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Note

Affinity chromatography on immobilized S-adenosyl-L-homocysteine

Purification of a furanocoumarin O-methyltransferase from cell cultures of *Ruta graveolens* L.

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To a large extent, the present interest in affinity chromatography for enzyme purification originates from the paper of Cuatrecasas *et al.*[1]. This study, in which the name affinity chromatography was used for the first time, stimulated an extensive use of this method in the isolation of enzymes, their inhibitors, antibodies, antigens, nucleic acids and a great number of other products, as evidenced by many literature references. The ever increasing number of commercially available insoluble affinants is the best evidence of the rapid development and important role of this method[2].

Agarose, a polydextran carrier, is the most common support in affinity chromatography to date. Cuatrecasas[3,4] has reported the preparation of a great number of agarose derivatives and various groups can therefore be employed for binding. These derivatives are available now as commercial products and include, for example, AH-Sepharose 4B (agarose with covalently bound 1,6-diaminohexane) which binds compounds through their carboxyl groups via a soluble carbodiimide. In this communication we wish to report an affinity system in which S-adenosyl-L-homocysteine was coupled to AH-Sepharose 4B by the carbodiimide coupling procedure (Fig. 1). The potential usefulness of this affinity system is illustrated by results of its application to the purification of an O-methyltransferase which catalyses the transfer of a methyl group from S-adenosyl-L-methionine to a phenolic furanocoumarin in *Ruta graveolens*[5]. Mack and Slaytar[6] have recently used a similar affinity system for the purification of an indole ethylamine N-methyltransferase from *Phaoaris tuberosa*.

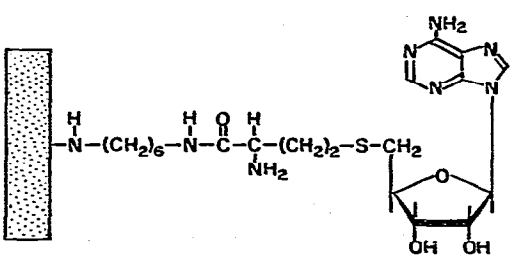

Fig. 1. Structure of S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B. The shaded rectangular area to the left represents the Sepharose matrix.

MATERIALS AND METHODS

Materials

These were obtained as follows: AH-Sepharose 4B from Pharmacia (Uppsala, Sweden); S-adenosyl-L-homocysteine from Sigma (St. Louis, Mo., U.S.A.); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl from BioRad Labs. (Richmond, Calif., U.S.A.); [$^{14}CH_3$]S-adenosyl-L-methionine (Amersham, Chicago, Ill., U.S.A.) had a specific activity of 58 mCi/mmole.

Methods

Enzyme isolation. Cells from cultures of *R. graveolens*, maintained as previously described[7], were harvested 6 days from transfer, at which time the O-methyltransferase activity was near its maximum[5]. A crude extract of the enzyme was prepared[5] and filtered through a G-25 Sephadex column (26 × 1.4 cm) equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.5) at 4°. The Sephadex eluate was used in the experiments reported here.

Activity assay. Fractions emerging from the chromatographic columns were monitored for O-methyltransferase activity against bergaptol (5-hydroxypsoralen) as the substrate, with [$^{14}CH_3$]S-adenosyl-L-methionine as the methyl donor. Enzyme activity was expressed as nmoles of [$^{14}CH_3$]bergapten (5-methoxypsoralen) formed per hour at 30°, under standard assay conditions[5].

Protein determination. Protein concentration was determined by the newly developed procedure of Bradford[8,9]. Bovine γ-globulin was used as the protein standard.

Preparation of the affinity matrix: carbodiimide coupling of S-adenosyl-L-homocysteine to AH-Sepharose 4B. All operations were performed at room temperature unless otherwise stated. Freeze-dried AH-Sepharose 4B (2 g) was swelled in an excess of 0.5 *M* sodium chloride solution (50 ml) and allowed to stand for 15 min after gentle stirring. The gel was washed with 0.5 *M* sodium chloride solution (400 ml) in order to remove the lactose and dextran present originally in the Sepharose 4B powder. Sodium chloride was then removed by washing the gel with 100 ml of deionized water (previously adjusted to pH 4.6). The ligand, S-adenosyl-L-homocysteine, was dissolved in 8 ml of deionized water (pH 4.6) to give a final concentration of 3.85 mg/ml, and added to the gel. The pH was 5.6 after mixing, and was adjusted to 4.6 by addition of dilute HCl. Solid carbodiimide powder (230 mg) was added, in small batches at regular intervals, to the slurry to give a final concentration of 0.1 *M*. The pH was maintained at 4.6–6.0 for 1 h, and the reaction was allowed to proceed for another 24 h. The gel was finally washed with deionized water (pH 4.6) and stored at that pH at 4°. Before use, the gel was equilibrated with 0.05 *M* Tris–HCl buffer, (pH 7.5) at 4° and packed into a column.

RESULTS AND DISCUSSION

The ligand, S-adenosyl-L-homocysteine, was selected as an ideal derivative for coupling to AH-Sepharose 4B for the purification of O-methyltransferase, since it has a strong inhibitory effect in S-adenosyl-L-methionine-homocysteine methyltransferase systems, including the O-methyltransferase reactions[10,11]. When the Sephadex G-25 eluate containing the crude O-methyltransferase from *R. graveolens* cell cultures

was applied to the ligand-bound AH-Sepharose 4B column at the optimal pH of the enzyme (Tris–HCl buffer, pH 7.5) and the affinity column developed at this pH, a protein peak containing no methyltransferase activity appeared between 1 and 2.5 elution volumes (Fig. 2). No O-methyltransferase appeared in the eluate even after more than 4 elution volumes of buffer had passed through the column. It appeared, therefore, that inactive protein passed through the column unretarded, but that the O-methyltransferase was retarded owing to its interaction with the S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B. As shown also in Fig. 2, the O-methyltransferase began to be eluted about one elution volume after the developing solvent was changed to 0.1 *M* acetic acid (pH 3). No additional protein was eluted when the column was washed with excess of 0.1 *M* acetic acid (pH 3). The specific activity of the enzyme was constant across its elution profile, showing that the enzyme was free of any inactive protein contaminants. This chromatographic procedure resulted in a 50-fold increase in the specific activity of the O-methyltransferase.

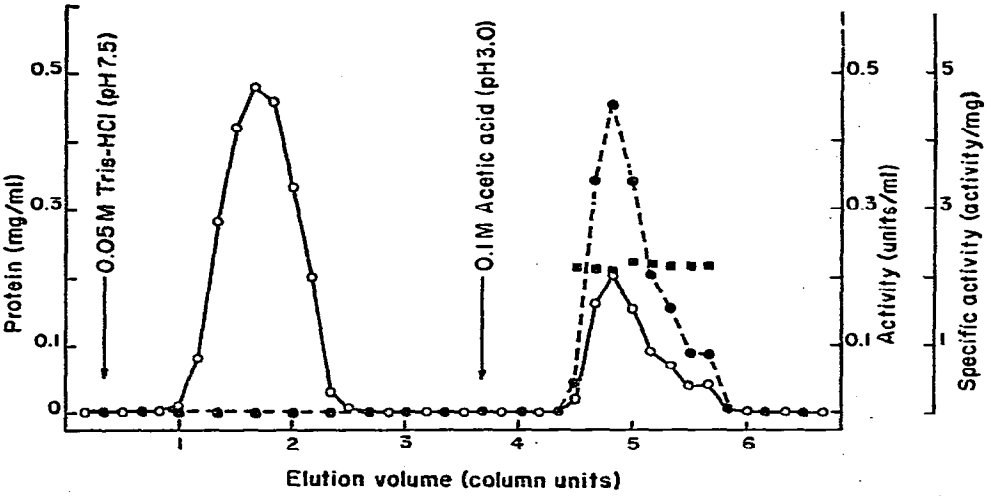

Fig. 2. Affinity chromatography of O-methyltransferase on a column (15 × 0.7 cm) of AH-Sepharose 4B coupled with S-adenosyl-L-homocysteine. Elution volume (in column volume units) was measured from the time of application of the sample, and the flow-rate was 28 ml/h. ○—○, Protein; ●—●, O-methyltransferase activity; ■, specific activity. Since O-methyltransferase from *R. graveolens* cell cultures is believed to be unstable at low pH[5], fractions eluted in 0.1 *M* acetic acid were collected in test tubes containing 0.5 ml of 0.05 *M* Tris–HCl buffer (pH 7.5) in order to raise the pH immediately.

In the preparation of sorbents for affinity chromatography, the introduction of functional groups into the agarose gel carrier is usually by treatment with cyanogen bromide followed by reaction with a bifunctional alkylamine. Recently it has been observed that coupling of aliphatic amines to agarose by the cyanogen bromide reaction yields isourea linkages which are positively charged at pH 7 (ref. 12). The presence of these charged sites in affinity gels causes a significant non-specific adsorption of proteins[12,13]. In order to test for non-specific adsorption in our "affinity gel" due to ionic binding, the same G-25 Sephadex eluate containing the O-methyltransferase was chromatographed on a "control gel". This gel was composed of

Sepharose 4B substituted with the spacer-arm assembly, and bearing no bio-specific ligand (S-adenosyl-L-homocysteine). As shown in Fig. 3, the adsorption–elution behaviour of the O-methyltransferase on this "control gel" is entirely different from its behaviour on the "affinity gel" (see Fig. 2). The active O-methyltransferase was found to be unretarded and eluted in 0.05 *M* Tris–HCl buffer (pH 7.5). The specific activity varied across the elution profile (Fig. 3) indicating the presence of other inert proteins. These results demonstrate clearly, again, that the process involved in the separation is related to the functional ability of the enzymatic binding site for the specific ligand (S-adenosyl-L-homocysteine) attached to AH-Sepharose 4B.

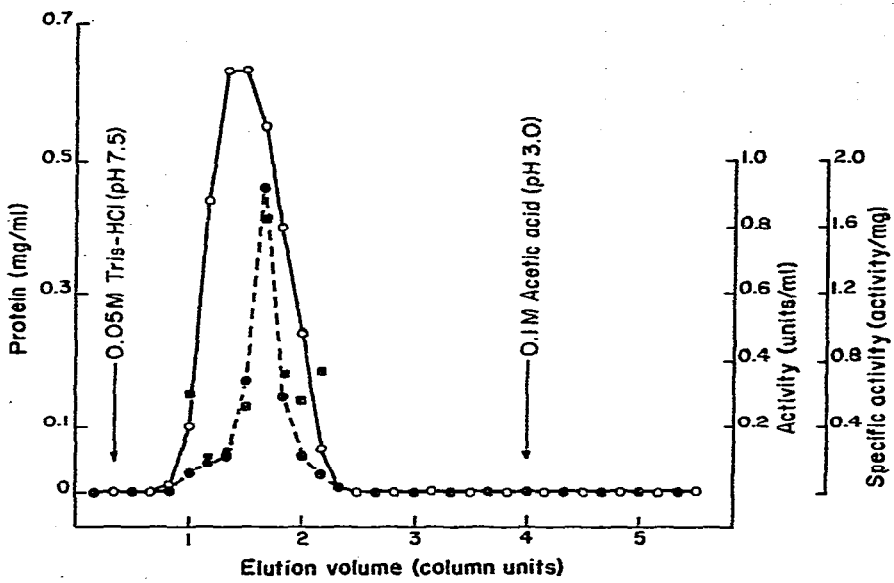

Fig. 3. Chromatography of O-methyltransferase on a ligandless "control gel", *i.e.*, matrix-spacer arm assembly bearing no bio-specific ligand (S-adenosyl-L-homocysteine). The size of the column was 13 × 0.7 cm and the flow-rate was 22 ml/h. Elution volume was measured from the time of application of the sample. ○—○, Protein; ●—●, O-methyltransferase activity; ■, specific activity.

All experiments were carried out using a buffer, 0.05 *M* Tris–HCl (pH 7.5), as the column eluent. The columns were washed with about ten elution volumes of buffer between chromatography of successive samples and were re-usable within two weeks of preparation. Chemical stability of our "affinity gel" under operating conditions seems likely to be a problem, however, because the adsorbent lost its characteristic binding ability after two weeks in operation at pH 7.5 and 4°. This could have been due to exposure of the bio-specific ligand to the action of degradative enzymes presumed to be present in the crude extracts of *R. graveolens* cell cultures.

Finally, the significance of affinity chromatography using S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B as described above could go well beyond the purification of O-methyltransferases. This follows because many multi-substrate enzymes employ, as one of their substrates, a ligand common to many other enzymes (for example: ATP, NAD^+). It has been suggested that immobilized forms of such substrates, or their analogues, would constitute "general ligands" whose wide spe-

cificity would make them useful in the purification of a wide range of enzymes[14–16]. The ligand, S-adenosyl-L-homocysteine, may therefore be another example of a "general ligand" for methyltransferases, as is NAD^+ among dehydrogenases. If this is so, it should be possible to apply our affinity system also for the purification of other methyltransferases, such as N-methyltransferases.

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NOTE ADDED IN PROOF

While this paper was in press, Kim *et al.*[17] reported the use of essentially the same affinity system described above for the purification of an S-adenosylmethionine: protein carboxyl O-methyltransferase from calf brain.

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