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Note

The incorporation of carboxyl groups into dextran and cross-linked agarose by *O*-succinylation

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Bioaffinity chromatography has been extensively used for the isolation of antibodies, enzymes, hormone receptors, lectins, and other binding proteins[1]. The technique is based on the specific interaction of the proteins with ligands that are, in turn, covalently attached to insoluble matrices. Polysaccharide matrices commonly used in this procedure lack the necessary functional groups for ligand attachment, and therefore the groups must be chemically introduced into the matrix. The activation of polysaccharide-based, chromatographic gels by alkaline cyanogen bromide[2] enabled covalent coupling of the activated gel directly to compounds containing primary amino groups. Coupling of such bifunctional compounds as 1,6-diaminohexane or 6-aminohexanoic acid, followed by covalent ligand-binding, provides spacer arms between the ligand and the matrix, enhancing the accessibility of the bound ligand. A variety of other procedures for introducing reactive groups has since been developed[1].

Matrices derivatized with amino groups can be *N*-succinylated with succinic anhydride. The reaction provides free carboxyl groups, instead of free amino groups, and has the advantage of extending the spacer arms by several carbon atoms[3]. The *N*-succinylation of Sepharose CL-6B preactivated with (3-aminopropyl)triethoxysilane (to introduce free amino groups) resulted in significant *O*-succinylation, in addition to the expected *N*-succinylation[4]. This observation offered the possibility of designing a derivatizing procedure devoid of many of the problems usually encountered in preparing bioaffinity matrices in the laboratory, namely, toxic and unstable reagents, expensive chemicals, and instability of the activated matrix. Therefore, it was decided to investigate the conditions for *O*-succinylation, and its applicability to bioaffinity chromatography.

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EXPERIMENTAL

Succinylation of polysaccharides. — Dextran (mol. wt. 9400; 100 mg) dissolved in water (1.0 mL), and succinic anhydride (100 mg) dissolved in 25% acetone (4.0 mL), were separately chilled in an ice bath, combined, and the pH maintained at 6.0 by the dropwise addition of 20% NaOH until the pH stabilized (10 to 15 min). After being kept for 5 h at 5°, the mixture was exhaustively dialyzed against water, and then lyophilized.

Sepharose CL-6B (Pharmacia; 40 mL of packed gel) was successively washed on a sintered-glass funnel with water (500 mL) and acetone (200 mL), and then air-dried. Dried gel (1.0 g) suspended in water (25 mL), and succinic anhydride (200 mg) dissolved in 40% acetone (10 mL), were combined after chilling, and the pH was adjusted to 6.0 as described for dextran. The reacted gel was kept for 5 h at 5°, and then successively washed on a sintered-glass funnel with 25% acetone (500 mL) and water (500 mL). The gel (succinylated Sepharose CL-6B) was stored, as a suspension in water, at 5°.

Quantitation of esters. — Esters were quantitated spectrophotometrically by a hydroxylamine–ferric chloride method[5]. Ethyl acetate, dissolved in 5% methanol, was used as the standard.

^{13}C-N.m.r. spectra of succinylated dextran. — The ^{13}C-n.m.r. spectra were recorded by use of a JNM/FX60Q n.m.r. spectrometer operating at 15.04 MHz and equipped with a 10-mm, ^{1}H–^{13}C dual probe, an NM-3975 foreground–background unit, and an NM-5471 temperature controller. The spectra were recorded at 70° by using the pulsed, fast Fourier-transform method, employing the deuterium resonance of the sodium phosphate in D_2O buffer, pH 7.2, for the internal lock[6]. The chemical shifts were determined relative to external 1,4-dioxane, taken as 67.40 p.p.m. relative to tetramethylsilane.

Bioaffinity chromatography. — Succinylated Sepharose CL-6B (410 mg, dry weight) was suspended in water (12 mL), and the pH was adjusted to 4.7. Solutions of D-tryptophan methyl ester (D-TMe, Sigma Chemical Co.; 25 mg in 3 mL of H_2O, pH 4.7) and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (EDAC, Sigma Chemical Co.; 40 mg in 3 mL of H_2O, pH 4.7), prepared just before use, were added to the hydrated gel, and the suspension was automatically rocked for 24 h at 25°. The Sepharose CL-6B-D-TMe was used to prepare a column (0.9 × 10 cm). The gel was successively washed with the following solutions (20 mL of each): M NaCl; 0.1M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), pH 7.6; M NaCl; 0.05M sodium formate, pH 3.0; M NaCl; and 0.05M Tris–0.02M $CaCl_2$, pH 7.6 (application buffer). α-Chymotrypsin (Sigma Chemical Co.; 3.5 mg in 0.7 mL of application buffer) was applied to the column, and the column was washed with the application buffer (24 mL). The bound chymotrypsin was eluted with 0.1M acetic acid–2mM $CaCl_2$, pH 3.0. The flow rate of the column was maintained at 0.5 mL/min, and 2-mL fractions were collected. The elution of the column was monitored at 280 nm, to determine which fractions contained protein.

Assay of chymotryptic activity. — Chymotryptic activity of selected, column fractions was ascertained by incubating a mixture of the column eluate (1.0 mL, adjusted to pH 7.6), 0.06M Tris–0.03M $CaCl_2$ buffer, pH 7.6 (1.7 mL), dimethyl sulfoxide (0.1 mL), and 2.5mM *N*-glutaryl-L-phenylalanine 4-nitroanilide (0.2 mL) for 15 min at 37°. The product, 4-nitroaniline, was determined spectrophotometrically[7] at 420 nm.

RESULTS AND DISCUSSION

The *O*-succinylation of soluble dextran and Sepharose CL-6B was quantitated by spectrophotometric determination of the newly formed ester bonds. The extent of ester formation was dependent on the concentration of succinic anhydride used (see Table I). Analysis of *O*-succinylated dextran by ^{13}C-n.m.r. spectroscopy showed the

TABLE I

EFFECT OF THE CONCENTRATION OF SUCCINIC ANHYDRIDE ON THE EXTENT OF *O*-SUCCINYLATION OF DEXTRAN

Reaction ratio (by weight) of succinic anhydride:dextran	*Ester groups incorporated (μmol of ester:μmol of* D-*glucose residues)*
2:1	1:3
1:1	1:5
1:2	1:8.6

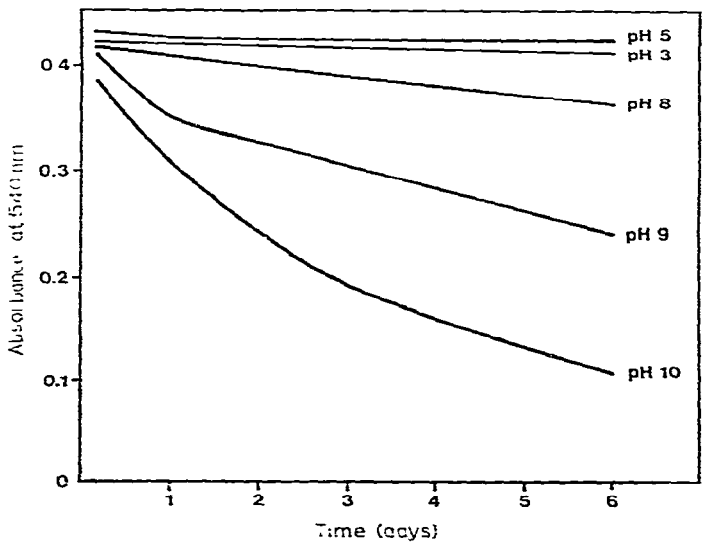

Fig. 1. Stability of succinyl ester bonds at various pH values. (Succinylated dextran was incubated at 25°, and aliquots were periodically removed for ester quantitation.)

presence of several carbonyl resonances, esters and carboxyl groups, in two groupings respectively centered at 174.8 and 179.9 p.p.m., in addition to the strong resonances, indicative of the methylene carbon atoms, at 30.6 and 31.6 p.p.m. The broad range of the carbonyl resonances indicated that ester formation probably involved the various available hydroxyl groups of the polysaccharides.

The possible application of succinylated Sepharose CL-6B in bioaffinity chromatography would be dependent on the pH stability of the ester linkage. The short-term stability of the succinylated Sepharose CL-6B was excellent throughout the pH range of 3 to 8 (see Fig. 1). At pH 8, the ester content of the succinylated dextran decreased by 12% during the 6-day period, and the hydrolysis followed first-order kinetics. At pH 9 or above, extended storage resulted in significant cleavage of ester bonds (see Fig. 1). Storage in a refrigerator or freezer would, presumably, significantly lessen the hydrolysis.

A competitive inhibitor of chymotrypsin, namely, D-tryptophan methyl ester (D-TMe), was coupled to succinylated Sepharose CL-6B *via* the carbodiimide procedure, in order to determine the utility of the newly incorporated *O*-succinyl groups in the preparation of a bioaffinity gel. The chromatography of chymotrypsin on the bioaffinity column showed binding of the enzyme at pH 7.6, and its subsequent elution at pH 3.0 (see Fig. 2). The small amount of unbound protein eluted in fractions 4 and 5 (see Fig. 2) was devoid of enzymic activity, and was, possibly, denatured enzyme. All of the active enzyme applied to the column was bound. The ability of the bioaffinity gel to bind the enzyme effectively was influenced by the quantity of

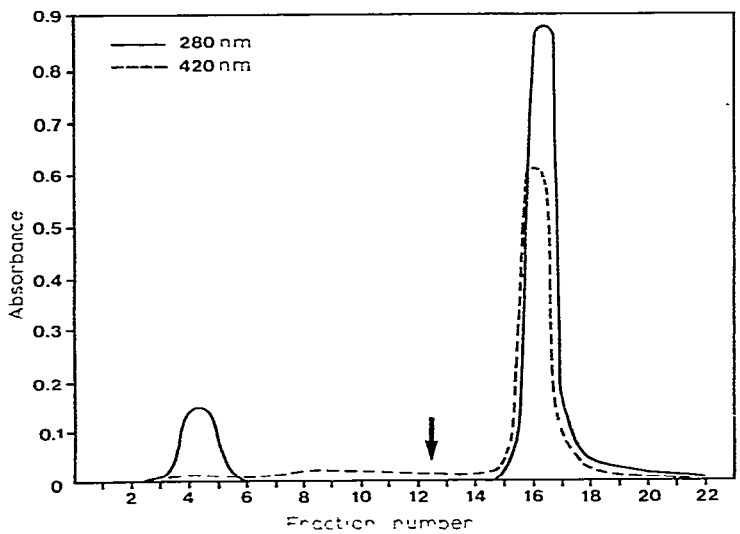

Fig. 2. Bioaffinity chromatography of chymotrypsin on a column of Sepharose CL-6B D-TMe. [Fractions (2.0 mL) were collected, and diluted 1:2 with the appropriate buffer, for absorbance readings at 280 nm. The absorbance at 420 nm is due to 4-nitroaniline released during the assay of chymotrypsin. The arrow indicates the point at which the change in buffer was made (pH 7.6 to pH 3.0).]

D-TMe incorporated. For example, efficient binding of the enzyme occurred when a D-TMe gel prepared by treating Sepharose CL-6B with succinic anhydride at a weight ratio of 5 : 1 was used; this gel had one *O*-succinyl group for every 30 monosaccharide residues. If the weight ratio of succinic anhydride to Sepharose in the reaction was increased to 1 : 1, and the product was coupled with D-TMe, the gel was less efficient in binding chymotrypsin. Bioaffinity gels prepared in this way allowed continuous bleeding of the enzyme from the column, even at pH 7.6. It is postulated that the closer packing of the succinyl-D-TMe groups interfered sterically with the approach of the enzyme.

If Sepharose CL-6B was succinylated, but the product was not subsequently coupled with D-TMe, the gel showed no retention of chymotrypsin. This observation indicated that the retention of the enzyme by the gel was not due to an ion-exchange effect with uncovered carboxyl groups, but was due to interaction of the enzyme with D-TMe.

In studies on the affinity chromatography of bacterial β-D-galactosidase (EC 3.2.1.23), a correlation between the length of the spacer group (connecting the ligand to the polysaccharide matrix) and the enzyme–ligand affinity had been reported[8]. A dramatic increase in the adsorption of the enzyme to the gel was observed if amino groups containing spacer arms were elongated by succinylation[8]. In light of the findings reported here, the enhancement of binding may actually have been due to the formation of *O*-succinyl–ligand groups, thereby providing more sites of attachment for the enzyme, rather than to the greater length of the spacer group. In later work concerning the *N*-succinylation of agarose derivatized with amino groups, the need to wash the coupled product with 0.1M NaOH (to remove "labile carboxyl groups") was recognized[9]. It now appears that these labile groups are attributable to *O*-succinylation.

The industrial application of esterification reactions between acid anhydrides and polymers of D-glucose has been reported. For example, adhesives and thickening agents have been produced from the esterification of starch with succinic anhydride[10], and the reaction between starch and propanoic anhydride affords products useful as paint additives[11]. However, these reactions are conducted at high temperatures under anhydrous conditions, and there do not appear to be any references to *O*-succinylation of polysaccharides under the mild, aqueous conditions under which *N*-succinylation occurs.

Although the ester bond is more labile than the amide bond at alkaline pH, the simplicity of the *O*-succinylation reaction without prior activation of the polysaccharide makes it an appealing adjunct to the methods of matrix modification and bioaffinity chromatography.

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