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APPLICATION OF 1,1'-CARBONYLDIIMIDAZOLE-ACTIVATED AGAROSE FOR THE PURIFICATION OF PROTEINS

II. THE USE OF AN ACTIVATED MATRIX DEVOID OF ADDITIONAL CHARGED GROUPS FOR THE PURIFICATION OF THYROID PROTEINS

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SUMMARY

The use of 1,1'-carbonyldiimidazole-activated agarose for biospecific affinity chromatography is described. Activation of agarose with this carbonylating reagent gives a matrix devoid of additional charged groups. Conditions for the coupling of a range of ligands and leashes have been evaluated. The efficient purification of bovine trypsin, human thyroglobulin and sheep thyroid membrane glycoproteins demonstrates the suitability of the new activated matrix for affinity chromatography.

INTRODUCTION

Since its introduction in 1968 by Anfinsen and co-workers[1], affinity chromatography has become a widely used technique for the isolation and purification of numerous water-soluble biologically active molecules[2]. This technique exploits the biological specificity of those subtsances which can form stable, reversible complexes with matrix-bound ligands. In these circumstances, non-complementary constituents in a mixture pass through an immobilised adsorbent unretarded whilst leaving behind adsorbed molecules, having an affinity for the ligand, to be eluted after some appropriate modification in the chromatographic conditions. The ligand can be coupled either directly to a suitably activated insoluble matrix such as a beaded agarose or via a leash or spacer which generally has the effect of increasing the efficiency of the affinity adsorbent[1]. The most widely used method for the activation of polysaccharide supports involves treatment of the matrix with aqueous cyanogen bromide[3] at alkaline pH, which can still present difficulties in handling and reproducibility despite recent improvements in the procedure[4,5]. Ligands or suitable leashes, containing free amino groups, are subsequently coupled to the imidocar-

bonate activated-matrix. Under these coupling conditions, the matrix–ligand or matrix–leash derivatives which are formed involve positively charged N-substituted isoureas and N-substituted imidocarbamates. These derivatives can behave as "ion-exchange" groups resulting in enhanced non-specific adsorption to the affinity columns[6,7]. In response to this problem there has been a resurgence of interest in new methods for the activation of polysaccharides in order to eliminate potential non-specific interferences due to the activating linkage. We have recently shown[8] that 1,1′-carbonyldiimidazole (CDI) and related carbonylating reagents are suitable for the activation of cross-linked agaroses, and other polysaccharides, for use in affinity chromatography. The intermediate activated matrix (an imidazolyl carbamate), formed under these conditions, couples smoothly with N-nucleophiles such as free amino groups present in ligands or leashes to give a nonbasic urethane (N-alkyl-carbamate) derivative. This method thus provides supports for affinity chromatography which are devoid of addition charged groups by the activation linkage. In this study, we describe the application of CDI-activated cross-linked agarose for the purification of a variety of proteins.

EXPIERMENTAL

Materials

Cross-linked agarose (Sepharose CL-6B) was purchased from Pharmacia (Uppsala, Sweden), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulphonate (CMC) from Merck (Rahway, N.J., U.S.A.) and CDI from Pierce (Rockford, Ill., U.S.A.). All other chemicals were of reagent grade. Trypsin (Type III), soyabean trypsin inhibitor (Type II-s) and *p*-aminobenzamidine hydrochloride came from Sigma (St. Louis, Mo., U.S.A.) and concanavalin A from Pharmacia. Crude human thyroglobulin was extracted from human thyroids by the method of Salvatore *et al.*[9] and chromatographed on Sepharose CL-4B using a 150 m*M* NaCl–10 m*M* Tris–HCl, pH 8.0, buffer as reported previously[10]. The sheep thyroid plasma membrane glycoproteins, possessing binding activity for human thyroid stimulating autoantibodies present in sera of patients with Graves' disease, were isolated by a solubilisation procedure to be reported. Binding activity was determined by the MacKenzie mouse bioassay[11]. Antisera to human thyroid extracts, thyroglobulin and serum proteins were prepared by established methods.

Activation and coupling procedures

The activation of Sepharose CL-6B with CDI and the coupling of ligands and leashes were carried out by methods similar to those reported previously[8]. In brief, Sepharose CL-6B was washed sequentially with water, dioxane–water (3:7), dioxane–water (7:3) and dioxane (20 ml of each for 3 g of moist gel cake) and the washed gel suspended in dioxane. Activation with CDI (0.93 mmol reagent for 3 g of moist gel cake) was carried out for 15 min to give an activated gel containing 46.4 μmol active groups per ml moist gel. The above material, after treatment with 6-aminohexanoic acid overnight at 4° and pH 10 (1 *M* Na_2CO_3) gave no reaction with trinitrobenzenesulphonate and by titration analysis the product contained 0.44 mmol/g dry gel of COOH end-groups. The 6-aminohexanoic acid derivative of Sepharose CL-6B was then treated at pH 4.7 for 24 h with *p*-aminobenzamidine in

the presence of CMC. Soyabean trypsin inhibitor, concanavalin A, anti-whole human serum, bovine thyrotrophin, porcine insulin, human immunoglobulin IgG and 3,3',5-triiodothyronine were attached directly to CDI-activated Sepharose CL-6B using a 0.5 *M* NaCl–1 *M* Na_2CO_3 buffer (pH 9.0), 1 *M* Na_2CO_3 buffer (pH 10) or a 0.1 *M* borate buffer (pH 8.5). Since the activation yields are high, the coupling of large amounts of ligand requires high concentrations of buffers to prevent pH changes. In a separate experiment, soyabean trypsin inhibitor was coupled to Sepharose CL-6B via the 6-aminohexanoic acid leash with CMC.

Affinity chromatography

Affinity chromatography was generally carried out at 20° using 1–30 ml of appropriate affinity adsorbent packed in Pasteur pipettes or glass columns of 16 mm I.D.

(i) A sample of crude trypsin (20 mg) in 500 m*M* NaCl–50 m*M* Tris, pH 8.0 (8 ml) was loaded onto a soyabean trypsin inhibitor–Sepharose CL-6B column from 1 g of moist cake activated with 0.15 g CDI, and 15 mg of soyabean trypsin inhibitor equilibrated in the same buffer. Desorption of the bound trypsin was carried out with 500 m*M* NaCl–3 m*M* HCl. The chromatographic profile is shown in Fig. 1. Trypsin was analysed directly by UV spectroscopy at 280 nm.

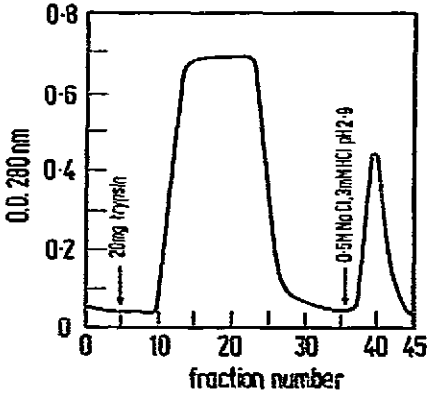

Fig. 1. Purification of crude bovine trypsin with a soyabean trypsin inhibitor-affinity column. Crude bovine trypsin (20 mg) in 500 m*M* NaCl–50 m*M* Tris, pH 8.0, was loaded onto a soyabean trypsin inhibitor–Sepharose CL-6B column (from 1 g of moist cake activated with 0.15 g CDI and 15 mg soyabean trypsin inhibitor) equilibrated in the same buffer. At the point indicated, the buffer was changed to 500 m*M* NaCl–3 m*M* HCl to elute the bound trypsin.

(ii) Sepharose CL-4B chromatographed human thyroglobulin (15 mg) in 20 m*M* Tris–HCl, pH 7.2 was loaded onto a rabbit anti-whole human serum–Sepharose CL-6B column (10 ml) (prepared from CDI-activated Sepharose CL-6B and 10 ml rabbit anti-whole human serum coupled in the usual bicarbonate buffer, pH 9.0) equilibrated in the same buffer. The non-bound protein peak and the adsorbed protein peak recovered when the eluent was changed to 200 m*M* glycine–HCl–500 m*M* NaCl, pH 2.8, were collected, dialysed and lyophilised. The recovered proteins were analysed by crossed immunoelectrophoresis (Fig. 2).

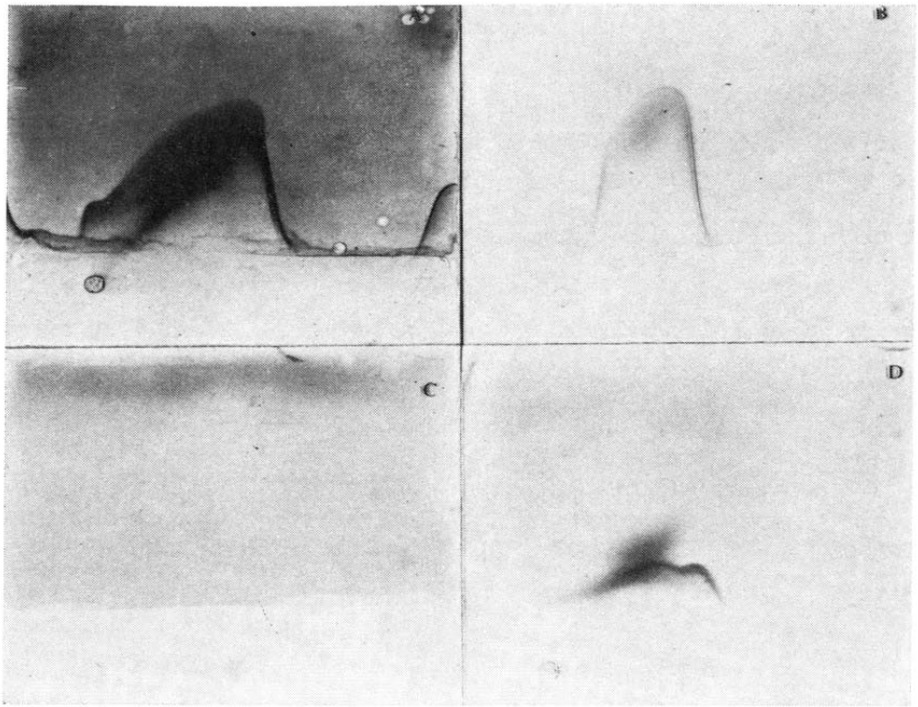

Fig. 2. Crossed immunoelectrophoresis of Sepharose CL-4B fractionated human thyroglobulin (A), affinity purified human thyroglobulin (B) and (C) and human serum protein impurities recovered from the anti-whole human serum adsorbent (D). The antibody containing gels were: 100 μl of rabbit anti-human thyroid extract serum (A and B) and 100 μl of rabbit anti-human whole serum (C and D). Electrophoresis in the second dimension was carried out at 0.5 V/cm for 18 h.

(iii) A sample of sheep thyroid membrane proteins (100 mg) in 150 m*M* NaCl–1 m*M* $MnCl_2$–1 m*M* $MgCl_2$–1 m*M* $CaCl_2$–0.02% NaN_3, pH 7.4 (5 ml) was loaded onto a concanavalin A–Sepharose CL-6B column (from 30 ml activated gel containing *ca.* 50 μmol active groups per ml and 120 mg concanavalin A) equilibrated in the same buffer. After the non-bound components had been eluted (150 ml), 100 m*M* methyl α-D-mannoside was added to the running buffer. The chromatographic and activity profile is shown in Fig. 3.

Subsequent to the completion of these studies, Pierce Chemical Co. has agreed to make the activated matrix commercially available as Reacti-Gel (6X).

RESULTS AND DISCUSSION

The preparation of biospecific adsorbents for affinity chromatography requires efficient activation and coupling methods for the attachment of ligands to suitable matrices. Because of its good chromatographic properties and mechanical stability, particularly when cross-linked with epihalohydrins and other cross-linking reagents[12], beaded agarose has been used most frequently as the inert carrier matrix. Although a variety of activation procedures and coupling strategies are now available the rigorous restraints dictated by the nature and stability of the moeity to be purified,

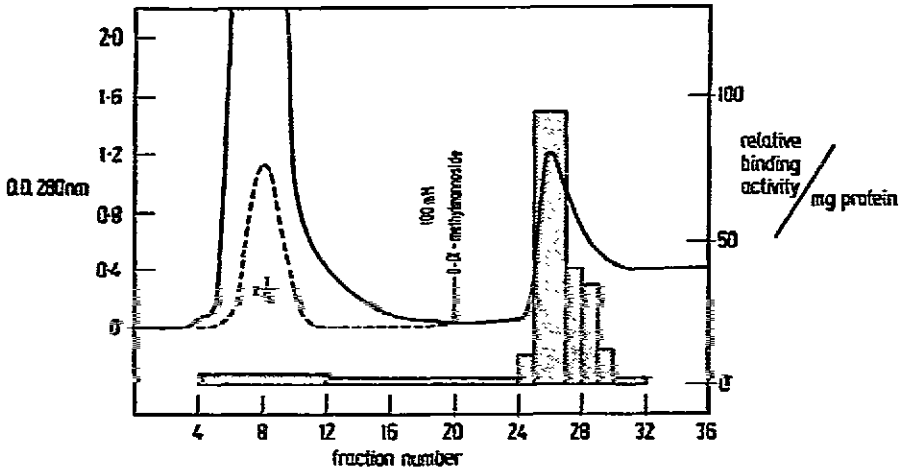

Fig. 3. Fractionation of partially purified soluble sheep thyroid plasma membrane proteins on a column of concanavalin A–Sepharose CL-6B prepared by the CDI approach. The arrow indicates the start of elution with 100 m*M* methyl α-D-mannoside. Relative binding activity for a standardised Graves' serum containing thyroid stimulating autoantibodies was determined by the MacKenzie mouse bioassay[11], protein concentration were determined by the Bradford assay[22].

associated with the requirements for a suitable affinity system of high activation yields, the absence of introduced non-specific charged groups and the formation of very stable linkages between the ligand and matrix, limit the suitability of many of the previously published methods[13,14]. Although it does introduce non-specific charged groups into the carrier support which can affect the biological specificity of the column, the activation of polysaccharide supports with alkaline cyanogen bromide has been up till now the simplest and most widely used method. Treatment of cross-linked agarose with CDI in dioxane or acetone at room temperature for 15 min is an alternative and very efficient activation procedure which, however, satisfies all the above requirements.

In order to determine the suitability of CDI-activated Sepharose CL-6B for use in the preparation of biospecific adsorbents, a preliminary series of coupling experiments was carried out. The results of these experiments are found in Table I. These examples were designed to demonstrate that biologically active molecules could be linked to the polysaccharide matrix by the CDI method under conditions which allowed retention of their activity. Besides the proteins examined, a series of coupling experiments with amino-compounds of different pK_a values were carried out to determine the comparative reactivities of the CDI-activated matrix towards amines and water. The amino-compounds selected were 6-aminohexanoic acid ($pK_a \approx 11$), glycine (pK_a 9.8), glycylglycine (pK_a 8.1) and 3,3',5-triiodothyronine (pK_a 10.0). Above pH 11 the coupling yields decreased rapidly presumably due to hydrolysis of the active groups. However, in the range pH 8–10, good coupling yields were obtained provided the buffer capacity was sufficiently high to prevent significant pH changes. The CDI-activated matrix is relatively stable to hydrolysis. At pH 9.0 and pH 10.0, the total time to hydrolyse all the active groups is 30 h and 10 h, respectively. At the completion of coupling a ligand to the CDI-activated matrix, unreacted active groups

TABLE I

Compound	*Concentration on the insoluble support (μmol/g)*
Glycine	194*
6-Aminohexanoic acid	130*
Glycylglycine	80*
3,3′,5-Triiodothyronine‡	52**
Porcine insulin‡‡	4.5***
Bovine thyroid stimulating hormone‡‡‡	3.6***
Human IgG	1.5***
Human thyroglobulin†	0.6***

* The figures were obtained by potentiometrical titration under nitrogen of the end groups on the matrix using 2 *M* KOH from pH 3 to 11.7 with a Radiometer TTT2 automatic titration assembly.

** Determined by N elemental analysis.

*** Determined by amino acid analysis of a 0.6 g sample which had been hydrolysed with 6 *N* HCl, 110°, 24 h.

‡ One ml of gel completely neutralised a 1:100 dilution of rabbit anti-triiodothyronine serum.

‡‡ Porcine insulin can couple[21] covalently to Sepharose CL-6B through Phe B1 or Lys B29, depending on the reaction conditions. The adsorbent used is a mixture of both coupling sites. One ml of gel neutralises a 1:100 dilution of guinea pig anti-insulin serum. The bound antibody could be eluted from the adsorbent by lowering the pH of the reaction buffer (50 m*M* phosphate– 500 m*M* NaCl, pH 7.8) with 1 *M* HCl.

‡‡‡ One ml of gel completely neutralises a 1:32 dilution of a rabbit anti-bovine-TSH serum.

† The binding capacity of this adsorbent for human thyroglobulin autoantibodies is identical to the corresponding CNBr-activated matrix, *ca.* 4 mg/ml gel.

can be blocked with ethanolamine. Generally, this blocking step is not necessary if coupling reactions are left for more than 12 h at pH 10.0 and 48 h at pH 9.0. Where possible, couplings should be carried out at a pH which is at least one unit above the pK_a value of the ligand or leash. It was apparent from these studies that coupling yields, based on the available active groups on the matrix, were of the order of 45%. Although similar coupling yields are observed with CNBr-activated polysaccharides, it should be borne in mind that the activation yields, *i.e.*, the number of active groups introduced into the matrix, obtained with CNBr are significantly lower, *e.g.*, 2% yield based on CNBr reagent[15] whilst 40–45% yields were reproducibly obtained with the CDI reagent. A comparison of the coupling rates and yields of CNBr- and CDI-activated Sepharose CL-6B with simple ligands and with soyabean trypsin inhibitor suggest that CNBr-activated matrices is somewhat more reactive than the corresponding CDI-activated material. However, other considerations indicate that the CDI-matrix is superior for the preparation of affinity supports. Firstly, CDI activation can give a matrix of much higher substitution than can be achieved with CNBr. Such an activated matrix should be extremely useful for coupling small amounts of proteins particularly in circumstances where a biologically active ligand is difficult to obtain and the yield based on protein is more important than that based on the matrix. Secondly, preliminary results indicate that CDI-activated agarose couples more efficiently with higher concentrations of simple ligands. This property of the CDI-activated matrix, which allows the preparation of "high density leash" matrices without introducing ion-exchange groups, will be described in more detail elsewhere.

Illustrative of the use of CDI-activated agarose, are the results obtained with the well known model affinity chromatographic system, namely, the purification of trypsin using different ligands. Fig. 1 shows the elution profile for the bioaffinity separation of a commercial bovine trypsin preparation on a soyabean trypsin inhibitor–Sepharose CL-6B column. The adsorbent used in this separation had a trypsin capacity of 5.5 mg/ml gel. The corresponding *p*-aminobenzamidine–6-aminohexanoic acid–Sepharose CL-6B and soyabean trypsininhibitor–6-aminohexanoic acid–Sepharose CL-6B adsorbents had a trypsin capacity of 12.4 and 3.6 mg/ml gel, respectively. The capacity of the soyabean trypsin inhibitor column prepared by direct reaction of the protein with CDI-activated Sepharose CL-6B compared favourably with values obtained by Hixon and Nishikawa[16] with similar matrices activated by the CNBr method whilst the *p*-aminobenzamidine–6-aminohexanoic acid–Sepharose CL-6B (CDI method) affinity support has a trypsin capacity nearly ten times greater than the *p*-aminobenzamidine–hexamethylenediamine–Spheron P300 (CNBr method) affinity support[17]. It is also interesting to compare these results with the different trypsin capacities obtained with the *p*-aminobenzamidine– and soyabean trypsin inhibitor–6-aminohexanoic acid–Sepharose CL-6B adsorbents prepared by the CDI method. As has been noted previously with adsorbents prepared with small ligands coupled to activated matrices, *e.g.*, sulphanilamide for the purification of carbonic anhydrase[18,19], the presence of an interlocking leash allows high capacities presumably by avoiding steric interactions with the matrix. In the experiment reported above, dissociation of the trypsin-inhibitor complex was achieved with an 500 m*M* NaCl–3 m*M* HCl. These conditions do not permit resolution of the different active forms of trypsin. This should however be possible using pH gradient elution conditions similar to those used with bisoxirane-coupled soyabean trypsin inhibitor agarose[19].

Fractionation of thyroid extracts by gel chromatography yields thyroglobulin preparations contaminated with small, but significant amounts of serum proteins[10,20]. Antisera raised in rabbits or guinea pigs with these thyroglobulin preparations contain antibodies to these impurities as well as to thyroglobulin. However, the antigenic serum proteins can be removed simply with a rabbit anti-whole serum immunoadsorbent prepared from CDI-activated Sepharose CL-6B. In this case, the thyroglobulin emerges as the non-bound protein peak, whilst the serum proteins can be subsequently eluted with 200 m*M* glycine–HCl–500 m*M* NaCl, pH 2.8. Using this method, human thyroglobulin of high purity can be obtained. The crossed immunoelectrophoresis of the Sepharose CL-4B partly purified human thyroglobulin and the affinity purified human thyroglobulin, together with the recovered serum proteins, shown in Fig. 2, clearly indicates the absence of the serum protein impurities in the human thyroglobulin sample after affinity chromatography. Antisera raised with human thyroglobulin preparations purified in this manner fail to react with normal serum proteins and are consequently well suited for sensitive radioimmunoassay measurement of circulating levels of human thyroglobulin in normal and pathological sera.

As a further example of the use of a biospecific adsorbent derived from CDI-activated agarose, the purification of solubilised thyroid plasma membrane glycoproteins, which show binding activity for thyroid stimulating autoantibodies, may be included. Using a concanavalin A–Sepharose CL-6B column, previously

activated with CDI, a partially purified sheep binding protein preparation was resolved into a non-bound major inactive fraction and a bound minor active fraction (Fig. 3). In view of the possible involvement of plasma membrane glycoprotein antigens and the membrane receptor for thyrotrophin in the aetiology of Graves' autoimmune disease, much effort has recently been devoted to the purification of soluble thyroid glycoproteins. The above procedure provides a very facile approach for the preparation of relatively large quantities of these molecules.

In summary, the data presented here shows that CDI-activated agarose can be used for the preparation of biospecific adsorbents. An important feature of this new method is its ability to provide supports for affinity chromatography which are devoid of charge groups caused by the linkage. Stable, high capacity adsorbents can be easily and reproducibly prepared. Their specific use for the efficient isolation of a variety of different proteins described above clearly indicate that this new approach is a potentially useful alternative to existing methods for the purification of biologically active substances by affinity chromatography.

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