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PREPARATION AND CHARACTERIZATION OF A DEXTRAN–AMYLASE CONJUGATE*†

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ABSTRACT

Bacillus amyloliquefaciens alpha-amylase was attached to dextran after activation of the polysaccharide by using a modification of the cyanogen bromide method. The soluble dextran–amylase conjugate was purified by molecular-sieve chromatography. The conjugated enzyme has greater stability than the unmodified enzyme at low pH values, during heat treatment, and on removal of calcium ions with a chelating agent. Attachment of dextran to alpha-amylase did not alter the Michaelis constant of the enzyme acting on starch. The polysaccharide–enzyme conjugate probably consists of a cross-linked aggregate of many dextran and many enzyme molecules, in which a proportion of the enzyme molecules, although not inactivated, are unable to express their activity, except after dextranase treatment.

INTRODUCTION

Naturally occurring glycoproteins[2–5] and carbohydrate-free enzymes that have been covalently attached to insoluble polysaccharide supports[6,7] often have stability properties that are superior to those of carbohydrate-free enzymes. Carbohydrate–protein conjugates may show, for example, exceptional heat-stability, as well as resistance to inactivation by proteolytic degradation and other unfavorable conditions. We therefore predicted that conjugates prepared by coupling of enzymes to soluble carbohydrate polymers might show the improved stability of the corresponding insoluble conjugates and naturally occurring glycoproteins. Preliminary experiments have indicated[8] that this is, indeed, the case. In this paper, the protocol for synthesis and purification of a soluble dextran–amylase conjugate is described and evidence supporting the covalent attachment of carbohydrate and protein is presented. The properties of the modified and native enzymes are discussed and the molecular organization of the product is considered. Soluble enzyme–polysaccharide conjugates may

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be of value when stabilization is required, but where immobilization is unnecessary or even a disadvantage, for example, in the enzyme therapy of metabolic disorders[9,10].

MATERIALS AND METHODS

Materials. — *Bacillus amyloliquefaciens* alpha-amylase (4 times crystallized, sold as *Bacillus subtilis* alpha-amylase, but see Refs. 11 and 12) was obtained from Sigma Chemical Co, dextran (molecular weight $\sim 10^6$) from Fisons Pharmaceuticals Ltd. (Loughborough, England), and soluble starch from the Baker Chemical Co. Amylopectin was prepared by 1-butanol fractionation of a dispersion of potato starch[13]. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, and dextranase (36 U/mg, from a penicillium species) from Sigma Chemical Co.

Methods. — Reducing sugars resulting from enzyme action were determined (as D-glucose) by using the 3,5-dinitrosalicyclic acid method[14]. Protein concentrations were determined by the method of Lowry *et al.*[15]. Dextran concentrations were measured by the phenol–sulfuric acid method[16].

Except where otherwise stated, alpha-amylase activity was determined by measuring the release of reducing sugars from soluble starch in digests (2.0 ml) containing substrate (10 mg), buffer (sodium acetate, pH 5.5, 25mM), calcium chloride (1.25 mg/ml), and a suitable amount of enzyme, incubated at 25°. One unit (U) of activity is the amount of enzyme which releases 1 μmol of reducing sugar (as D-glucose equivalents) per min under these conditions. Specific activities are expressed as U/mg of protein.

Chromatography was performed at 4° on a column (90 × 1.5 cm) of Sephadex G-100, prepared according to the manufacturer's literature, and eluted with 50mM acetate buffer (pH 5.0) containing 5mM calcium chloride. Samples (3.2 ml) were collected automatically.

Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, according to Weber and Osborn[17].

RESULTS

Synthesis and purification of the dextran–amylase conjugate. — To a stirred solution of dextran (0.5 g) in water (50 ml), cyanogen bromide (0.1 g) was added, followed, after 15 min, by a second portion of cyanogen bromide (0.1 g). The pH was maintained at 10.7 during this process by addition of aqueous sodium hydroxide. Forty-five minutes after the second addition of cyanogen bromide, solid alpha-amylase (170 mg) was added and the pH immediately adjusted, by addition of aqueous sodium carbonate, to 9.2. Coupling was allowed to proceed during 24 h at 4°, the solution was then dialyzed overnight, and the product (0.55 g) was isolated by lyophilization.

A sample (50 mg) of the product was chromatographed on Sephadex G-100, and the column fractions were analyzed for carbohydrate, protein, and alpha-amylase activity. Most of the enzyme activity was eluted at the void volume of the column,

associated with dextran (Fig. 1a). A control run, in which appropriate amounts of dextran and alpha-amylase were mixed and subjected to chromatography on the same column, showed that the unconjugated dextran–amylase mixture separated under such conditions (Fig. 1b). Thus, it was concluded that the alpha-amylase had been

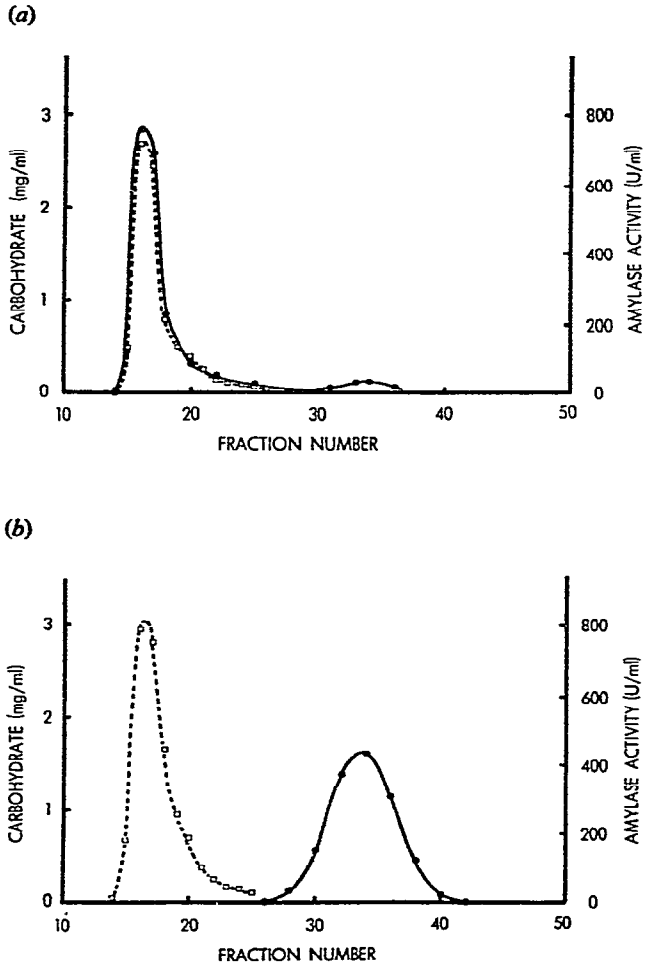


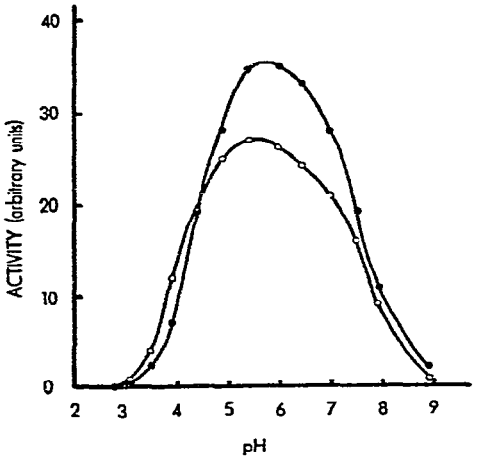
Fig. 1. Sephadex G-100 column chromatography of (*a*) dextran–amylase conjugate and (*b*) dextran-amylase mixture. In each case, the sample applied to the column contained ~15 mg of alpha-amylase and 35 mg of dextran. Distribution of alpha-amylase activity is shown by ●——●, and the distribution of carbohydrate by □——□.

covalently attached to the dextran. Fractions 15–19 from the column were combined. Analysis showed the conjugate to contain 70% of carbohydrate and to have ~30% of the specific activity of the native enzyme.

Properties of the dextran–amylase conjugate and native alpha-amylase. — (*a*) *Polyacrylamide gel electrophoresis.* Under denaturing conditions, the native

enzyme migrated as a single band corresponding to a polypeptide of molecular weight ~50,000, whereas the conjugate showed a band at the top of the gel, indicating that its molecular weight was too large to permit penetration of the gel. Only a trace amount of protein with mobility corresponding to that of unmodified alpha-amylase was observed.

(*b*) *pH Optima and stability.* pH-Activity curves for *Bacillus amyloliquefaciens* alpha-amylase and its dextran conjugate were determined by measurement of the amount of reducing sugars released in digests (2.0 ml) containing substrate (10 mg), buffers (citrate–phosphate, 1.0 ml) of various pH values, and an appropriate amount of suitably diluted enzyme (1.3 U). The pH-activity curves for both forms of the enzyme were closely similar (Fig. 2).



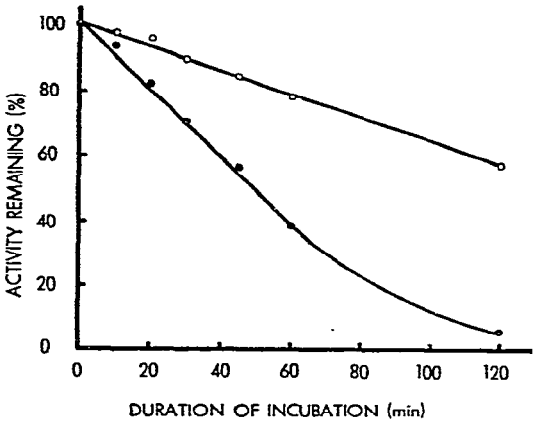


Fig. 2. Dependence of activity of alpha-amylase (●) and dextran–amylase conjugate (○) on pH. For experimental details, see the text.

Fig. 3. Inactivation of alpha-amylase (●) and dextran–amylase conjugate (○) during incubation in the presence of EDTA (10mM) at 37°. For experimental details, see the text.

To determine the effect of pH on the stability of the alpha-amylase and its dextran-conjugate, samples (1.3 U) were preincubated at room temperature for 30 min in McIlvaine[18] buffers (50 μl) of various pH values. The activity remaining after preincubation was then determined by addition of starch (1.0 ml, 10 mg/ml) and buffer (McIlvaine, pH 6.0, 1.0 ml), and determination of the amount of reducing sugars released in a 5-min period. Above pH ~4.5, the stability of the conjugated enzyme was closely similar to that of the native enzyme. However, at lower pH values, the conjugate was more resistant to inactivation than the native enzyme. Thus, at pH 4.4, 3.9, and 3.5, there was, respectively, 7, 15, and 20% less activity lost from the conjugate than from the native enzyme.

(*c*) *Stability in presence of EDTA.* Native and conjugated alpha-amylases (20 U/ml) were incubated at 37° in 20mM Tris/HCl buffer (pH 7.5) containing EDTA

(10mM). Samples were removed at intervals for determination of residual alpha-amylase activity. The results are shown in Fig. 3.

(*d*) *Heat stability.* Solutions of alpha-amylase and its dextran conjugate (5 μg of protein/ml) in water were heated at 65°, and samples removed at intervals for activity determinations. The results are shown in Fig. 4. In a control experiment, alpha-amylase was heated under the above conditions but in the presence of unconjugated dextran (14 μg/ml). The rate of activity loss under the latter conditions was identical to that in the absence of dextran. There was no restoration of enzyme

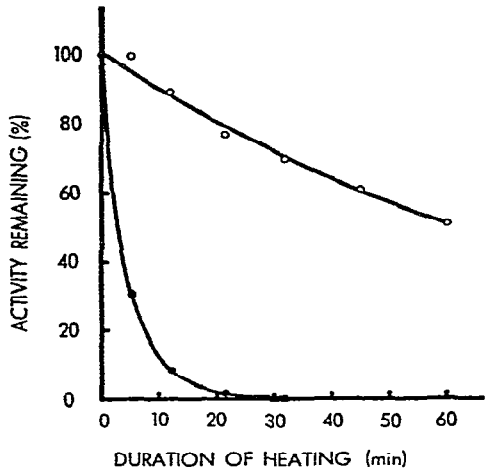


Fig. 4. Heat inactivation at 65° of alpha-amylase (●) and dextran–amylase conjugate (○). For experimental details, see the text.

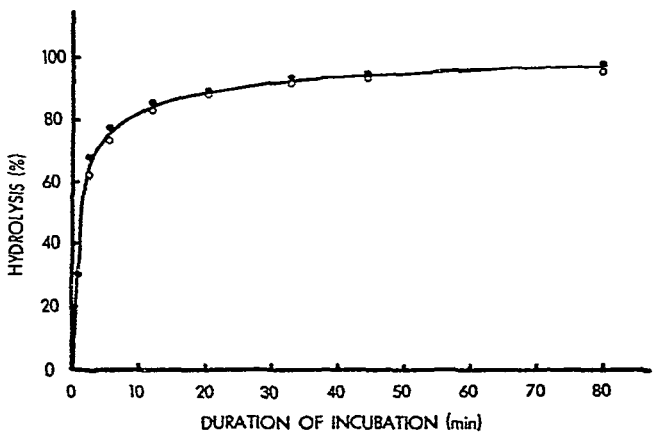


Fig. 5. Time course of hydrolysis of amylopectin by alpha-amylase (●) and dextran–amylase conjugate (○). The extent of hydrolysis is expressed in terms of conversion into maltose. For experimental details, see the text.

activity on storage of samples of heat-treated (30 min) native and conjugated amylases at 0° for 60 min.

(*e*) *Time course and kinetics of substrate degradation.* Digests were prepared containing amylopectin (5 mg/ml), acetate buffer (pH 5.5, 25mM), calcium chloride (1.25 mg/ml), and native or conjugated alpha-amylase (2.6 U/ml). Samples were removed at intervals for reducing-sugar determinations. The results are shown in Fig. 5.

The rates of release of reducing sugars from amylopectin were determined in digests containing substrate (0.025–2.5 mg/ml), acetate buffer (25 mM, pH 5.5), calcium chloride (2.5 mg/ml), and enzyme (native or conjugated, 0.11 U/ml). The Michaelis constants for the native and conjugated enzymes were determined from Lineweaver–Burk plots (Fig. 6) and were 0.58 mg/ml for both enzymes.

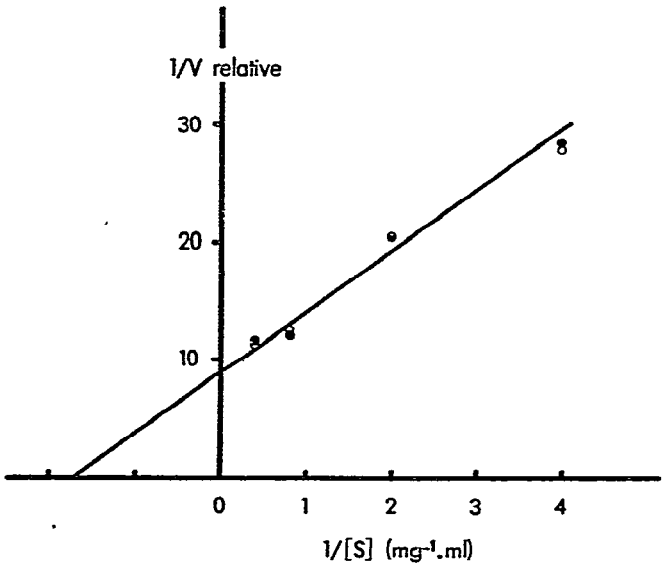


Fig. 6. Lineweaver–Burk plots for alpha-amylase (●) and dextran–amylase conjugate (○) acting on soluble starch. For experimental details, see the text.

Effect of dextranase treatment on the activity of the conjugate. — A sample of conjugated alpha-amylase (1 Unit) was treated at 37° for 1 h with dextranase (18 U) in a digest (1.0 ml) containing 100mM acetate buffer (pH 5.5) and 20mM calcium chloride. A similar amount of alpha-amylase was also treated under these conditions. Following dextranase treatment, alpha-amylase activity was determined in the usual manner. The action of dextranase increased the activity of the conjugate by 59%, *i.e.*, to ~48% of the activity of the native enzyme, but did not affect the activity of native alpha-amylase.

DISCUSSION

Synthesis of soluble dextran–amylase conjugate

During initial attempts to activate soluble dextran with concentrations of cyanogen bromide similar to those used for activation of insoluble polysaccharides[19,20], rapid, irreversible precipitation of the polysaccharide took place during the activation procedure. This was considered to be the result of cross-linking of polysaccharide chains by side-reactions[21]. Kågedal and Åkerström[22] experienced similar problems during activation of soluble dextran, prior to coupling with compounds of low molecular weight and insulin, and found that the insolubilization during activation could be prevented by using lower concentrations of cyanogen bromide. We also have found that such conditions give satisfactory results.

Gel chromatography of the product on Sephadex G-100 showed that the enzyme activity emerged from the column at the void volume, associated with dextran (Fig. 1a). Only a trace of activity was present at the normal position of elution of the amylase, indicating that virtually all the enzyme had been conjugated. The interaction between amylase and dextran clearly involves covalent linkages, as a mixture of alpha-amylase and dextran was separated by gel chromatography under the same conditions (Fig. 1b).

Further evidence for the covalent attachment of amylase and dextran came from examination of the behavior of a sample from the excluded peak (Fractions 15–19, Fig. 1a) from the G-100 column, during electrophoresis under denaturing conditions. Protein stains showed that this did not migrate into the polyacrylamide gel, indicating that it is of very high molecular weight. On the other hand, the native enzyme migrated as expected of a protein with molecular weight of ~50,000.

The specific activity of the alpha-amylase in its conjugated form, after purification, was ~30% of that of the native enzyme. It is likely that the loss of activity is due both to inactivation during coupling and to unfavorable steric interactions between the carbohydrate moieties on the enzyme and the macromolecular substrate. The involvement of the latter phenomenon is revealed by the effect of dextranase treatment on the activity of the conjugate (*vide infra*).

Comparison of properties of native and dextran-conjugated alpha-amylase

A study of the effect of pH on the activity of the native and conjugated enzyme showed this to be closely similar for both forms of the enzyme (Fig. 2). This finding is not surprising in view of the uncharged nature of both the substrate and the carbohydrate attached to the enzyme. Most alpha-amylases are unstable in weakly acidic solution[23], for reasons which have not yet been explained at the molecular level. The conjugated enzyme was stabilized to a small, but significant, extent against acid inactivation.

In the presence of EDTA, most alpha-amylases are inactivated; this is considered to be the result of unfolding of the enzyme molecule after chelation of its essential calcium, followed by proteolytic degradation by traces of contaminating

protease[23]. In the presence of 10mM EDTA at 37°, the conjugated enzyme was significantly more stable than the native enzyme (Fig. 3). This may be because the presence of carbohydrate prevents unfolding after removal of calcium, or because the presence of carbohydrate substituents attached to the polypeptide chain causes unfavorable steric interactions that prevent proteolysis of the unfolded enzyme. An alternative explanation is that the trace of protease present in the alpha-amylase preparation is also conjugated to dextran, rendering it inactive towards protein substrates. We have observed such a phenomenon after coupling of bovine-pancreatic trypsin to dextran; the conjugate has high activity towards ester substrates, but low activity towards proteins[1]. Unfortunately, the inherent stability of the *Bacillus amyloliquefaciens* alpha-amylase to proteolytic degradation by trypsin, pronase, subtilisin, and thermolysin precluded a comparison of the effect of proteolytic enzymes on the native and conjugated forms of the enzyme.

The most marked effect resulting from attachment of carbohydrate is on the heat-stability characteristics of the alpha-amylase. Native alpha-amylase has a half-life at 65° of 2.5 min; after coupling, this increased to 63 min (Fig. 4). The enhanced stability may result from a change in the degree of hydration of the enzyme molecule, this being influenced, in turn, by the presence or absence of carbohydrate[24]. An alternative explanation is that the carbohydrate serves to maintain the tertiary structure of the enzyme, possibly by cross-linking different parts of the polypeptide chain. This would result from reaction of two activated monosaccharide residues in a single dextran molecule with the ε-amino groups of two lysine residues in a single enzyme molecule. Despite the effect of attached carbohydrate in stabilizing the enzyme against heat denaturation, the carbohydrate in the conjugated form of the enzyme did not appear to assist renaturation of the heat-inactivated enzyme.

The effect of carbohydrate on the interaction of alpha-amylase with starch was examined in two different ways. Firstly, comparison was made of the time course of substrate degradation by equal concentrations of the native enzyme and the enzyme–dextran conjugate. In the initial stages of reaction, alpha-amylase acts on macromolecular material, in the intermediate stages on megalosaccharide substrate, and in the later stages on small oligosaccharides. It might, therefore, be expected that significant differences in the rates of product formation with time by the two forms of the enzyme would be apparent if attachment of carbohydrate affected the interaction with macromolecular substrate. However, the time-course of starch degradation by native and conjugated alpha-amylases was identical (Fig. 5), indicating that carbohydrate attachment does not affect the relative rates of hydrolysis of macromolecular substrates and those of low molecular weight. The second piece of evidence against unfavorable steric interactions between the attached carbohydrate and the macromolecular substrate came from examination of the kinetics of substrate hydrolysis by native and conjugated enzyme. Lineweaver–Burk double-reciprocal plots (Fig. 6) showed the Michaelis constant to be the same for both forms of the enzyme.

It is difficult, at first sight, to reconcile the above findings with the observed 59% increase in the activity of the conjugated form of the enzyme after dextranase treat-

ment. This latter observation suggests that some potentially active enzyme is unable to express its activity because of the presence of carbohydrate. These observations are only compatible if the conjugate consists of a mixture of fully active, enzyme molecules, and some potentially active molecules whose activity is completely masked by carbohydrate before dextranase treatment. It is possible to see how such a situation could arise when the nature of the conjugate is considered. Few conjugated "molecules" are likely to consist of one dextran molecule attached to a single enzyme molecule. The amount of cyanogen bromide used for dextran activation is sufficient to activate many monosaccharide residues in each dextran molecule, and each activated dextran molecule thus has the ability to cross-link polypeptide chains both intra- and, more significantly, inter-molecularly. In all probability, aggregates consisting of very many enzyme molecules and many dextran molecules are produced during the coupling step. It may then be envisaged that the enzyme molecules at the surface of such an aggregate would be fully active and that those in the interior would be able to interact with substrate only after partial breakdown of the aggregate by dextranase treatment.

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