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Note

The incorporation of amino groups into cross-linked Sepharose by use of (3-aminopropyl)triethoxysilane

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The use of (3-aminopropyl)triethoxysilane (APTS) for the covalent binding of amino groups to glass for the purpose of immobilizing proteins on the glass surface was introduced by Weetall[1] in 1969. The reagent has been used extensively in this respect, because of the great interest in enzyme immobilization[2]. The reaction of APTS with other inorganic surfaces, such as nickel oxide[3] and certain metallic semiconductors[4], has been documented. However, a literature search revealed no reports on the reaction of APTS with polysaccharide-based matrices, such as the agarose gels used for chromatographic analyses. The covalent attachment of amino or carboxyl functional groups to such gels provides sites to which other compounds can, in turn, be attached for the purpose of affinity chromatography. The potential of APTS as a reagent for introducing free amino groups into polysaccharide gels was, therefore, of interest.

The study now described showed that the reaction between APTS and cross-linked agarose (Sepharose CL-6B, Pharmacia) results in the incorporation of amino groups into the agarose in high yield. The reaction is conveniently carried out, and can be completed in the course of one day. Additionally, it was shown that the amino groups couple readily with proteins through reaction with glutaraldehyde.

EXPERIMENTAL

(3-Aminopropyl)triethoxysilane and chlorotrimethylsilane (CTMS) were purchased from Pierce Chemical Company. 2,4,6-Trinitrobenzenesulfonic (picrylsulfonic) acid, sodium salt, was supplied by Aldrich Chemical Company. Alpha-chymotrypsin was purchased from Sigma Chemical Company.

Reaction of the gel with APTS. — Sepharose CL-6B (several grams) was washed

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on a sintered-glass funnel with water, followed by ten 15-mL portions of acetone, and then air-dried.

A 10% solution of APTS in dry acetone was prepared by mixing APTS (1.0 mL), CTMS (0.05 mL), and acetone (8.95 mL). To the dried polymer (1.0 g) in a screw-capped tube was added the 10% APTS reagent (4.0 mL), and the mixture was kept for 5 h in a water bath at 50° with periodic mixing. At the end of this time, the supernatant liquor was decanted, and fresh reagent (4.0 mL) was added. The mixture was kept for an additional 4 h at 50°, with periodic mixing. The gel was thoroughly washed with acetone (ten 20-mL portions) on a sintered-glass funnel, with suction, to remove the excess of the reagent, and then air-dried.

Quantitation of gel-bound amino groups. — Amino groups were quantitated spectrophotometrically with picrylsulfonate by a modification of the procedure described by Lowe and Dean[5]. Samples (25 mg each) of dried, reacted gels were each suspended in 2M acetic acid (2.0 mL) and heated in a boiling-water bath for 2 h, to solubilize the samples. 2M NaOH (2 mL) followed by saturated sodium borate (4.0 mL) were then added to the solutions. To each solution was added a 6% aqueous solution (0.1 mL) of picrylsulfonate, sodium salt, and each was kept for 4 h at 24°, and diluted 1:20 with 50% acetic acid: the absorbance at 340 nm was then determined. Amino groups were quantitated by employing a standard curve constructed from known amounts of APTS that had reacted with picrylsulfonate exactly as described for the gel samples.

Elemental analysis for nitrogen was performed on the reacted gels by Atlantic Microlab, Inc., Atlanta, Georgia.

Immobilization of chymotrypsin. — Samples of APTS-reacted Sepharose and unreacted Sepharose (100 mg of each) were separately suspended in 2.6 mL of 0.1M potassium phosphate buffer, pH 7.5, to which was added 25% glutaraldehyde (0.4 mL). The suspensions were kept for 5 h at 37° with occasional mixing, and then thoroughly washed with water on a sintered-glass funnel, with suction, to remove unreacted glutaraldehyde.

To each of the washed samples was added 2.0 mL of the 0.1M potassium phosphate buffer, pH 7.5, containing 6 mg of α-chymotrypsin (mL). The samples were gently rocked mechanically for 17 h at 4°. Unbound chymotrypsin was removed by centrifugally washing the gels with eight 8-mL portions of M sodium chloride–0.1M sodium acetate buffer, pH 4.0, followed by six washings with 0.06M Tris, pH 7.6.

Test for immobilized enzyme-activity. — Enzymic activity was tested by suspending 10 mg each of the washed gels in 3.0 mL of the assay buffer (0.06M Tris, pH 7.6, containing 0.03M $CaCl_2$) to which was added dimethyl sulfoxide (0.12 mL). The suspensions were preincubated for 10 min at 37°, and then 0.25 mL of the substrate [0.9 g/100 mL of *N*-glutarylphenyl-L-alanine *p*-nitroanilide (GPNA) dissolved in Me_2SO] was added. The activity was monitored by determining the increase in absorbance at 420 nm as the yellow *p*-nitroaniline was released by the enzyme. Absorbances were determined on the supernatant liquors following separation from the gels by centrifugation.

The presence of immobilized enzyme was also ascertained by elemental analysis for nitrogen.

RESULTS AND DISCUSSION

Quantitation of incorporated amino groups. — The colorimetric method for the estimation of incorporation of amino groups, based on the reaction of the derivatized gel with picrylsulfonate, indicated a loading of 352 μmol of aminoalkyl groups/g of dry gel. The nitrogen content, determined by elemental analysis, was found to be 0.65%, equivalent to 450 μmol/g of dry gel. The reason for the difference in results between the two analytical methods has not been investigated. A possible explanation, however, may be that the presence of reacted picrylsulfonate groups may hinder the approach of unreacted reagent, resulting in the failure of some amino groups to combine with the picrylsulfonate.

The incorporation of more than 400 μmol of N/g of dry gel represents a considerably greater loading with aminoalkyl than the 80–90 μmol reported for porous glass[5].

Determination of immobilized enzyme. — The amount of chymotrypsin bound to the gel was established by subtracting the nitrogen content of the APTS-reacted Sepharose from the nitrogen content of the APTS-Sepharose that had been coupled with chymotrypsin. The percentage of nitrogen in the enzyme-coupled gel was found to be 0.89%. The (aminoalkyl)ated preparation (no chymotrypsin) contained, as indicated previously, 0.65% of N, resulting in 0.24% of N being attributed to the protein. Assuming the protein-nitrogen content to be 16%, it was, therefore, established that 15 mg of the enzyme was bound per g of Sepharose.

Retention of enzymic activity by the immobilized enzyme was clearly demonstrated. Reaction of the enzyme–gel preparation with GPNA as the substrate resulted in an increase in the absorbance (A) at 420 nm that was linear throughout the first 10 min of incubation. The ΔA/min during this period of time was 0.08. The unreacted Sepharose control, which had also been allowed to react with glutaraldehyde and chymotrypsin, was essentially devoid of enzymic activity. The specific activity of the bound enzyme was not determined.

In this study, it was found that chymotrypsin can be readily coupled to (3-aminopropyl)diethoxysilyl*-Sepharose CL-6B, with retention of activity. It was also observed that the (aminoalkyl)silylation of the Sepharose gel was affected by the temperature of the reaction and the concentration of the APTS reagent. Chlorotrimethylsilane is routinely used as an adjunct in the formation of trimethylsilyl ethers for analysis by gas–liquid chromatography, and may function similarly in this reaction.

A convenient method for the introduction of amino groups into Sepharose gel is described. The derivatized gel was shown to be a suitable matrix for the immo-

*Or, if cross-linked, containing one, or no, ethoxy group.

bilization of chymotrypsin. The possibility of applying this reaction to the production of modified gels for the immobilization of a broad range of substances is suggested.

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REFERENCES

1 H. H. WEETALL, *Science*, 166 (1969) 615–617.
2 S. P. COLOWICK AND N. O. KAPLAN, *Methods Enzymol.*, 44 (1976).
3 H. H. WEETALL AND L. S. HERSH, *Biochim. Biophys. Acta*, 206 (1970) 54–60.
4 I. HALLER, *J. Am. Chem. Soc.*, 100 (1978) 8050–8055.
5 C. R. LOWE AND P. D. G. DEAN, *Affinity Chromatography*, Wiley, New York, 1974, pp. 253–254.