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An Improved Method for the Purification of Carbonic Anhydrase Isozymes by Affinity Chromatography

Methods for purifying carbonic anhydrase (EC 4.2.1.1.) isozymes, carbonic anhydrase B (or I) and C (or II), by affinity chromatography have been described by Falkbring *et al.* (1) and Whitney (2), in which affinity gels are formed by coupling, respectively, *p*-aminobenzenesulfonamide, and *p*-(aminomethyl)benzenesulfonamide to Sepharose polysaccharides by means of cyanogen bromide activation. Attempts to repeat these methods in our laboratory were not satisfactory as the reproducibility and yields were variable. It is possible that the susceptibility of the linkage to esterase hydrolysis by carbonic anhydrase could account for this variability. It has been suggested (3) that the linkage given by cyanogen bromide activation is of an ester type, i.e., —C—O—CO—NH—*R*, where *R* represents a functional group, and it was possible to hydrolyse this linkage by heating at about 80°C and pH values between 8 and 12. When purified carbonic anhydrase at pH 9.0 was passed through columns of this type of gel, cleavage of the sulfonamides occurred; and in particular, the cleavage products were readily observed when highly colored azosulfonamides were coupled. Also the total distance of the coupled inhibitor from the gel matrix, given by these methods, does not exceed the depth of the active site cleft (4). Therefore, to overcome these factors, gels were prepared by coupling sulfonamides to CM Sephadex using a water-soluble carbodiimide (5) to form a peptide bond between the carboxyl and amino groups. Two sulfonamide inhibitors were used, *p*[(2,4-diaminophenyl)azo]benzenesulfonamide (Prontosil), and *p*-(aminomethyl)benezenesulfonamide. The azosulfonamide coupled gel had the advantages of a higher capacity and a red-colored product that enabled visual estimation of coupling efficiency.

For recent reviews of carbonic anhydrase isozymes see Lindskog *et al.* (6), and Carter (7).

METHODS

CM Sephadex was obtained from Pharmacia and all other reagents from Aldrich. Prontosil was prepared by diazotization of *p*-aminobenzenesulfonamide and coupling to *m*-phenylenediamine as previously described (8). The affinity gel was prepared by coupling the sulfonamide to CM Sephadex by the method of Hoare and Koshland (5). Ten grams of CM Sephadex (C50 coarse grade) was added to 300 ml of 50% ace-

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tone containing 5 g of the sulfonamide. The pH was adjusted to 4.75, and 5 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (freshly dissolved in 10 ml of H_2O) was added dropwise to the stirred suspension, maintaining pH 4.75 by the addition of 1 M H_2SO_4. The reaction mixture was gently stirred overnight at 22°C. The gel was washed in a 600 ml coarse Buchner funnel using, progressively, acetone, 50% acetone, and hot water at pH 10, until free of excess inhibitor.

The capacities of the gels were determined in the following manner. Approximately 2 ml of affinity gel equilibrated in 0.1 M Tris–SO_4/0.2 M Na_2SO_4 pH 9.0 was equilibrated with 90 mg of purified human carbonic anhydrase B and then transferred to a 1.5 cm i.d. column. The excess enzyme was eluted and the amount determined using a molar extinction value of 49,000 M^{-1} cm^{-1} at 280 nm. The bound enzyme was eluted with 0.4 M KI/0.1 M Tris–SO_4, pH 7.0, and the amount determined as above. Finally the gel was transferred to a tared 20 ml Buchner funnel, dried by repeated washing with acetone, and the quantity of dry gel determined. In order to determine the amount of coupled inhibitor the spectra of the two types of affinity gel, in 0.1 M Tris–SO_4/0.2 M Na_2SO_4 at pH 7.5, were recorded between 700–200 nm using a Beckman recording spectrophotometer and 1 mm cells against a reference of uncoupled CM Sephadex in the same buffer. The spectra of the free inhibitors, at pH 7.5, and several concentrations were also recorded using 1 cm cells.

For the large-scale preparation of human carbonic anhydrase isozymes, 250 ml of washed, packed red cells was lysed with water (1:2) and the pH adjusted to 6.0 with 1 N H_2SO_4 to precipitate the cell stroma, which was removed by centrifugation at 16,000*g* for 30 min. The stroma-free lysate was extensively dialyzed against water, the pH adjusted to 9.0 with 0.2 N NaOH, and any further precipitate removed by centrifugation at 16,000*g* for 30 min. The lysate was pumped through a column (2.5 × 20 cm) of affinity gel equilibrated in 0.2 M Tris–SO_4 buffer pH 9.0, at a flow rate of 35–40 ml/hr. Nonspecifically bound protein was eluted with 0.1 M Tris–SO_4/0.2 M Na_2SO_4 buffer solution at pH 9.0, until the absorbance at 280 nm was zero. Carbonic anhydrase B, the low activity isozyme, was specifically eluted with 0.4 M KI/0.1 M Tris–SO_4 pH 7.0, and carbonic anhydrase C, the high activity isozyme, with 0.2 M KCN/0.1 M Tris–SO_4/0.2 M Na_2SO_4 pH 7.5. The eluted enzymes were tested for purity by starch gel electrophoresis (9), and by immunodiffusion on agar plates (10). To check for the completeness of binding of the isozymes, the eluted hemolysate was tested for carbonic anhydrase by a specific esterase assay (11). Carbonic anhydrase isozymes from the red cells of rhesus macaque, chimpanzee, dog, cat, pigeon, chicken, and duck were purified in a similar way, except that the avian red cells were processed in the presence of 10 mM cysteine and were lysed using a 1:3

ratio with water. Alternatively, a batchwise procedure was used for the large-scale purification of carbonic anhydrase. The dialyzed, stroma-free lysate at pH 9.0 was equilibrated with affinity gel for at least 3 hr, and the gel washed free of the majority of nonspecifically bound protein in a Buchner funnel with 0.1 M Tris–SO_4/0.2 M Na_2SO_4 pH 9.0. The gel–enzyme complex was transferred to a column to complete the washing and the isozymes eluted as above.

RESULTS

Two separate batches of affinity gel were used by coupling Prontosil, and also two batches by coupling *p*-(aminomethyl)benzenesulfonamide, to C50 CM Sephadex. The swollen gel capacity was estimated from that for the dry gel by assuming that 1 g of dry gel gave 18 ml of swollen gel, at pH 9.0, in 0.1 M Tris–SO_4/0.2 M Na_2SO_4. The spectra of the free and coupled Prontosil showed a broad absorption maximum at 500–400 nm, and a sharper minimum at 300 nm. Because of the high absorbance of the Prontosil-coupled gel at 500–400 nm the absorption minimum at 300 nm was used to estimate coupling yield. From the free Prontosil a molar extinction coefficient was determined of 34,000 M^{-1} cm^{-1} at 300 nm and pH 7.5, and this was used to calculate the amount of coupled inhibitor. The spectra of the free and coupled *p*-(aminomethyl)benzenesulfonamide both showed a characteristic peak at 267 nm, and this wavelength was used to quantitate the coupled inhibitor, using a molor extinction coefficient of 7400 M^{-1} cm^{-1} at 267 nm and pH 7.5. The capacities coupling yields and binding efficiencies are shown in Table 1. The results obtained for the amounts of coupled inhibitor indicate that of the total carboxyl groups on the Sephadex ion exchanger about 2.4%

TABLE 1
CARBONIC ANHYDRASE CAPACITIES AND BINDING EFFICIENCIES OF THE AFFINITY GELS

Affinity gel	Enzyme capacity (mg/g dry gel)	Enzyme capacity (mg/ml gel[a])	Coupled inhibitor (μmoles/ml gel)	% Binding efficiency[b]
CM-Prontosil	300	17	6.5	9
	420	23	5.5	14
CM-*p*$NH_2CH_2C_6H_4SO_2NH_2$	244	14	27.5	2
	324	18	35.0	2

[a] Based on estimate that 1 g of dry gel gives 18 ml of swollen gel.

[b] Defined as % ratio of observed carbonic anhydrase capacity (μmoles/ml gel) to coupled inhibitor (μmoles/ml gel).

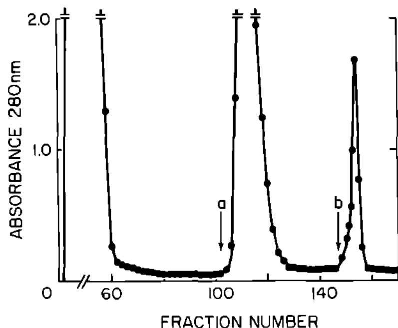

FIG. 1. Affinity chromatography of 250 ml of packed human red cells (800 ml of treated hemolysate) using a CM-Prontosil gel, and a 2.5 × 20 cm column with a 38 ml/hr flow rate and 19 ml fraction volumes. (a) Indicates the first elution of KI, and (b) the first elution of KCN. Fractions 106–123 contain pure carbonic anhydrase B, and its secondary isozyme and fractions 148–156 pure carbonic anhydrase C. For details see the text.

are coupled in the Prontosil gel, and about 12.2% in the *p*-(aminomethyl)benzenesulfonamide gel.

Figure 1 shows the elution profile obtained from the purification of 250 ml of packed human red cells, which gave a yield of 360 mg of carbonic anhydrase B and 55 mg of carbonic anhydrase C. These preparations of the major isozyme forms also included much smaller amounts of their respective secondary isozymes. Starch gel electrophoresis of the pooled concentrated peaks, followed by staining for protein, and CO_2 hydratase and esterase activity, showed that the first peak contained pure carbonic anhydrase B, and the second pure carbonic anhydrase C. Also immunodiffusion gels using anti-human carbonic anhydrase B and anti-human carbonic anhydrase C, prepared from rabbit serum, indicated that each peak contained only the one component isozyme. The eluted hemolysate showed no carbonic anhydrase activity when assayed with *p*-nitrophenyl acetate. Carbonic anhydrases from red cells of the rhesus macaque, chimpanzee, dog, cat, pigeon, chicken, and duck, were also purified by this method. Of these species only the rhesus macaque and chimpanzee possess both high and low activity isozymes, and their red cells were treated in an identical manner to those of human to give pure carbonic anhydrase B and carbonic anhydrase C isozymes and their respective secondary isozymes. The other species have only one major form of carbonic anhydrase and the eluting conditions were as follows: dog, pigeon, and duck, 0.4 M KI/0.1 M Tris–SO_4 7.0; cat and chicken, 0.2 M KCN/0.1 M Tris–SO_4/0.2 M Na_2SO_4 pH 7.5. A further indicator of the purity of these preparations was the single sequences obtained, from C-terminal analyses using carboxypeptidase, of cat, dog, chicken, pigeon, and duck isozymes (12). The starch gel patterns of

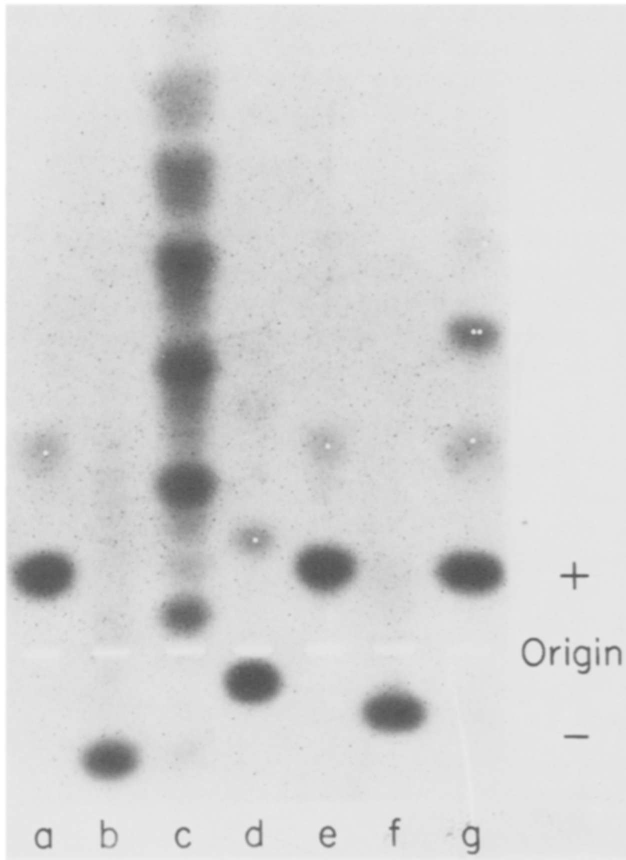

FIG. 2. Starch gel electrophoresis patterns of carbonic anhydrase isozymes purified by affinity chromatography. Vertical electrophoresis was at 4°C in 20 mM sodium borate buffer, pH 8.5, and 0.3 M sodium borate bridge buffer, pH 8.0 (containing 30 mM NaCl), for 21 hr at 8 V/cm. Gels were stained with 0.4% nigrosin. (a) Human carbonic anhydrase B; (b) human carbonic anhydrase C; (c) human carbonic anhydrase C eluted with KOCN; (d) rhesus macaque carbonic anhydrase B; (e) chimpanzee carbonic anhydrase B; (f) cat carbonic anhydrase; (g) dog carbonic anhydrase. The secondary isozymes are indicated by single white dots, and a variant of the dog enzyme by a double white dot.

some purified carbonic anhydrases are shown in Fig. 2. The elution of carbonic anhydrase C with cyanate ion gave multiple bands, probably due to its reaction with amino groups (13), resulting in a net reduction in positive charge for each reacted amino groups.

DISCUSSION

This method gives a stable, high capacity affinity gel suitable for the purification of carbonic anhydrase from a variety of sources. The two gel

types described have been reused several times with no loss of efficiency, with the azo-derived gel having the advantages of higher capacity, and a colored product that enables coupling to be assessed visually. The separation of the secondary isozymes from the major forms of the isozymes requires the use of ion-exchange columns, but these can be small and efficient as only pure isozymes will be chromatographed. The separation and characterization of these secondary isozymes has been discussed by Funakoshi and Deutsch (14). The higher yields of the azo gels can be explained by the length of the coupled inhibitor. Erlich *et al.* (4), using spin-labeled sulfonamides, investigated the active site clefts of the human isozymes and concluded that they were about 140 nm deep. The ligand of the azo-coupled gel is about 183 nm long and therefore is well in excess of this value, whereas the *p*-aminobenzenesulfonamide derived gel produces a ligand of about 125 nm, and this may account for its low ratio of binding capacity to coupled inhibitor. The use of cyanate to release bound enzyme as in the method of Falkbring *et al.* (1) is not appropriate because of its reaction with amino groups. For large-scale purifications, e.g., >100 ml of packed red cells, batchwise procedures are recommended for the initial binding of enzyme.

Finally, if the linkage given by the cyanogen bromide coupling procedure is susceptible to esterase hydrolysis, then any affinity matrix prepared with this coupling technique could be altered when used with starting material containing esterase activity.

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