ISOLATION AND PURIFICATION OF BIOPOLYMERS BY AFFINITY CHROMATOGRAPHY. IV. PREPARATION AND SOME PROPERTIES OF TREHALOSE IMMOBILIZED ON EPOXY-ACTIVATED SEPHAROSE [1]

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A description is given of the synthesis and some properties of a new affinity-adsorbent — "trehalose—epoxy-activated Sepharose 6B" (TES). The chromatographic behavior on TES of acid α-glucosidase (AG) and of concanavalin A (CA) has been studied. Washed epoxy-activated Sepharose 6B (1 g) was stirred at 40°C with a solution of 2.27 g of α,α'-trehalose in 3 ml of 0.1 N NaOH for 16 h. The gel was washed with 100 ml of distilled water and was stirred with 3 ml of 1 M ethanolamine at 20°C for 16 h and the TES obtained was washed with water. When an unpurified extract of human liver or a purified AG preparation was chromatographed in several buffer systems and water, no adsorption of the enzyme on the TES was observed. CA biospecifically binds to TES and can be eluted quantitatively with 0.1 M methyl α-D-glucoside or D-glucose. Some aspects of the affinity chromatography of biopolymers having an affinity for α-D-glucopyranosyl residues on TES are discussed.

The covalent addition of ligands of different natures to solid supports containing reactive epoxy groups is a widespread and promising method of obtaining affinity adsorbents [2]. The advantages of this method are obvious: 1) as a rule, there is no necessity for the preliminary chemical modification of the ligand, since not only NH_2 groups but also OH and SH groups can participate in the addition reaction; 2) the ligand is attached to the support through a hydrophilic and uncharged spacer; and 3) the simple ether bond between the spacer and support ensures a high stability of the adsorbent.

All that has been said above relates in full measure to ligands of carbohydrate nature. There has already been a considerable number of publications describing the immobilization of carbohydrate ligands on epoxy-activated Sepharose [3]. This question has been investigated in most detail in a recent study by Uy and Wold [4]. It has been shown, in particular, that under fairly severe conditions of attachment only nonreducing mono- and oligosaccharides retain stability, while reducing derivatives undergo substantial changes and transformations leading to chemically inhomogeneous adsorbents. Furthermore, it has been established that the primary OH groups of carbohydrates are more reactive than secondary, and attachment takes place to the extent of more than 90% at the primary OH groups of the ligand [4].

In the light of these results, in solving the problem of obtaining affinity adsorbents with nonreducing α-D-glucopyranose as ligand it appeared desirable to immobilize α,α'-trehalose on epoxy-activated Sepharose 6B. Other methods of covalently binding trehalose with polymers (such as proteins [5]) are associated with a complex and multistage chemical process directed to the selective protection of the hydroxy groups of the disaccharide. In the present paper we describe the preparation and some properties of the affinity adsorbent "trehalose—epoxy-activated Sepharose 6B" (TES).

The addition of the trehalose to the epoxy-Sepharose was carried out in 0.1 N caustic soda at 40°C for 16 h. Preliminary experiments had shown that under these conditions trehalose is fairly stable; its properties (chromatographic mobility, angle of optical rotation) did not change appreciably over 16 h in 0.1 N caustic soda at 40°C. For immobilization we used a large excess of disaccharide (~2 mmole/ml of gel) to prevent the cross-linkage of the Sepharose beads and to ensure the single-point attachment of the ligand. The structure of the adsorbent obtained is shown in the following schematic formula:

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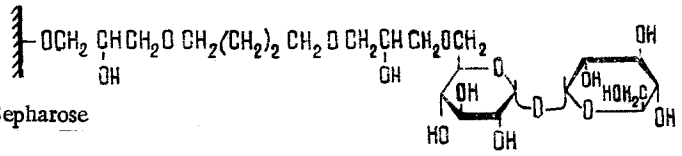

It must be mentioned that the possible minor product formed by the addition of trehalose to epoxy-Sepharose at one of the secondary hydroxy groups also ensures the presence on the adsorbent of a nonreducing α-D-glucopyranose residue with the condition of single-point attachment of the ligand.

The amount of trehalose in the adsorbent was determined from the amount of D-glucose formed after acid hydrolysis of the TES. According to the results obtained, the TES contained 5-6 μmole of ligand per ml of gel.

We first made an attempt at the biospecific purification of acid α-glycosidase (γ-amylase, E.C. 3.2.1.3) from human liver on the adsorbent obtained. However, when an unpurified liver extact or a purified preparation of the enzyme [6] was added to a column containing TES in several buffer systems or in water no appreciable adsorption of γ-amylase whatever was found. It is known that trehalose is a weak competitive inhibitor of this enzyme (K_i $5.4\text{-}5.7 \cdot 10^{-3}$ M [7]). It has been shown on the basis of the affinity chromatography (AfC) of α-fucosidase [1] and of other glycosidases [8] that AfC on ligands with low affinities for the biopolymer to be isolated is effective only with the participation of nonspecific hydrophobic and electrostatic interactions. Since TES contains no hydrophobic or charged groups, the affinity of γ-amylase for the immobilized ligand is, in itself, probably, insufficient for adsorption on the affinity sorbent and true AfC becomes impossible. It is appropriate to mention that an adsorbent with another α-D-glucoside ligand — p-aminophenyl α-D-glucopyranoside attached to CH-Sepharose — binds acid α-glucosidase strongly [9]. This fact can be explained by the nonspecific binding of the enzyme with this charged and hydrophobic adsorbent, as is confirmed by the impossibility of using for elution solutions containing 20% of isopropanol and 1 M NaCl [9].

On the other hand, concanavalin A (ConA) — a lectin having a high affinity for terminal nonreducing glucose — was biospecifically bound to the TES. Figure 1 shows a model experiment on the chromatography of Con A and of bovine serum albumin (BSA) (ballast protein) on TES. The quantitative desorption of Con A with 0.1 M methyl α-D-glucopyranoside confirms the biospecific nature of the binding of the lectin with the TES. The capacity of the TES determined at definite saturation with Con A amounted to 20-25 mg of Con A/ml of gel. Two years after the preparation of the absorption it still bound with about 10 mg of Con A per 1 ml of gel.

Thus, TES is a stable and effective affinity adsorbent for the biospecific purification of proteins having a high affinity for nonreducing glucopyranosyl residues.

EXPERIMENTAL

Epoxy-activated Sepharose 6B was obtained from Pharmacia Fine Chemicals (Sweden) and trehalose from Chemapol (Czechoslovakia). Con A was isolated from a flour made from the beans of *Canavalia ensiformis* [10]. The isolation of the acid α-glucosidase, its purification by chromatography on Sephadex G-200, and the determination of its activity were performed as described by Belen'kii et al. [6]. The concentration of protein was determined from the absorption in the UV region at 280 nm.

The amount of trehalose in the TES was determined after hydrolysis of the adsorbent with 2 N HCl at 100°C for 4 h, neutralization, and determination of the glucose by the glucose oxidase method [11].

Synthesis of the Adsorbent TES. Epoxy-activated Sepharose 6B (1 g) was washed with 100 ml of distilled water and was added to a solution of 2.27 g of α,α'-trehalose [monohydrate, mp 96°C, $[\alpha]_D^{20}$ +178° (c 1; water)] in 3 ml of 0.1 N NaOH. The suspension was slowly stirred with a magnetic stirrer at 40°C for 16 h. Then the gel was washed with 100 ml of distilled water and it was stirred with 3 ml of 1 M ethanolamine at 20°C for 16 h. The resulting adsorbent was washed with water (100 ml). It can be stored for a long time in aqueous suspension in the presence of 0.02% of sodium azide.

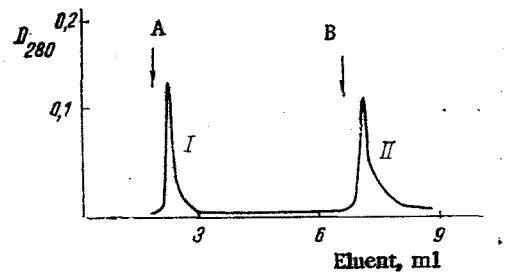

Fig. 1. Chromatography of concanavalin A and of bovine serum albumin on the "trehalose–epoxy-activated Sepharose" adsorbent. The chromatographic conditions are given in the text. Arrow A shows the beginning of the introduction of the sample and B the moment of addition of 0.1 M to the buffer solution; 1) BSA; 2) Con A.

Chromatography of an Unpurified Extract of Human Liver or of Purified Acid α-Glucosidase [6] on TES. Chromatography was performed by using as the equilibrating buffer solution (EBS): 1) a 0.1 M sodium acetate buffer solution, pH 4.8, with 1 mM disodium ethylenediaminetetraacetate (EDTA); or 2) 25 mM sodium chloride with 1 mM EDTA, pH 6.7; or 3) water. The protein solution added to the column (V = 2 ml) was previously dialyzed against the corresponding EBS. In no case was adsorption of the enzyme on the TES observed.

Affinity Chromatography of Con A on TES. A solution of 5 mg of BSA and 5 mg of Con A in 1 ml of 0.01 M sodium acetate buffer solution, pH 5.5, containing 1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ was deposited on a column of TES (V = 2 ml) equilibrated with the same buffer solution. The column was washed with EBS at the rate of 20 ml/h (the BSA was eluted). The Con A was eluted by the addition to the EBS of 0.1 M methyl α-D-glucoside or 0.1 M D-glucose (Fig. 1). The yields of the biopolymers were quantitative.

D. M. Belen'kii took part in the experimental work and in a discussion of the results.

SUMMARY

1. A new biospecific adsorbent "trehalose–epoxy-activated Sepharose 6B" has been synthesized.

2. The chromatographic behavior on this adsorbent of acid α-glucosidase and of concanavalin A has been studied.

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