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Preparation of Modified Agarose Gels Containing Thiol Groups

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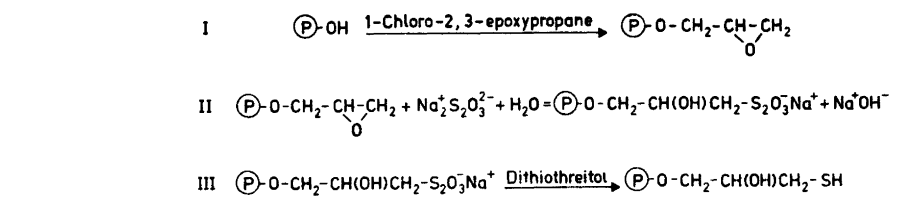
A simple and efficient route for preparation of thiol-agarose derivatives of various degrees of substitution is described. The technique for introducing thiol-groups in the agarose gel network involves the following steps: (a) introduction of epoxide groups; (b) treatment by sodium thiosulfate to convert epoxide groups into *S*-alkyl-thiosulfate structures; (c) reductive cleavage of the alkyl thiosulfate structures by dithiothreitol.

Chemists concerned with biochemical research are to an increasing extent making use of immobilized biological activity as a means of conversion and separation. Many adsorbents for bioaffinity chromatography are based on beaded agarose gels [1] and enzymes have been immobilized by their attachment to such gels.[2] Thiol-group containing agarose gels would be of particular interest in this connection on account of the variety of reactions thiol groups may undergo even under mild conditions in water solution. Products containing S – C, S – S, S – O, and S – metal bonds may thereby be formed.

Thiolated agarose gel beads have been prepared by Cuatrecasas [3] from ω-amino alkyl agarose by reaction with homocysteine thiolactone. The ω-aminoalkyl derivatives were prepared by the cyanogen bromide technique. The thiolated products were, upon further derivatization, used as adsorbents for bioaffinity chromatography. Brocklehurst *et al.*[4] prepared thiolated agarose by direct coupling of glutathione to cyanogen bromide activated agarose gel without protecting the thiol group. The glutathione gel was derivatized with 2,2′-dipyridyl-disulfide and the disulfide derivative used for the preparation of fully active papain from dried powder of papaya latex. The chromatographic procedure used involved specific formation of a disulfide linkage between the thiol gel and the active site thiol-group of papain and subsequent breaking of the disulfide by means of a low-molecular weight thiol. The technique was described as covalent chromatography. Subsequently, Carlsson *et al.* demonstrated the usefulness of glutathione-agarose for the immobilization of urease.[5] In this connection it should be mentioned that Eldjarn *et al.*[6] prepared a thiolated Sephadex derivative which, after mercuration, was used to selectively isolate thiol-proteins from proteins not containing thiol groups. The thiolated Sephadex was also used to prevent autooxidation of biologically important thiols in biological materials.[7]

We wish to report a simple and efficient route for the preparation of thiol-agarose derivatives of various degrees of substitution. The thiolated agarose gels described above have a thiol content of < 50 μmol/g dried polymer. With the presented procedure, substitution degrees of 800 – 900 μmol/g are obtained. The technique is mild enough not to destroy the gel beads and involves chemical cross-linking of the gel to make it more stable to leakage. The attachment of thiol groups *via* extended hydrocarbon chains can be performed. The method is based on the reactions given in Scheme 1.

The initial epoxidation step was performed essentially according to Sundberg and Porath.[8] As reagents 1-chloro-2,3-epoxypropane (epichlorohydrin) or, in order to get an extended arm, 1,4-bis(2,3-epoxypropoxy)butane have been applied. Introduction of epoxide structures was governed by varying the amount of reagent. The epoxide gel was then treated with a sodium thiosulfate solution to transform the epoxide structures to *S*-alkyl-thiosulfate structures.[9] The reaction is accompanied by a release



Scheme 1.

of OH^-, which can be analytically utilized for determining the degree of substitution by titration with hydrochloric acid (Table 1). Finally the thiosulfate ester gel was reduced by dithiothreitol.[10] The products were analyzed for sulfur and thiol groups (Table 1).

While the thiosulfate ester gels are stable, the thiol gels oxidize when stored in water suspension with the formation of S—S-structures. The oxidation is particularly rapid when the gel is highly substituted with thiol groups. Such gels, having partially lost their original content of thiol groups can become fully restored to the original thiol group content by reduction with dithiothreitol.

Experiments show that the thiol groups of the thiolated agarose gels are so reactive that the reactions with thiol groups which are known to occur in solution are possible to perform even on the gel and under such mild conditions that the fragile gel structure is preserved. The thiolated gels have been utilized in this laboratory as a medium for sorption processes based on thiol-disulfide interchange reactions and on metal-complex formation reactions. They have been applied as electron exchangers and as water-insoluble metal-complexing agents. The possibility of attaching ligands *via* stable thioether bonds to the gel carrier has been utilized to prepare adsorbents for bioaffinity chromatography. The experimental details will be given in separate communications.

In this paper we want to exemplify the use of the thiolated agarose by immobilizing urease according to the technique of Carlsson *et al.*[5] The thiol gel is transferred to a 2-pyridyl-disulfide gel by means of 2,2′-dipyridyl-disulfide. The content of the reactive 2-pyridyl-disulfide structures was determined by Kjeldahl analyses (Table 2). A solution of a urease preparation was passed through a column of the disulfide gel. An enzyme conjugate of high protein content and high catalytic activity was obtained (Table 2). The column with immobilized enzyme could then be used for continuous hydrolysis of urea. During the immobilization step the urease preparation was purified by a factor of about 8.

Table 1. Preparation of thiolated agarose gels (Sepharose 6B). Beads of agarose were activated to various degrees of substitution with 1-chloro-2,3-epoxypropane or 1,4-bis(2,3-epoxypropoxy)-butane by variation of the amount of reagent. The epoxide-activated gels were treated with sodium thiosulfate solution and the obtained *S*-alkyl-thiosulfate structures were finally reduced with dithiothreitol. Analytical data.

	"Epoxide-activation" (ml reagent/3 g gel)	Epoxide gel; μmol epoxide structure/g dried product	Thiol gel; μmol S/g dried product	Thiol gel; μmol SH groups/g dried product
1-Chloro-2,3-epoxy-propane	(0.45	670	690	660
	(0.15)	310	220	170
	(0.075)	70	55	50
1,4-Bis(2,3-epoxy-propoxy)butane	(5)	1050	850	860
	(1.2)	460	370	410
	(0.3)	120	105	110

Table 2. Chemical fixation of urease to thiolated agarose (Sepharose 2B) by activation with 2,2′-dipyridyl-disulfide.

Carrier	Thiol-Sepharose
Thiol content, μmol/g	440
Content of 2-Py−S−S−, μmol/g	270
Content of urease in conj., mg/g conj.	320
Activity, μmol NH_3/min mg	
free urease	84
bound urease	695

METHODS*

Preparation of epoxide-activated agarose gel beads.[8] *(a) By 1-chloro-2,3-epoxypropane.* Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) was washed on a glass filter with distilled water and sucked free from interstitial water. The gel beads (3 g) were suspended in 2.4 ml 1 M sodium hydroxide solution. 1-chloro-2,3-epoxypropane (various amounts; see Table 1) was added slowly under stirring at room temperature, whereupon the temperature was increased to 60 °C and maintained for 2 h. The activated gel was washed with distilled water on a glass filter until neutral. The product is not stable enough to store.

(b) By 1-4-bis(2,3-epoxypropoxy)butane. Sepharose 6B (3 g), washed as above, was suspended in 1.6 ml 2.5 M sodium hydroxide solution, and 12 mg sodium borohydride was added. 1,4-bis(2,3-epoxypropoxy)butane (EGA-Chemie, Steinheim, Switzerland) (various amounts; see Table 1) was slowly added under stirring. Reaction time 6 h at room temperature. The activated gel was washed with distilled water until neutral, with 20 ml acetone and finally with water. The product is not stable enough to store.

Epoxide group analyses. The reaction between epoxide groups and sodium thiosulfate which proceeds with release of OH^- can be used to determine the amount of epoxide structures in the gel. An amount of activated gel corresponding to 10 − 100 μmol of epoxide groups was suspended in water to a volume of 10.0 ml suspension. The pH of the suspension was corrected to 7.0. The suspension was stirred and 1.00 ml was transferred to a plastic titration vessel by means of an Oxford pipette with plastic tip. One ml of 2 M sodium thiosulfate solution (pH 7) was added to the gel suspension. The pH was kept constant by addition of 50 mM HCl by means of a pH-stat. The reaction mixture was stirred. The time for titration was 30 − 120 min. The dry weight content of the gel suspension was determined by transferring 5.00 ml gel suspension to a small glass filter and washing with water and water/acetone mixtures of increasing concentration of acetone. The gel was dried over P_2O_5 at 100 °C *in vacuo* for 20 h and weighed.

Preparation of S-alkyl-thiosulfate agarose gel beads. Epoxide activated Sepharose 6B (3 g) was washed on a glass filter with 0.5 M phosphate buffer (4.1 g $NaH_2PO_4.H_2O$ + 2.8 g $Na_2HPO_4.2H_2O$ dissolved in 100 ml distilled water, pH = 6.3). The gel was sucked free from interstitial buffer and suspended in the same buffer to a final volume of 6 ml. A solution of 2 M sodium thiosulfate (3 ml) was added and the reaction mixture was shaken for 6 h at room temperature. The gel was washed free from sodium thiosulfate with distilled water. The thiosulfate ester gel, suspended in distilled water, is suitable for storage.

Reduction of S-alkyl-thiosulfate gel. The thiosulfate ester gel (3 g) was suspended in 0.1 M. sodium bicarbonate solution. Total volume 6 ml. Dithiothreitol was used in at least twofold excess. The value of epoxide content could thereby be used. Dithiothreitol (*threo*-1,4-dimercapto-2,3-butandiol, Aldrich Chem. bo., Milwaukee, Wisc., USA) was dissolved in 3 ml 1 mM EDTA-solution. The reaction time was 30 min at room temperature. The gel was washed on a glass filter with 30 ml 0.1 M sodium bicarbonate solution (1 M in sodium chloride and 1 mM in EDTA) and finally with 100 ml 1 mM EDTA-solution.

The thiolated gel was normally used directly. For a couple of days the gel can be stored in 0.01 M deaerated sodium acetate buffer of pH 4 (1 mM in EDTA). Upon storage of a highly substituted thiol gel under these conditions for 1 month, a decrease of the thiol content by 80 % was found. Upon renewed reduction by dithiothreitol the original thiol content was obtained.

Thiol group analyses. Thiol content was determined by means of 2,2′-dipyridyl-disulfide according to Brocklehurst *et al.*[4] but 0.1 M sodium bicarbonate buffer was used instead of Tris buffer. The results are given in Table 1. The thiol content could also be determined by means of Ellman's reagent. The sulfur content of the gel was determined according to Gustavsson.[11]

Preparation of 2-pyridyl-disulfide-agarose gel beads. Thiol agarose gel beads were prepared from Sepharose 2B according to the procedures described above by means of 1-chloro-2,3-epoxypropane (0.15 ml reagent per 3 g gel). The thiol gel (3 g) was washed on the glass filter with 60 % acetone-40 % 0.05 M sodium bicarbonate solution (1 mM in EDTA). The gel was suspended in the same solvent to a total volume of 5 ml. A 0.3 M solution of 2,2′-dipyridyl-disulfide (10 ml) in the above-mentioned solvent system was prepared. The suspension of thiol was rapidly added to the solution of 2,2′-

* The preparations described have also been performed on a 10-fold larger scale.

dipyridyl-disulfide. The mixture was stirred during the reaction; reaction time 1 h at room temperature. The product was washed with 60 % acetone in water and finally with 1 mM EDTA solution. The degree of substitution was determined by nitrogen determination according to Kjeldahl. The product is stable to storage.

Immobilization of urease. A reactor column was prepared by pouring a suspension of 2-pyridyl-disulfide-Sepharose 2B into a Perspex tube (⌀ 10 mm). The height of the packed gel bed was 22 mm. The gel was washed for 4 h with 0.1 M Tris-HCl buffer of pH 7.2 (0.1 M in potassium chloride and 1 mM in EDTA), flow rate 5.1 ml/h. The tube was connected to a peristaltic pump. A solution of urease (Sigma type IV) (150 mg) in 50 ml of the above Tris buffer system was prepared and passed through the column. The column was washed with the buffer until A_{280} and A_{343} had decreased to the base line level.

The activities of free and bound urease were determined.[5] The protein content of the gel and the protein concentration of the solutions of free urease were determined by amino acid analyses (Table 2).

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