IMMOBILIZATION OF LIGANDS FOR BIOSPECIFIC AFFINITY CHROMATOGRAPHY VIA THEIR HYDROXYL GROUPS. THE CYCLOHEXAAMYLOSE-β-AMYLASE SYSTEM

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1. Introduction

Several methods for the immobilization of ligands for biospecific affinity chromatography have been developed [1–3]. However, there is a constant need for new procedures as increasingly sophisticated systems come under study. There are few direct methods available for the immobilization of hydroxyl group-containing substances like carbohydrates. These substances have been immobilized by copolymerization [4] and cross-linking [5] procedures, and after conversion to amino [6–11] or aldonic acid [12] derivatives. In special cases, unsubstituted polysaccharide matrices have served as biospecific adsorbents for lectins and carbohydrate-metabolizing enzymes [13–20].

In this study is demonstrated a procedure for the direct binding of hydroxyl group-containing ligands to Sepharose 6 B which has been previously activated by a bifunctional epoxide. The method is exemplified by the immobilization of cyclohexaamylose and the use of the resulting adsorbent for biospecific affinity chromatography of sweet potato β-amylase.

2. Experimental

2.1. *Materials*

Sepharose 6 B, activated by 1,4-bis(2,3-epoxypropoxy)-butane and freeze-dried (epoxy-activated Sepharose 6 B), was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cyclohexaamylose (Schardinger α-dextrin), α-amylase (Type II A from *B. subtilis*, EC 3.2.1.1), and β-amylase (crystalline, sweet potato; EC 3.2.1.2) were products of Sigma Chem. Co., St. Louis, Mo. Crystalline bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., Eastbourne, England. Soluble starch (after Zulkowsky) and 3,5-dinitrosalicyclic acid were from Merck AG, Darmstadt, BRD. All other chemicals were of reagent grade. A Uvicord III (LKB, Stockholm, Sweden) with a 3 mm cell was used for UV-monitoring.

2.2. *Immobilization of cyclohexaamylose (CHA)*

Two 4 g batches of freeze-dried epoxy-Sepharose were reconstituted in distilled H_2O for one hr on glass filters. The gels were transferred to 0.1 M NaOH and excess liquid removed by suction. One batch was reacted with a solution of 300 mg CHA in 12 ml 0.1 M NaOH at 45°C for 16 hr. The other batch was incubated with 12 ml 0.1 M NaOH at 45°C and served as a control. The incubations were carried out on a shaker in a water bath.

After incubation the gels were washed on glass filters with distilled H_2O for 30 min and with glucose (25 mg/ml H_2O) for 30 min, again with distilled H_2O for 30 min, and finally, with 50 mM Na acetate buffer, pH 4.8, for 2 hr. The gels were packed in 9 × 150 mm glass columns and equilibrated with the acetate buffer.

2.3. *Affinity chromatography*

0.4 ml of β-amylase suspension was desalted on a 16 × 60 mm column of Sephadex G-25 in 50 mM acetate buffer, pH 4.8. The protein concentration of the eluate was adjusted to 0.75 mg/ml. To 2 ml of this solution, 3 mg of salt-free BSA was added, whereafter the sample was chromatographed on a 9 × 80 mm column of CHA-Sepharose as shown in fig. 1a. Enzyme activity could be determined in the fractions without prior removal of cyclohexaamylose.

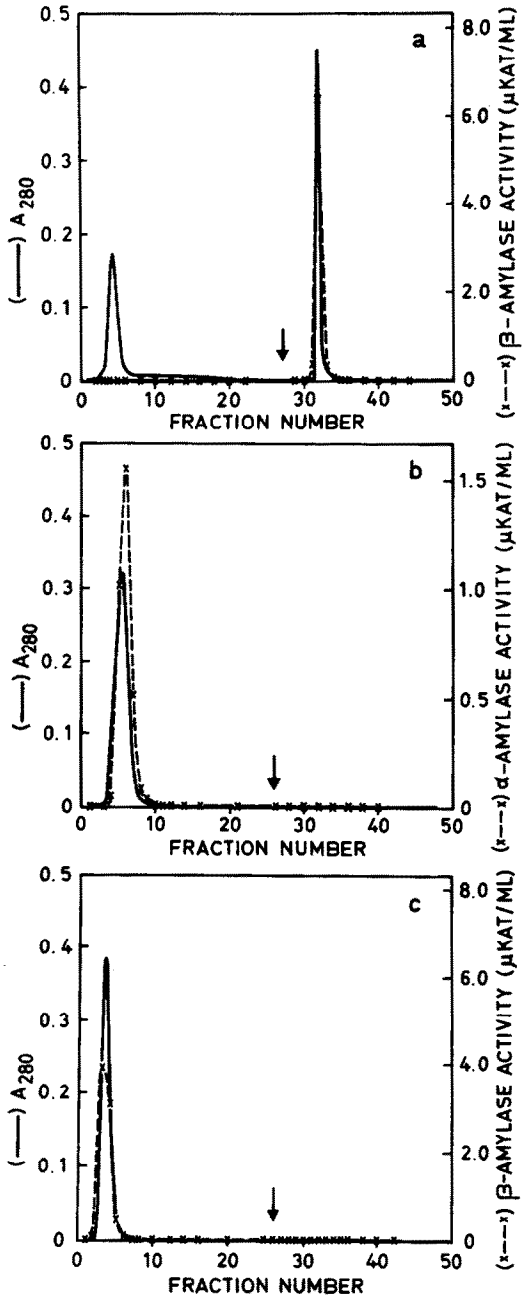

Fig. 1. Biospecific affinity chromatography on epoxy-activated Sepharose 6 B substituted with cyclohexaamylose (CHA). In all experiments the buffer was 50 mM Na acetate, pH 4.8, the flow rate 16 ml/hr, and the fraction volume 2 ml. An arrow indicates the position where CHA (10 mg/ml) was added to the buffer. a) Fractionation of 1.5 mg β-amylase and 3 mg bovine serum albumin (BSA) on a 9 × 80 mm CHA-Sepharose column. b) Fractionation of 2 mg α-amylase and 3 mg BSA on the same column as above. c) Fractionation of 1.5 mg β-amylase and 3 mg BSA on a 9 × 80 mm column containing epoxy-activated Sepharose which had been incubated under coupling conditions in the absence of CHA.

A control experiment was performed on the CHA-Sepharose column using as sample a mixture of 3 mg BSA in 1 ml of Na-acetate buffer and 2 mg α-amylase in 1 ml of Na phosphate buffer, pH 6.5, adjusted to a final pH of 4,8. The conditions were the same as in the previous experiment (see fig. 1b).

As an additional control, a mixture of β-amylase and BSA (of the same composition as in the first experiment) was passed through a 9 × 80 mm column packed with the reference gel which had not been reacted with CHA. The conditions were the same as above. The result is shown in fig. 1c.

2.4. *Activity determinations*

Enzyme activities were determined using a modification of the assay described by Bernfeld (21). α-Amylase solutions were diluted in 50 mM Na phosphate buffer, pH 6.9, and mixed at 25°C with equal volumes of 1% soluble starch in the same buffer. In the case of β-amylase, 50 mM Na acetate buffer, pH 4.8, was used instead. Aliquots were withdrawn, incubated with 3,5-dinitrosalicylate at 100°C, and their content of reducing sugar determined spectrophotometrically at 500 nm. Absorbance changes were converted to amounts of maltose using a standard curve. One katal is defined as the amount of activity that produces one mole of reducing groups from starch per second [22].

2.5. *Fractionation of crystalline β-amylase*

A sample of desalted β-amylase (1.5 mg in 2 ml acetate buffer) was divided in two parts. One half was chromatographed on CHA-Sepharose under the conditions given in fig. 1. The protein which was eluted by CHA was collected and dialyzed. Determinations of activity and UV-adsorption at 280 nm were carried out on the solutions containing fractionated and unfractionated enzyme, aliquots of which were subsequently submitted to amino acid analysis. The extinction coefficients ($E_1^{\%}{}_{cm}$ at 280 nm) obtained in this way for fractionated and unfractionated enzyme were

22.1 and 22.7, respectively (cf. ref. [23]). From these data, the specific activities given below were calculated.

2.6. *Capacity determination*

A 1 ml column of CHA-Sepharose was saturated with β-amylase using a 0.5 mg/ml solution in 50 mM acetate buffer, pH 4.8, at a flow rate of 8 ml/hr. The enzyme was displaced by CHA (10 mg/ml). The capacity of the column was determined from the activity and from the absorbance at 280 nm of the eluted and dialyzed protein fraction. These two figures agreed very well (see below).

3. Results and discussion

Agarose gels, after activation by epichlorohydrin or bifunctional epoxides (oxiranes), are capable of binding substances containing free amino groups. In weakly alkaline medium (pH 8–10) peptides and proteins are readily immobilized [24]. At high pH (> 11) epoxide-substituted agarose also reacts with substances containing hydroxyl groups. The latter reaction can be employed for the synthesis of adsorbents for biospecific affinity chromatography by immobilization of carbohydrates and alcohols.

The experiments reported here were performed using Sepharose 6 B, which had been activated by1,4-bis (2,3-epoxypropoxy)-butane. The latter compound functions as a spacer arm between the matrix and the ligand (see fig. 2). The importance of using spacers has been stressed by several workers [1,25]. This spacer is hydrophilic, and so non-specific adsorption of substances to the matrix, which sometimes becomes a problem when dealing with hydrocarbon spacer arms [25], is less likely to occur.

Cyclohexaamylose, which was used here as the ligand, is a specific inhibitor of β-amylase (K_i for the sweet potato enzyme = 1.7×10^{-4}, ref. [26]). The adsorbent obtained by incubation of epoxy-activated

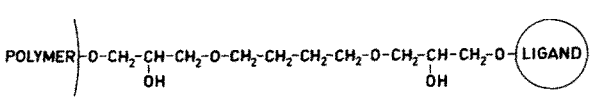

Fig. 2. The structure of the linkage between polymer and hydroxyl group-containing ligand obtained by use of the bifunctional epoxide, 1,4-bis(2,3-epoxypropoxy)-butane.

Sepharose with CHA at pH 13 had a binding capacity of 3.0 mg crystalline β-amylase per ml of sedimented gel. The binding of the enzyme was bio-specific, which is evident from the following. A CHA-Sepharose column separates albumin from β-amylase. The former protein passes the column unretarded and the latter is subsequently eluted by addition of CHA (10 mg/ml) to the starting buffer (fig. 1a). Furthermore, α-amylase, which is closely related to β-amylase in substrate specificity, is not retarded by the column (fig. 1b). Finally, the binding of β-amylase is not caused by non-specific interaction of the protein with spacer arms on the matrix. In fig. 1c is shown an experiment where β-amylase passes unretarded through a bed of epoxy-activated Sepharose which has previously been incubated under coupling conditions in the absence of CHA.

When a sample of commercial β-amylase was applied to a CHA-Sepharose column, a small amount of material absorbing at 280 nm passed unretarded. The removal of this contamining material (which contained glucosamine, in contrast to the adsorbed enzyme) resulted in an increase in the specific activity of the enzyme from 19.5 μkat/mg to 20.4 μkat/mg.

Inhibition studies on β-amylase led to the suggestion that the active site is complementary to CHA in size [27]. Nevertheless CHA molecules with one (in some cases possibly more) modified residues can bind the enzyme. However, during immobilization of ligands having only one specific site for binding of the complementary biological molecule, there is always a possibility that residues which are essential for binding become substituted or sterically blocked. In such cases, when an enzyme or a lectin to be purified is specific for a certain monosaccharide, a corresponding oligosaccharide can always be attempted as ligand in order to facilitate interaction with unsubstituted carbohydrate residues.

A decreased degree of specificity during adsorption can also be compensated for if displacement of the bound molecules is carried out using a highly specific eluant (so-called affinity elution). The eluant then contains a 'competitive counter-ligand' [25], i.e. the ligand or a substance of closely related structure. This type of elution is necessary in the systems discussed here. It is rather common in 'affinity chromatography' work to use pH shifts and salt gradients for the elution of bound substances; however, hydrophobic interaction

or ion exchange may well be responsible for the binding effects observed in such systems.

The procedure described here for the immobilization of CHA could be generally applied to hydroxyl group-containing ligands. Conjugates prepared by this technique have several attractive properties in addition to the above mentioned hydrophilic spacer arm between matrix and ligand. No charges are introduced by the coupling reaction. Consequently there will be little adsorption caused by ionic interactions provided that a matrix of low charge density, e.g. Sepharose, is used. Also the spacer is connected to both polymer and ligand via ether linkages which are fairly strong and resistant to hydrolysis.

Certain ligands may not tolerate the high pH necessary for binding via their hydroxyl groups to an epoxy-activated matrix in aqueous medium. Preliminary studies in this laboratory using mixtures of water and organic solvents as coupling media indicate that this problem could be overcome. This work will be reported elsewhere.

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