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Purification of carbonic anhydrase isoenzymes by high-performance affinity chromatography and hydrophobic interaction chromatography

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ABSTRACT

Isoenzymes of carbonic anhydrase were purified by a combination of affinity chromatography and hydrophobic interaction chromatography. Immobilization of sulfonamides on an epoxy-activated support provided a stationary phase for affinity chromatography which was stable to hydrolysis by carbonic anhydrase. A first purification step allowed the isolation of enzymes directly from homogenates of human erythrocytes and rat stomach. Without any further preparation, except the addition of ammonium sulfate to the eluate from affinity chromatography, the isoenzymes could be separated by hydrophobic interaction chromatography with very high recovery of protein and retention of enzymatic activity.

INTRODUCTION

Sulfonamides are specific inhibitors of carbonic anhydrases [1]. It is possible to purify carbonic anhydrases by affinity chromatography by coupling *p*-aminobenzol sulfonamide to Sepharose polysaccharides by means of cyanogen bromide activation [2,3]. It has been suggested that the linkage given by cyanogen bromide activations is of the ester type [4]. It is possible to hydrolyze this linkage by heating at about 80°C at pH between 8 and 12. Owing to esterase activity, carbonic anhydrase is able to cleave the sulfonamide groups, which decreases reproducibility and yields [5]. Moreover, the total distance of the coupled inhibitor from the gel matrix, given by this method, does not exceed the depth of the active-site cleft [6]. To overcome these problems the sulfonamide was immobilized on an

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epoxy-activated spacer which was bound to a polymer support [7] to form a structure which is resistant to hydrolysis.

Usually further separation of isoenzymes of carbonic anhydrases is accomplished by ion-exchange chromatography on diethylaminoethyl (DEAE) columns. The elution of carbonic anhydrases during affinity chromatography is performed with salt-containing buffers. Because the amount of salt required is considerable the enzyme fraction has to be dialyzed in preparation for ion-exchange chromatography at the expense of time and loss of enzymes. Hydrophobic interaction chromatography omits this intermediate step and reveals an excellent resolution of the isoenzymes with very high recovery of protein and activity. Moreover, the performance of affinity chromatography and hydrophobic interaction chromatography in a high-performance liquid chromatographic (HPLC) system makes isolation methods quicker, simpler and more reproducible.

EXPERIMENTAL

Chemicals

All chemicals were of analytical reagent grade from Merck (Darmstadt, Germany) or Sigma (Munich, Germany).

High-performance liquid chromatography

The HPLC system consisted of two pumps, a programmer, a spectrophotometer, a loop injection valve (all from Bio-Rad, Munich, Germany) and a Frac-100 collector (Pharmacia-LKB, Freiburg, Germany).

Columns and chromatographic conditions

As a ligand for affinity chromatography, *p*-aminomethylbenzol sulfonamide (Merck) was immobilized on an epoxy-activated polymer support (Eupergit C 30N™, Röhm Pharma, Weiterstadt, Germany; particle size 30 μm, column dimensions 60 × 8.0 mm I.D.), giving a ligand density of approximately 300 μmol ligand per g support. We found a binding capacity of 6 mg carbonic anhydrase per g support. Eupergit C 30N is a bead polymer of methacrylamide, N-methylene-bis-methacrylamide, glycidyl methacrylate and allyl glycidyl ether [8]. The oxirane groups of the glycidyl residue act as active components and covalently bind compounds containing amino, mercapto or hydroxy groups [9] (Fig. 1). Binding bufferA contained 0.1 mol/l Tris–HCl, pH 8.3. Washing buffer B consisted of buffer A supplemented with 1 mol/l NaCl. Elution was achieved by buffer C (0.1 mol/l sodium acetate, pH 5.5, and 1 mol/l NaCl). Flow-rates were 1.0 ml/min.

Hydrophobic interaction chromatography was performed with stationary supports carrying phenyl groups (TSK Phenyl-5-PW™, Bio-Rad; particle size 10 μm, column dimensions 80 mm × 7.5 mm I.D.) and propyl groups (Euramid™; Säulentechnik Knauer, Berlin, Germany; particle size 7 μm, column dimensions

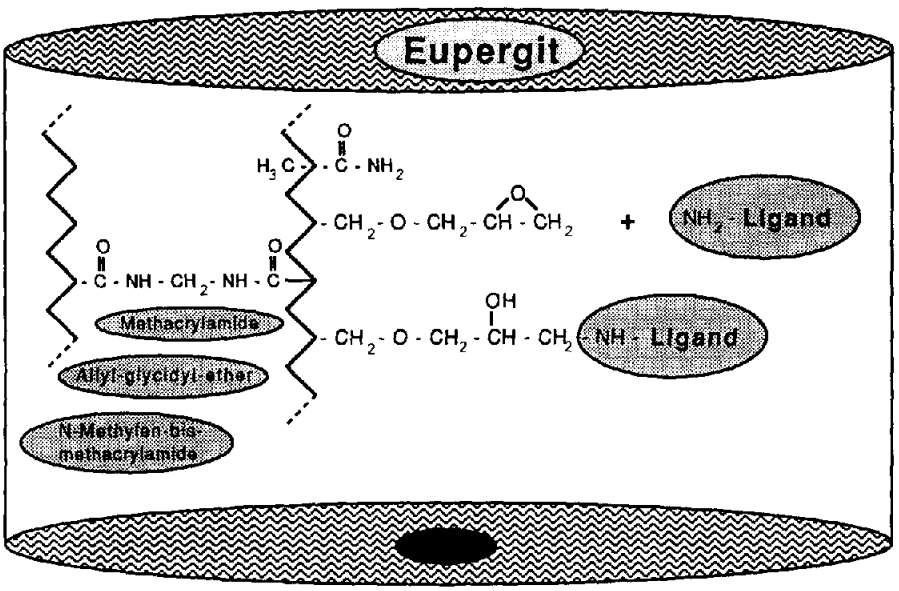

Fig. 1. Chemistry of Eupergit supports and of the process of ligand binding. Eupergit consisted of a polymer matrix with an epoxy-activated surface. Ligands containing amino groups could be immobilized by amino bonds which were stable to hydrolysis.

60 mm × 8.0 mm I.D.). Binding was achieved with buffer A containing 20 mmol/l Tris–HCl, pH 7.0, and 1.5 mol/l ammonium sulfate. A gradient from 100 to 0% ammonium sulfate was used for elution. Flow-rates were 0.3 ml/min.

Reversed-phase chromatography was performed on a C_4-Nucleosil™ column (Säulentechnik Knauer; particle size 5 μm, column dimensions 120 mm × 4.0 mm I.D.). Binding was accomplished with buffer A (0.1% trifluoroacetate, pH 2.0) and elution was carried out by a gradient from 0 to 100% buffer B (0.1% trifluoroacetate, pH 1.3). A flow-rate of 0.6 ml/min was used.

Protein determination

Protein recovery was determined by measuring the protein concentration before and after separation according to the procedure of Lowry *et al.* [10].

Determination of CO_2-hydration activity

The assay was performed at 0°C according to the method of Roughton and Booth [11] as modified by Wilbur and Anderson [12]. Samples were made up to 1 ml with water, and 2 ml of diethylbarbituric acid (22 mmol/l) containing bromothymol blue (50 mg/l) were added. After addition of 2 ml of CO_2-saturated water, the time taken for a visible color change to occur at pH 6.3 was recorded. Activity was calculated by the formula $[(t_0 - t_c)/t_c] \times v \times 16.15 = U/ml$ (v =

sample volume in μl; t_0 and t_c = recorded times for the uncatalyzed and catalyzed reaction).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of 10–25 μl were mixed with 3–7 μl of 500 mmol/l Tris–HCl buffer (pH 6.8) containing 10% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Samples were heated at 95°C for 4 min and 5–20 μg of protein were applied to each track. SDS-PAGE was performed according to the method of Laemmli [13], using the Bio-Rad mini system (Bio-Rad).

Isoelectric focusing

Samples of 20 μl containing 10–30 μg of protein were loaded onto a polyacrylamide gel (4% T, 3% C, 4.5% Servalyte pH 6–8 or 5–7, 1% Servalyte pH 4–9) with a pH range from 5 to 8. Focusing was performed for 4.5 h on a horizontal electrophoresis system (Multiphor™, Pharmacia-LKB).

Sample preparation

Human erythrocytes were washed with an equal volume of wash buffer (10 mmol/l Tris–HCl, pH 7.5, containing 0.15 mol/l NaCl) and diluted with 1.5 volumes of a hypotonic solution (10 mmol/l Tris–HCl) and then frozen at −20°C and thawed. The homogenate was centrifuged at 100 000 *g* for 60 min, and 10 ml were used for affinity chromatography.

RESULTS AND DISCUSSION

Bovine carbonic anhydrase was applied to a column prepared for affinity chromatography. After loading and washing, elution was achieved by a pH change at high salt concentration. The eluate contained 98% of the original activity and 86% of the original amount of protein (compare Fig. 2 and Table I). Loss of protein was due to previously denatured enzyme molecules which had lost their ability to bind to sulfonamides and ran through the column.

In the next experiment we tried to purify carbonic anhydrase from a natural source. A freshly prepared homogenate of human erythrocytes was subjected to affinity chromatography. Almost all the activity was found in the elution fraction. Moreover, the eluate contained highly purified carbonic anhydrase as shown by SDS–PAGE (Fig. 3). This procedure also allowed the purification of carbonic anhydrase from the stomach of rats, giving a similar chromatogram (not shown here). Purity of the eluted material was also monitored by SDS–PAGE (Fig. 3). Many chromatographic runs were carried out over several months using the same affinity chromatography column. During this period no apparent loss of resolution and recovery was observed, indicating a stable ligand-support structure which is resistant to hydrolysis by carbonic anhydrase.

Without any further preparation, 5 ml of the fraction eluted by affinity chro-

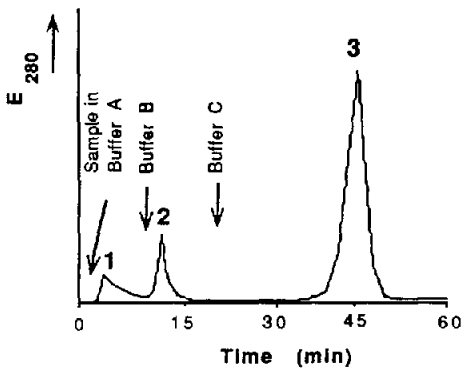

Fig. 2. Separation of native and denatured forms of bovine carbonic anhydrase by affinity chromatography. A 1.5-mg sample of bovine carbonic anhydrase was dissolved in 10 ml of buffer A (0.1 mol/l Tris HCl, pH 8.3) and applied to the column. After washing with buffer B (buffer A containing 1 mol/l NaCl), elution was achieved with buffer C (0.1 mol/l sodium acetate, pH 5.5, containing 1 mol/l NaCl). Chromatographic conditions: column *p*-aminomethylbenzol sulfonamide coupled to Eupergit C 30 N; particle size 30 μm; pore size 45 nm; column size 60 mm × 8.0 mm I.D.; flow-rate 1.0 ml/min; pressure 5 bar; detection at 280 nm; room temperature. For recovery see Table I.

matography were diluted with 5 ml of 3 mol/l ammonium sulfate and then directly applied to the hydrophobic interaction column. Binding of carbonic anhydrase to the column was achieved by using 1.5 mol/l ammonium sulfate in the binding buffer. A gradient from high to low salt concentration caused stepwise dissociation of the two isoenzymes of human carbonic anhydrase (Fig. 4). Recovery of protein and activity was in the range 85–99% (Table II).

Furthermore, hydrophobic interaction chromatography allowed the separation of four different forms of bovine carbonic anhydrase, which was verified by

TABLE I

RECOVERY OF CARBONIC ANHYDRASE FROM AFFINITY CHROMATOGRAPHY

Affinity chromatography was performed with bovine carbonic anhydrase (see Fig. 1). Recovery of protein and enzymatic activity for all isolated fractions was determined.

Fraction	Protein (μg)	Activity (U)	Specific activity (U/μg)
Sample	1500	248 888	166
Peak 1	110	546	7
Peak 2	152	169	1
Peak 3	1290	243 303	189
Recovery (%)	86	98	

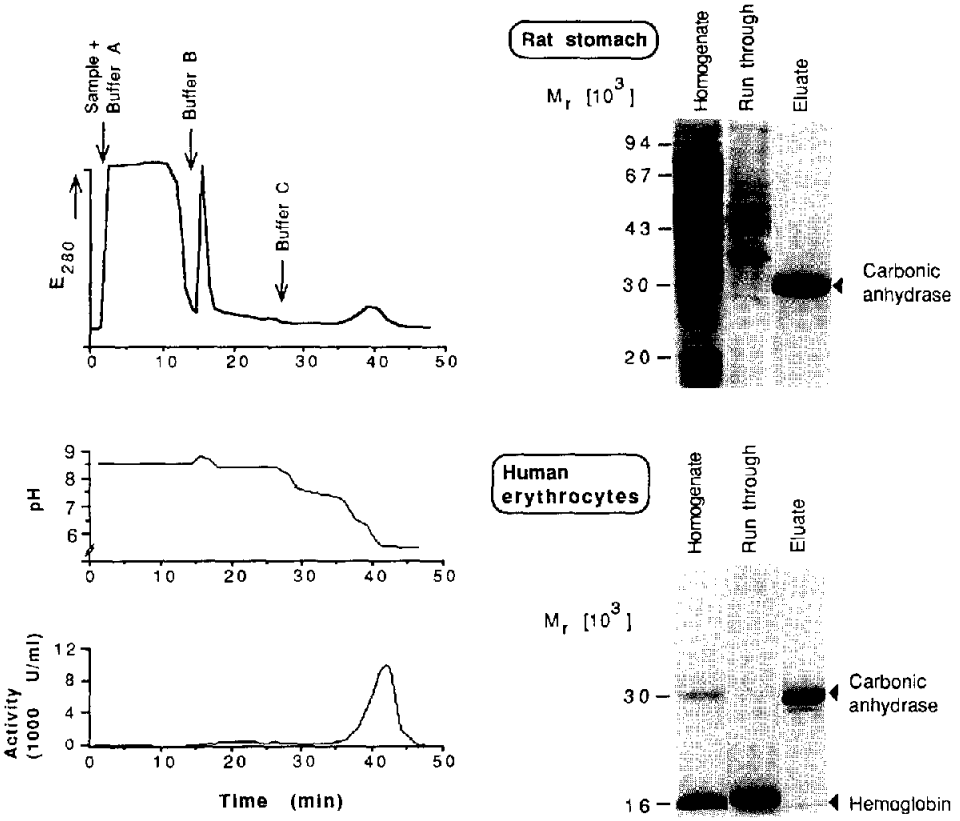

Fig. 3. Purification of carbonic anhydrase from human erythrocytes and rat stomach by affinity chromatography. The column was loaded with 10 ml of homogenate. Affinity chromatography of human hemolysate and rat stomach gave similar chromatograms shown on the left side. SDS-PAGE of sample and separated fractions is shown on the right. For chromatographic conditions see Fig. 2.

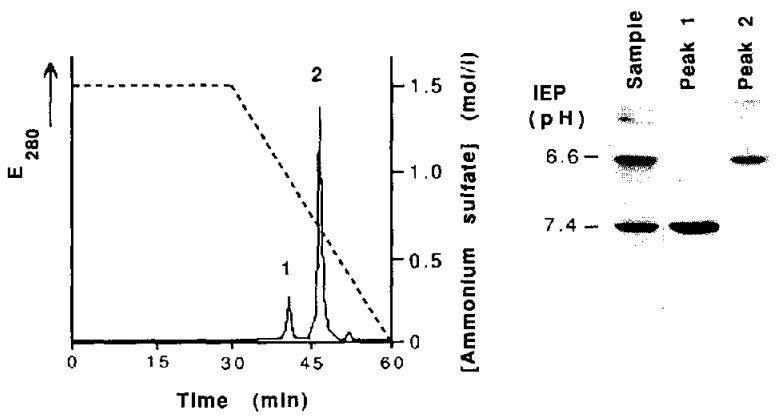

Fig. 4. Separation of isoenzymes of human carbonic anhydrase by hydrophobic interaction chromatography. A 320-μg sample of human carbonic anhydrase eluted during affinity chromatography in 5 ml of buffer C (0.1 mol/l sodium acetate, pH 5.5, containing 1 mol/l NaCl) was mixed with 5 ml of 3 mol/l ammonium sulfate and loaded onto the column. The gradient is shown in the figure. Chromatographic conditions: column TSK Phenyl-5-PW; particle size 10 μm, column dimensions 80 mm × 7.5 mm I.D.; flow-rate 0.3 ml/min; pressure 8 bar; detection at 280 nm; room temperature. For recovery see Table II.

TABLE II

RECOVERY OF HUMAN CARBONIC ANHYDRASE FROM HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The eluate of affinity chromatography was directly applied to hydrophobic interaction chromatography (see Fig. 3). Recovery of protein and enzymatic activity of two major carbonic anhydrase isoczymes was determined.

Fraction	Protein (μg)	Activity (U)	Specific activity (U/μg)
CA I + II	320	15 127	47
CA II	32	7 129	223
CA I	280	5 880	21
Recovery (%)	97	86	

isoelectric focusing (Fig. 5 and Table III). In contrast, only two major and one minor isoenzymes could be found in the human hemolysate, which might be the result of species-specific differences. For fractionation of the isoenzymes of carbonic anhydrase it was important to choose a concentration of ammonium sulfate which would not precipitate the enzyme or else a very poor resolution with strongly overlapping peaks was obtained. Marked precipitation of carbonic anhydrase occurred when ammonium sulfate concentrations exceeded 70% saturation, which is equal to about 2.8 mol/l.

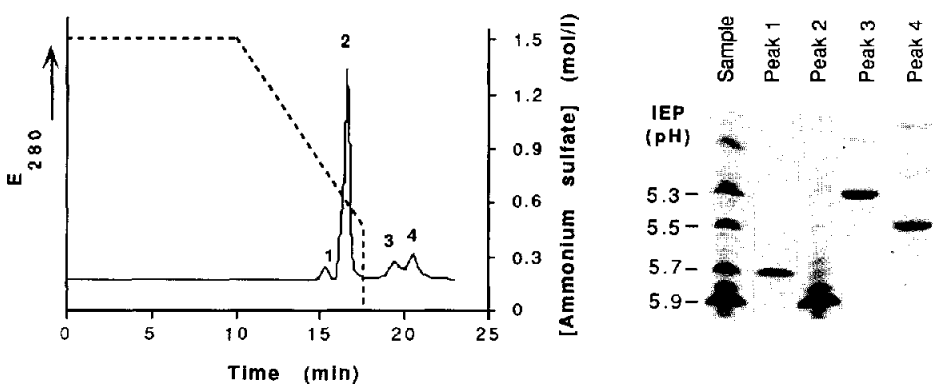

Fig. 5. Hydrophobic interaction chromatography of bovine carbonic anhydrase. A 1-mg sample of bovine carbonic anhydrase dissolved in buffer A (20 mmol/l Tris-HCl, pH 7.0, containing 1.5 mol/l ammonium sulfate) was applied to the column. Elution was carried out by a gradient from 1.5 to 0 mol/l ammonium sulfate (shown in the figure). Chromatographic conditions: column Euramid-HIC; particle size 7 μm; pore size 100 nm; column size 60 mm × 8.0 mm I.D.; flow-rate 0.3 ml/min; pressure 10 bar; detection at 280 nm; room temperature. For recovery see Table III.

TABLE III

RECOVERY OF BOVINE CARBONIC ANHYDRASE FROM HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Bovine carbonic anhydrase was applied to hydrophobic interaction chromatography (see Fig. 4). Recovery of protein and enzymatic activity of four carbonic anhydrase isoenzymes was determined.

Fraction	Protein (μg)	Activity (U)	Specific activity (U/μg)
Sample	1000	154 348	154
Peak 1	30	1 193	30
Peak 2	685	133 670	195
Peak 3	122	6 060	50
Peak 4	76	3 155	42
Recovery (%)	91	93	

Because of its hydrophobicity, carbonic anhydrase binds strongly to butyl groups of reversed-phase chromatographic matrices. In contrast to hydrophobic interaction chromatography, it was not possible to separate carbonic anhydrase isoenzymes by reversed-phase chromatography (Fig. 6). Moreover, the organic solvent acetonitrile caused denaturation of carbonic anhydrases, thus causing a complete loss of activity.

Two major forms and one minor form of human carbonic anhydrase and four different forms of bovine carbonis anhydrase could be separated by hydrophobic interaction chromatography. These forms differ with respect to their isoelectric

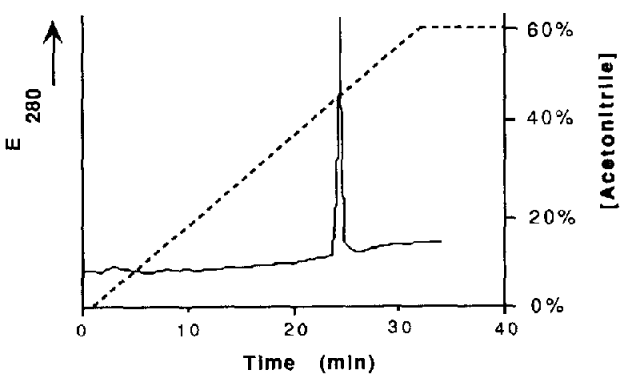

Fig. 6. Reversed-phase HPLC of bovine carbonic anhydrase. A 500-μg sample of bovine carbonic anhydrase dissolved in 200 μl buffer A (0.1% trifluoroacetate, pH 2.0) was loaded onto the column. The gradient was 0 to 100% buffer B (0.1% trifluoroacetate, pH 1.3, containing 60% acetonitrile). Chromatographic conditions: column Nucleosil-C_4; particle size 5 μm; pore size 30 nm; dimensions 120 mm × 4.0 mm I.D.; flow-rate 0.6 ml/min; detection at 280 nm; room temperature.

points and specific activities. The various carbonic anhydrases isolated from hemolysates are all related to two major forms, designated CA I and CA II. Probably all the different forms of isoenzymes are physiologically present in cells [14]. There are at present no data available that would provide an adequate basis for deciding whether all isoenzymes are formed from the two major isoenzymes, *e.g.* by random loss of amide groups [15] or by specific phosphorylation involving a cyclic AMP-dependent protein kinase [16] or whether they are single, unique species generated by post-translational modifications. Possibly there is a slow conversion of the two major isoenzymes to minor isoenzymes in living cells. This would suggest that older tissue cells, such as erythrocytes, should contain higher levels of the converted isoenzymes than younger ones.

The change from soft-gel chromatography to high-performance chromatography for purification of isoenzymes by affinity chromatography and hydrophobic interaction chromatography provided advantages due to shorter separation time and improved resolution. Moreover, hydrophobic interaction chromatography enabled fractionation of isoenzymes of carbonic anhydrase which so far has not been achieved by ion-exchange chromatography on DEAE–Sepharose. Further investigations must be carried out to decide whether a conversion from major to minor isoenzymes occurs *in vivo* or whether the appearance of minor isoenzymes is the result of artificial modifications caused by purification procedures.

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