adsorption, washing and recovery selected on the basis of the foregoing studies, resulted in a 17-fold purification of the enzyme with 44% recovery of activity. However, although the product was essentially free from other enzymic activities [(1 → 3)-β-D-glucanase, α-D-glucosidase], it was not homogeneous. It was still contaminated with a brown, pigmented material, large amounts of which are present in snail intestinal juice, and which possibly binds to the adsorbent by hydrophobic interaction with the aliphatic cross-links in the adsorbent. In previous work[24–26], we have observed that such pigments invariably appear in the exclusion volume on fractionation of Glusulase by chromatography on Bio-Gel P-60. Chromatography of the desorbed enzyme preparation is shown in Fig. 1*a*. The alpha amylase emerged from the column associated with little protein and was well separated from residual pigment. Chromatography of crude snail juice under the same conditions resulted in elution of the alpha amylase associated with a broad distribution of protein in the column effluent (Fig. 1*b*). After the purification by chromatography on Bio-Gel P-60, the alpha amylase was judged homogeneous by poly(acrylamide)-gel electrophoresis. The overall purification was 360-fold and the recovery 35%.

The extent of usefulness of the cross-linked glycogen adsorbent for purification of alpha amylases from different sources remains to be determined; efforts to adapt the procedure for the isolation of other alpha amylases are in progress.

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