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Received April 13, 1978

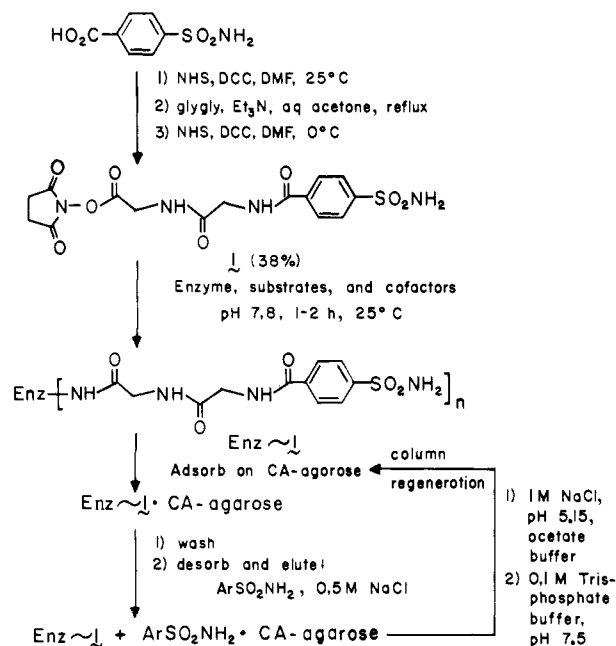
Generalized Affinity Chromatography: Enzyme-Sulfonamide Conjugates Can Be Isolated by Adsorption on Immobilized Carbonic Anhydrase¹

Sir:

The practicality of synthetic procedures involving enzymes as catalysts often depends on the ease with which these enzymes can be recovered for reuse.² Enzyme recovery is commonly facilitated by immobilization on an insoluble matrix.³ Immobilization is, however, not always practical, and in several circumstances it may be advantageous to employ a soluble enzyme: when a substrate, product, or cofactor of the enzyme is itself insoluble; when the enzyme is deactivated by immobilization; when association of the enzyme with other macromolecules is required for activity;⁴ or when diffusion of substrate through an immobilizing matrix is rate limiting. In principle, recovery of soluble enzymes from synthetic reaction mixtures can be accomplished by a number of conventional techniques for isolating proteins; in practice, each has disadvantages. Ultrafiltration may denature enzymes by shear at, or adsorption on, the filter face; further, it involves apparatus not commonly available in organic laboratories. Gel permeation and ion-exchange chromatographies and protein precipitation techniques are inconvenient when applied to large volumes of solution containing low concentrations of enzyme. Affinity chromatography requires both the development of an appropriate adsorbent for each enzyme,⁵ and a preliminary separation of reactants, products, and cofactors which compete with the affinity ligand for the enzyme active site.

To circumvent the limitations of conventional affinity chromatography for enzyme recovery from synthetic reaction mixtures, we have developed a variant on this technique which utilizes a single affinity column to isolate previously modified enzymes from solution. In this procedure, the enzyme of interest is first coupled covalently with an aryl sulfonamide moiety. The enzyme-sulfonamide conjugate may subsequently be isolated easily from reaction mixtures by adsorption on a column of immobilized carbonic anhydrase (CA, from bovine erythrocytes, E.C. 4.2.1.1) and desorption by treatment with solutions of either 0.01 M *p*-toluenesulfonamide or 1.0 M sodium chloride (Scheme I). We have selected carbonic anhydrase as the adsorbing protein for several reasons: it is commercially available and inexpensive; it is easily immobilized in good yield; it does not catalyze reactions likely to destroy probable reactants or products of enzyme-catalyzed synthetic processes; it binds a wide variety of sulfonamide derivatives at its active site with affinities sufficient to make biospecific adsorption easily practical, but not so high as to make desorption difficult or slow ($K_i \approx 10^{-7}$ M).⁶ Since aryl sulfonamides are readily manipulated synthetically, generation of appropriate reagents for the preparation of protein-sulfona-

Scheme I. Preparation and Affinity Adsorption of Enzyme-Sulfonamide Conjugates (Enz-1) (NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide)



mid conjugates is straightforward. We have found **17** to give good results (Scheme I).⁸

Compound **1** was coupled with three representative enzymes, glucose 6-phosphate dehydrogenase (G-6-PDH, from *Torula* yeast, E.C. 1.1.1.49), hexokinase (from Baker's yeast, E.C. 2.7.1.1), and lysozyme (from egg white, E.C. 3.2.1.17) (all from Sigma Chemical Co.) to yield enzyme-sulfonamide conjugates according to the following general procedure. A glass vial was charged with 200 μ L of 2 M HEPES buffer (pH 7.8), 50–100 μ L of solution containing enzyme (50–100 U of G-6-PDH or hexokinase; 10⁵ U of lysozyme), and 40–100 μ L of solution containing appropriate substrates and cofactors in concentrations well above their Michaelis constants.⁹ A 10- μ L aliquot of a solution of **1** (0.4 μ mol) in dimethyl sulfoxide was added to the vial, mixed, and allowed to remain at room temperature for 1–2 h. At the end of this coupling period, solutions of G-6-PDH, hexokinase, and lysozyme retained respectively 60, 90, and 90% of their original enzymatic activities.¹⁰ The reaction mixture was loaded onto a column containing CA immobilized on cyanogen bromide activated Sepharose 4B.¹¹ The column was washed with 50 mL of 0.1 M Tris-phosphate buffer (pH 7.5), to remove unbound enzyme, and then 50 mL of the same buffer containing 0.01 M *p*-toluenesulfonamide ($K_{i,C_7H_7SO_2NH_2} = 1.0 \times 10^{-7}$ M)⁶ and 0.5 M NaCl ($K_{i,Cl^-} = 0.05$ M)⁶ to elute the enzyme-**1** conjugate.¹²

Average yields of activities eluted by the sulfonamide-containing buffer were 50% for G-6-PDH, 80% for hexokinase, and 50% for lysozyme,¹⁴ based on the original activities of the native enzymes prior to the coupling reaction. The proteins eluted with sulfonamide were retained essentially quantitatively on reapplication to the column of immobilized CA, and are assumed to be modified by the covalent attachment of at least one sulfonamide moiety. Figure 1 illustrates representative chromatographic behavior of enzyme-**1** conjugates. These plots indicate that the retention volume of the conjugates is not related to that of the native enzymes and is apparently not influenced by the presence of a large quantity of an unfunctionalized protein (here, horseradish peroxidase) in solution. Moreover, elution of adsorbed enzyme-**1** conjugates from the CA column can be accomplished easily and in high yield.

Since column chromatography may be inconvenient in

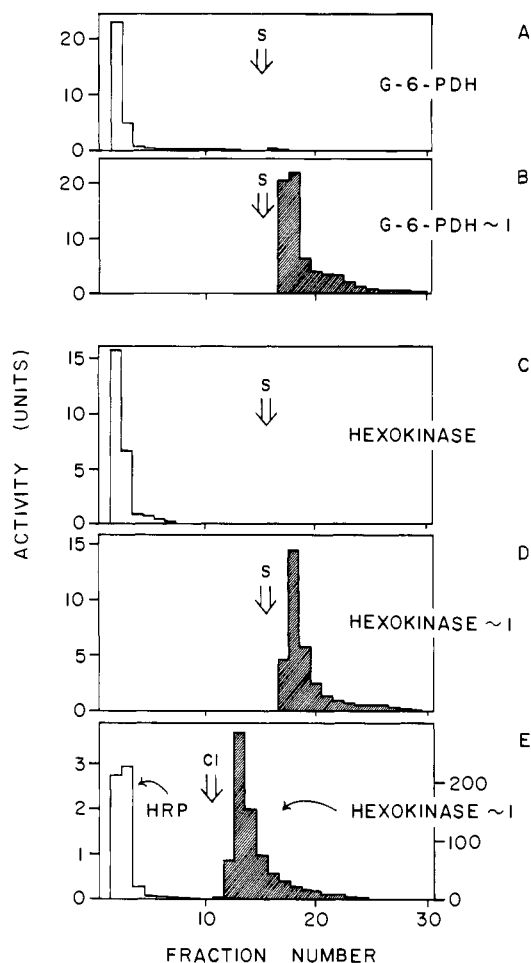


Figure 1. Chromatographic behavior of enzyme-sulfonamide conjugates on a 10-mL column of immobilized carbonic anhydrase ($0.2 \mu\text{equiv mL}^{-1}$): A, native G-6-PDH; B, G-6-PDH-I; C, native hexokinase; D, hexokinase-I; E, a mixture of hexokinase-I (0.03 mg) and horseradish peroxidase (HRP, 10 mg). Units of G-6-PDH and hexokinase activities are displayed on the left axis; units of HRP activity are displayed on the right axis. Each fraction has a 5-mL volume. The initial eluting buffer in each plot is 0.1 M Tris-phosphate (pH 7.5). At the position indicated by the arrow, the buffer is changed to S, 0.1 M Tris-phosphate (pH 7.5) containing 0.5 M NaCl and 0.01 M *p*-toluenesulfonamide; Cl, 0.1 M acetate buffer (pH 5.15) containing 1 M NaCl.

certain synthetic applications of enzymes, we established that the affinity of CA for sulfonamide moieties was sufficient that it was possible to isolate an enzyme-I conjugate by nonchromatographic adsorption. A 1.00-mL solution containing 16 U of hexokinase-I was added to a beaker containing 10 mL of CA-agarose ($\sim 0.2 \mu\text{equiv mL}^{-1}$) suspended in a total volume of 25 mL of 0.1 M Tris-phosphate buffer (pH 7.5); the nominal concentration of hexokinase-I in this mixture was $0.1 \mu\text{M}$. The mixture was stirred for 2 h at 6°C and then filtered and washed with 25 mL of 0.1 M buffer. The filtrate contained 0.6 U (4%) of the hexokinase-I. Buffer (25 mL) containing 0.01 M *p*-toluenesulfonamide and 0.5 M sodium chloride was added to the gel and the mixture stirred for 10 min at 25°C . The gel was filtered and washed with 25 mL of *p*-toluenesulfonamide/NaCl buffer; this filtrate contained 15.4 U (96%) of the hexokinase-I.

The potential of this generalized affinity chromatography system for the recovery of enzymes from synthetic reaction mixtures was demonstrated on a small scale for hexokinase-I and lysozyme-I. A solution of hexokinase-I (41 U) was transferred to a reaction flask containing ATP (3.0 mmol), glucose (3.3 mmol), and magnesium acetate (0.45 mmol) in 20 mL of water (pH 8.0). This reaction mixture was stirred at

room temperature and maintained between pH 7.8 and 8.3 by addition of 1 N NaOH solution until 78% of the glucose had been converted to glucose 6-phosphate. The reaction mixture was loaded on the CA column and eluted in two fractions with 50 mL of 0.1 M Tris-phosphate (pH 7.5) followed by 50 mL of 0.01 M *p*-toluenesulfonamide and 0.5 M NaCl in the same buffer; $95 \pm 5\%$ of the hexokinase-I was recovered in the fraction eluted by the second buffer solution. Addition of barium acetate (4.60 g, 18 mmol) to the first fraction yielded barium glucose 6-phosphate (0.832 g, 50%, 94% pure); no effort was made to optimize this yield. In a related experiment, lysozyme-I (22 000 U) was added to a turbid suspension of *Micrococcus lysodeikticus* (300 mg/L) in 0.05 M phosphate buffer (pH 9.0) and stirred at room temperature until a clear solution was obtained. Adsorption on and elution from the CA column afforded 100% recovery of the lysozyme-I conjugate in the biospecifically eluted fraction.

The isolation of enzyme-I conjugates by affinity chromatography over immobilized carbonic anhydrase is a particularly convenient, rapid, and gentle procedure. It should find use in organic syntheses based on enzymes and in other areas requiring the recovery of proteins from solution.

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- (12) The presence of 0.5 M NaCl in the toluenesulfonamide-containing buffer gave sharper elution profiles, probably both by reducing nonspecific ionic interactions between the protein and the column¹³ and by competing for the active site of CA.⁶ When it was inconvenient to remove the toluenesulfonamide from the recovered enzyme solution, elution with 1.0 M NaCl in 0.1 M acetate buffer (pH 5.15) also yielded sharp elution profiles. In this eluting system, both Cl^- and Ac^- ($K_i = 0.03 \text{ M}$) compete with the enzyme-I for the CA active site.
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Received December 15, 1977