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COVALENT COUPLING OF UNSATURATED COMPOUNDS TO THIOL AGRAROSE USING γ-RADIATION

A New Method for Preparation of Adsorbents for Affinity Chromatography

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Unsaturated compounds can be attached to thiol polymers by means of γ-radiation. By this method several agarose conjugates have been synthesized from thiol agarose gel and different ligand derivatives (testosterone, estradiol, 3-octenoic acid, and hexa-2,4-dienoic acid). The amount of fixed ligand is about 60–120 μmol/g of dried conjugate. The method described is a valuable complement to the currently available procedures for the preparation of adsorbents for affinity chromatography.

INTRODUCTION

Affinity chromatography has become a valuable tool for the isolation and purification of biologically active substances, and a great number of methods (1–3) have been described for the preparation of the affinity chromatography adsorbents used. The most attractive and satisfactory adsorbents have been prepared by covalent attachment of the ligands to an insoluble carrier, and for this purpose many organochemical reactions have been utilized. The available methods for covalent attachment of ligands depend on the presence of particular functional groups in the ligands to be coupled. Despite the fact that several different functional groups can be utilized (e.g., amino, carboxyl, hydroxyl, and thiol groups) it can still be a rather difficult task to immobilize certain ligands by means of existing coupling reactions. One source of difficulty might be that functional groups suitable for coupling are lacking or cannot be utilized because they are essential for maintaining the biological specificity of the ligand. However, derivatives containing a carbon–carbon double bond are available for

many ligands. In these cases coupling via the double bond is very attractive, but as far as we know this possibility has not yet been utilized for the preparation of affinity chromatography adsorbents.

However, it has been reported earlier that thiol-containing substances can add to carbon–carbon double bonds upon radiation (4, 5). We have utilized this reaction as the basis for a new coupling method for the preparation of affinity chromatography adsorbents, using thiol agarose gel and a suitable unsaturated derivative of the ligand to be coupled.

MATERIALS AND METHODS

Thiol agarose was prepared from Sepharose 2B (Pharmicia Fine Chemicals, Uppsala, Sweden) according to the method of Axén et al. (6). The agarose was reacted with 1,4-bis-(2,3-epoxypropoxy)butane, obtained from EGA Chemie, Steinheim, Switzerland (0.5 ml reagent/3 g gel). The epoxy-activated gel was treated with sodium thiosulfate and the obtained *S*-alkylthiosulfate structures were reduced with dithiothreitol (Sigma Chemical Company, St. Louis, Missouri) immediately prior to use. This procedure gave a thiol content of 165 μmol/g dried gel. The thiol content of the gel was determined with 2,2′-dipyridyl disulfide (Aldrich-Europe, Beerse, Belgium) according to Grassetti and Murray (7) with the application to determinations in gels introduced by Brocklehurst et al. (8). The bovine serum albumin (type II) used in the affinity chromatography experiment was purchased from Sigma Chemical Company.

For γ-irradiation a ^{60}Co source was used with a dose rate of 30 rads/sec. The temperature was kept at about 20°C. Amounts of hexa-2,4-dienoic acid (sorbic acid, obtained from Hopkin & Williams, Romford, England) and 3-octenoic acid (Koch-Light Laboratories, Colnbrook, England) immobilized on the thiol agarose were determined by pH titration as described by Rybák et al. (9).

The steroids were determined with use of radioactive compounds. [1,2,6,7(n)-^{3}H]Testosterone and [4-^{14}C]estradiol-17β were obtained from The Radiochemical Centre, Amersham, England. Before use the radioactive compounds were diluted with the corresponding unlabeled steroids obtained from Sigma Chemical Company to give specific activities of 5.7 and 5.4 mCi/mol, respectively. The radioactivity was measured with 0.5-g samples of gel suspended in 5.0 ml Instagel (Packard Instruments) in a Beckman LC 100 S liquid scintillation counter. Observed values were compared with a standard curve made with known amounts of radioactive steroids in 5.0 ml Instagel containing 0.5 g swollen Sepharose 2B.

RESULTS

Samples of freshly prepared thiol agarose gels (3 g) were carefully washed with 99% ethanol. The samples were then suspended in glass tubes containing 50 mg of unsaturated ligand derivative in 5 ml of 99% ethanol. The derivatives used were octenoic acid, sorbic acid, testosterone, and estradiol. Dissolved oxygen was removed from the samples by flushing nitrogen gas through the suspensions for 30 min. The tubes were then stoppered and exposed to 0.5 Mrad of γ-radiation. After irradiation the samples were carefully washed on a glass filter funnel with the following solutions: 99% ethanol; acetone; distilled water; 0.2 M formic acid/formate buffer, pH 3.0, containing 1 M KCl; 0.1 M ammonia/ammonium acetate, pH 9.2, containing 1 M KCl; and finally with distilled water. The amount of ligand fixed to the gel was then determined and the result is shown in Table 1. It is evident that 35–75% of the gel thiol groups were utilized for immobilization of ligands. Nearly all thiol groups not involved in the coupling of ligand were recovered after reduction of the gel conjugate with dithiothreitol (20 mM for 30 min at pH 8.0). Thiol groups remaining after irradiation (20% of original value), which may interfere in affinity chromatography experiments, may be masked by blocking with iodoacetamide or *N*-ethylmaleimide.

Parallel experiments were performed without irradiation under otherwise identical conditions. No reaction between the unsaturated compounds and thiol agarose was then detected (i.e., less than 1 μmol of ligand/g dry weight). This shows that irradiation was necessary for the induction of coupling and that the washing procedure employed was sufficient.

To investigate the involvement of thiol groups in the coupling reaction, the experiments were repeated with agarose (Sepharose 2B) containing no thiol groups. Irradiation of agarose in the presence of testosterone

TABLE 1. **Coupling of Unsaturated Ligand Derivatives to Thiol Agarose (165 μmol SH/g dried gel)**

Ligand derivative	Amount of ligand fixed to thiol agarose (μmol/g dried conjugate)
Hexa-2,4-dienoic acid	98
3-Octenoic acid	123
Testosterone	69
17β-Estradiol	59

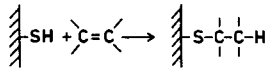

FIG. 1. Reaction scheme for the coupling of unsaturated carbon–carbon bond containing ligands to thiol agarose.

and estradiol gave conjugates containing only small amounts of coupled ligands (less than 5% of those obtained with thiol agarose).

The stability of the steroid conjugates upon storage in 50% ethanol at 4°C was tested by measuring the radioactivity in the supernatant. The loss of ligand from the gel conjugate after 5 months was less than 5%.

An affinity chromatography experiment was performed using the adsorbent prepared by coupling 3-octenoic acid to thiol-Sepharose as described earlier. To a 10-ml bed of the adsorbent 5 ml of 0.5% bovine serum albumin solution in 0.1 M phosphate buffer, pH 7.0, was applied. After washing with 0.5 M NaCl bound protein was desorbed by elution with 0.1 M phosphate buffer, pH 7.0, containing 8 M urea. Spectrophotometric measurement of the eluate at 278 nm showed that 17 mg albumin could be desorbed in that way. The capacity of this adsorbent is thus 95 mg albumin/g dried conjugate.

DISCUSSION

The results given herein show that compounds containing carbon–carbon double bonds can be immobilized on thiol agarose by γ-radiation. The coupling of unsaturated compounds to thiol agarose probably proceeds via a reaction involving radical species generated by the γ-radiation. We propose that the overall reaction results in the formation of a thioether bond, as shown in Fig. 1. The synthesis of thioethers from alkylthiols and unsaturated hydrocarbons by radiation has been described earlier (4, 5). Such reactions may also be initiated by chemical radical formation. However, γ-radiation is more effective and preferable from the technical point of view (5).

The chemical structures formed in the described process are very stable and leakage of ligand from the conjugates is low. The conjugates are well suited for use in affinity chromatography. In this sense the described procedure is a valuable complement to the currently available coupling methods, as it extends the possibilities for preparing suitable adsorbents by coupling the ligand via unsaturated carbon–carbon bonds.

NOTE ADDED IN PROOF

Thiol agarose is now commercially available (Pharmacia Fine Chemicals, Uppsala, Sweden). The use of this product should greatly facilitate the preparation of ligand conjugates according to the described procedure.

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