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PREPARING AND OPTIMIZING A NEWLY AFFINITY GEL FOR PURIFICATION OF CARBONIC ANHYDRASE ISOENZYMES

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ABSTRACT

Human carbonic anhydrase isozymes have been purified from the hemolysate, directly by using the original affinity gel in chemical structure of Sepharose 4B-L-tyrosine- sulfathiazole. Different solution buffers were used for obtaining the purified CA isozymes from the affinity column. Most suitable elution buffers were determined for CA I and CA II isoenzymes pH 6.3, 25 mM Na_2HPO_4/1.0 M NaCl and pH 5.6, 0.1 M $NaCH_3COO$ / 0.5 M $NaClO_4$ respectively. The purification values for CA I and CA II have been obtained as 635.71 and 666.71 folds with 28.41% and 46.19% yield respectively. The ionic strength and optimum pH values of the original affinity gel have been determined for each isozyme of CA. Maximum binding was achieved 0.3 ionic strength and pH 8.7 for both CA I and CA II. The single bands was indicated for each isoenzymes by SDS-polyacrylamide gel electrophoresis.

KEYWORDS:
Carbonic anhydrase, isozymes, affinity gel, affinity chromatography, purification.

INTRODUCTION

The carbonic anhydrase (CA, EC 4.2.1.1) contains a zinc ion which is important for catalysis. CAs catalyse the conversion of CO_2 to the bicarbonate ion and protons. The CA reaction is included in many physiological and pathological processes, including respiration, pH and CO_2 homeostasis, electrolyte secretion, gluconeogenesis, lipogenesis and ureagenesis; bone resorption; calcification; and tumorigenicity [1- 3]. Sixteen isozymes have been characterized up to now in mammals [1,2]. Carbonic anhydrase isoenzymes are sensitive to sulphonamides with different IC_{50} and Ki values. For example, CA II is more sensitive to sulphonamides than CA I [4]. Mammalian blood is cheapest and most speedily available from number of sources for CA isoenzymes [4-8]. Several purification methods known in the literature for CA isoenzymes [9-14].

Affinity chromatography is potent and practicable means of purifying proteins. [15] Affinity chromatography is a widely used method for CA isoenzymes with high yield and purification fold of the enzyme from various sources [9-14]. It is provided suitable ligands with a highly specific interaction for target protein [16-17]. The purification method is advantage of many proteins for specific and suitable ligands with particularity, high stability, and high volume [14]. Sulfonamides are used as ligands specific and strong inhibitors of CA in these methods [3, 4, 6, 8, 18, 19, 20]. In the literature have been described using a variety of matrices, spacer arms and ligands for affinity gels [9-14].

The present study had been synthesized an original affinity gel with Sepharose 4B used as a matrix, l-tyrosine used as a spacer arm was covalently attached to the matrix. Sulfathiazole is the ligand for affinity gel for carbonic anhydrase purification. The present study characterizes the accomplished purification of carbonic anhydrase from erythrocytes using an original affinity gel and to achieve high purification a selective elution step.

MATERIALS AND METHODS

Materials. Sepharose 4B, L-tyrosine, sulfathiazole, protein assay reagents, enzyme purification chemicals for buffers, enzyme assay chemicals for buffers and chemicals for electrophoresis were obtained from Sigma Aldrich Co. (Milan, Italy) and Merck Chem Co. (Milan, Italy). All other chemicals were of analytical grade.

Preparation of affinity gel. The affinity gel was prepared with 4 g cyanogens bromide (BrCN) in 1:1 dilution of Sepharose 4B and water. The mixture was adjusted to pH 11 with 4M NaOH in an ice bath and maintained at that pH for 10-15min. Mixture was transported to a Buchner funnel and washed with cold 0.1 M $NaHCO_3$ buffer pH 10 and was transported to a beaker in the same buffer. L-tyrosine solution in cold 0.1 M $NaHCO_3$ (pH 10)

buffer was coupled to BrCN activated Sepharose-4B-Ltyrosine. The suspension was completed by stirring with a magnet for 90 min [10]. The suspension was washed with 1L cold distilled water.[14, 20] The affinity gel was obtained by diazotisation of sulfathiazole and coupling of this compound to the Sepharose-4B-L-tyrosine. The reaction was stabilized at pH 9.5 in suspension which was mixed for 3h at room temperature. After incubation, the suspension was washed with 1L cold distilled water and 0.05 M Tris-SO_4 (pH: 7.5) buffer solution and was stored in a buffer solution containing 0.05 M Tris-SO_4 (pH: 7.5).

Purification of carbonic anhydrase from human erythrocytes. Human blood samples were obtained from the Balikesir University Faculty of Medicine, using tubes containing anticoagulant. The blood samples were centrifuged at 5000 rpm for 20 min and the plasma and buffy coat were removed. Erythrocytes were washed two times with NaCl (0.9%) and the erythrocytes were hemolyzed with cold water [14]. The tissue samples were centrifuged at 15000 rpm for 40 min at 4°C and the ghosts, intact cells and precipitate were removed. The pH of the hemolysate was adjusted to 8.5 with solid Tris. The hemolysate was applied to the Sepharose 4B-L-tyrosine-sulfathiazole affinity column equilibrated with 25 mM Tris /0.1 M Na_2SO_4 (pH: 8.5) buffer. The affinity gel was washed with 25 mM Tris/22 mM Na_2SO_4 (pH 8.5) buffer, and CA isozymes were eluted under different elution conditions.

Protein determination. The protein in the column eluates was determined with UV-VIS spectrophotometer by the absorbance at 280 nm. Protein quantitative determination was obtained by absorbance measurements at 595nm according to the Bradford method, using bovine serum albumin as the standard [21].

Enzyme assay. Carbonic anhydrase activity was assayed by the hydration of CO_2 according to the method of Maren method [22] which is based on the determination of the time required for the pH of solution decreasing from 10.0 to 7.4 due to the hydration of CO_2.

SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was applied after purification of the enzymes. It was performed in 10 and 3% acrylamide concentrations, containing 0.1% SDS according to the Laemmli procedure [23] for the running and stacking gel, respectively.

RESULTS AND DISCUSSION

Enzyme purification is laborious and time consuming process. For this reason, the purification method can be rapid and efficient. In this process, the enzymatic activity must not be reduced and the three-dimensional structure must not be denatured.

CA isoenzymes have been purified with different yields and purification folds from different sources [9-14]. Different purification methods have been used for CA isoenzymes [10,12,14] CA isoenzymes were purified first by Arslan et al. trough affinity chromatography, in their study, Sepharose-4B-Ltyrosine- p-aminobenzenesulfonamide gel and the enzyme was purified 416.8 times [10]. It was reported that EUPERGIT C250L- Ethylenediamine-4-isothiocyanato benzenesulfonamide gel were purified 184 times as a result of total hCA I and h CA II isoenzymes [12]. In another study, Sepharose 4B-Ethylenediamine-4-isothiocyanato benzenesulfonamide gel was used; the isoenzymes were purified approximately 672 and 580 times [14].

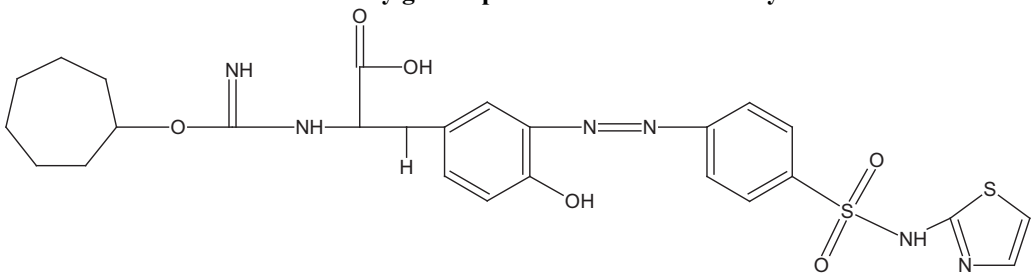
TABLE 1
Purification scheme of CA-I and CA-II from human erythrocytes by Sepharose-4B-L-tyrosine-sulfathiazole affinity chromatography

Purification Step	Volume (mL)	Total Activity (U)	Activity (U/mL)	Protein Amount (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)	Overall Yield (%)	Overall Purification (fold)
Hemolysate	25 ml	53	2.12	32.21	805.25	0.0658	100	-
Affinity Chromatography	3 mL	15.06	5.02	0.12	0.36	41.83	28.41	635.71

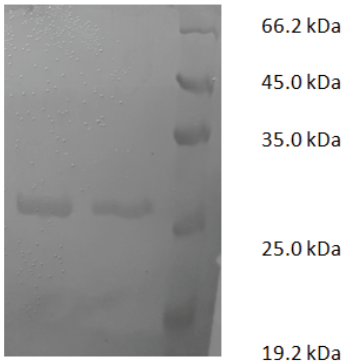
TABLE 2
CAII

Purification Step	Volume (mL)	Total Activity (U)	Activity (U/mL)	Protein Amount (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)	Overall Yield (%)	Overall Purification (fold)
Hemolysate	25 ml	53	2.12	32.21	805.25	0.0658	100	-
Affinity Chromatography	3 mL	24.48	8.16	0.186	0.558	43.87	46.19	666.71

SCHEME 1
The new affinity gel for purification of CA isoenzymes.



Sepharose 4B L-tyrosine Sulfathiazole



In study of Bozdag et al. (2015), hCA I enzyme was purified 672 times with 62 % yield and hCA II enzyme was purified 580 times with 43 % yield. In this study, hCA I enzyme was purified 635.71 times with 28.41 % yield and hCA II enzyme was purified 666.71 times with 46.19 % yield. It seems that the method of Bozdag et al. is better because approximately same purification fold with more yields for hCA I but in our study is better than the others with purification folds and yield for hCA II.

The present study, we design an original affinity gel for purifying the CA isoenzymes from human erythrocytes by using affinity chromatographic method. The novel affinity gel was prepared to purify the carbonic anhydrase isoenzymes. For this aim, the functional group $–NH_2$ of L-tyrosine was covalently attached to the matrix sepharose 4B by means of an amide bond. After that, L-tyrosine was attached to the activated gel as a spacer arm, and finally diazotised sulfathiazole was clenched to the meta position of L-tyrosine molecule as ligand. In this way, Sepharose-4B-L-tyrosine-Sulfathiazole affinity gel was obtained (Scheme 1). Sulfathiazole was chosen as a ligand, since it is a specific inhibitor of CA [24].

We synthesized Sepharose 4B- L-tyrosine-sulfathiazole affinity gel by change of washing and elution conditions for purifying the CA isoenzymes. The hemolysate was applied to the affinity column equilibrated with 25 mM Tris /0.1 M Na_2SO_4 (pH 8.5) buffer. The affinity gel was washed with 25 mM Tris/22 mM Na_2SO_4 (pH 8.5) buffer, and CA isoenzymes were purified under different elution status. The eluates were described by protein determination at 280 nm and assaying CO_2 hydratase activity for CA I and CA II. The isoenzymes CA I and CA II were purified up to 635.71 and 666.71 fold with a recovery ratio of 28.41% and 46.19% respectively in Table I. These values are better than some of the reported affinity gels, and equivalent to others [9-14]. Purified enzymes were acquired exhibiting a single band on SDS-PAGE, corresponding to a molecular weight of approximately 29 kDa (Figure 1).

We used only one chromatographic technique, Sepharose 4B-L-tyrosine-sulfathiazole affinity chromatography by change of elution conditions and binding capacities. CA I and CA II were eluted using the affinity gel with different elution buffers (Figure 2). The most suitable elution buffers were 1 M NaCl /50 mM Na_2HPO_4 (pH 6.3) for CA I and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6) for CA II. The binding capacities of the affinity gel for the CA I and CA II isozymes were determined at different ionic strengths (Figure 3) and pH buffers (Figure 4). For this purpose, elution buffers were used with different compounds at different concentrations. We used for elution CA I and CA II pH 6.3 and 5.6 buffers respectively. The binding capacities of the affinity gel for the CA I and CA II isoenzymes were found at different pH buffers in the range from 6.5-9.0. For specification of optimum ionic strength, enzyme activity was found using

different concentrations of $NaSO_4$, pH 6.3 and pH 5.6, in the range from 0.1 to 0.52. The enzyme was seen to exhibit the maximum binding was achieved ionic strengths around 0.3. So that we prepared the optimum pH for CA I and CA II, 50 mM Na_2HPO_4/1.0 M NaCl and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ buffers respectively. Buffers were prepared in the pH range of 6.5-9.0 (Figure 4), maximum binding was determined for CA I and CA II pH 8.7. These results displayed differences with other studies in the literature [9-14].

Sulfathiazole exhibited quite effective inhibition to CA I and CA II with 77.01 nM IC_{50} value [24]. In another study was seen with sulfanilamide for CA I and CA II isoenzymes as IC_{50} values of 24.53 nM and 100.73 nM, respectively [24]. IC_{50} values for sulfathiazole was effective inhibitor than sulfanilamide for CA II [24]. In this study, we used sulfathiazole for a ligand to synthesized affinity gel. The purification fold and yield Sepharose 4B-L tyrosine-sulfanilamide gel for CA I was better than CA II [10]. But, gel synthesized by us, purification fold and yield for CA II was better than CA I. In

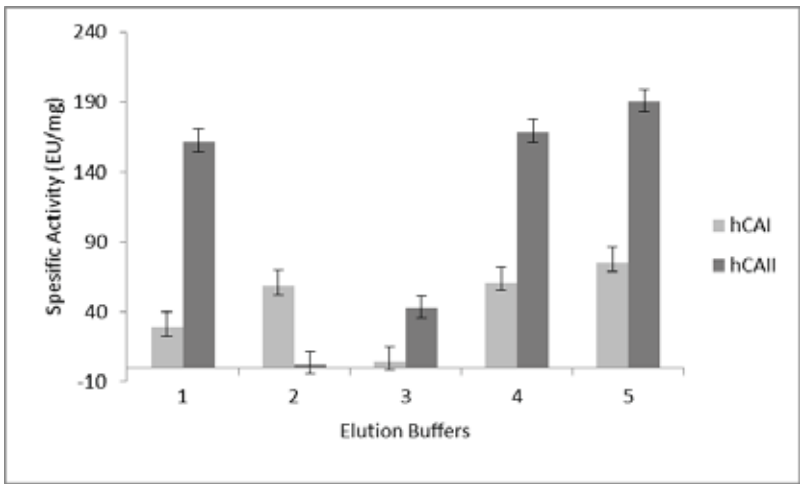


FIGURE 1
SDS-PAGE pattern (Lane 1 : hCA II, Lane 2: hCA I, Lane 3: Marker)

1. 50 mM Na_2HPO_4/1 M NaCl (pH:6.3)- 50 mM Na_2HPO_4/0.2 M KSCN (pH:6.3);
2. 0.1 M KI/ 0.1 M Tris-SO_4 (pH:7.0) -0.1 M $NaCH_3COO$/ 0.5 M $NaClO_4$ (pH:5.6);
3. 0.1 M KI/ 0.1 M Tris-SO_4(pH:7.0) - 50mM Na_2HPO_4 / 0.2 M KSCN(pH:6.3);
4. 50 mM Na_2HPO_4/1 M NaCl (pH:6.3)-0.1 M $NaCH_3COO$ / 0.5 M $NaClO_4$ (pH:5.6)
5. 1 M NaCl / 22 mM Na_2HPO_4 (pH:6.3), 0.1 M $NaCH_3COO$ / 0.5 M $NaClO_4$ (pH: 5.6)

FIGURE 2
Effect of different buffers for elution hCA I and hCA II

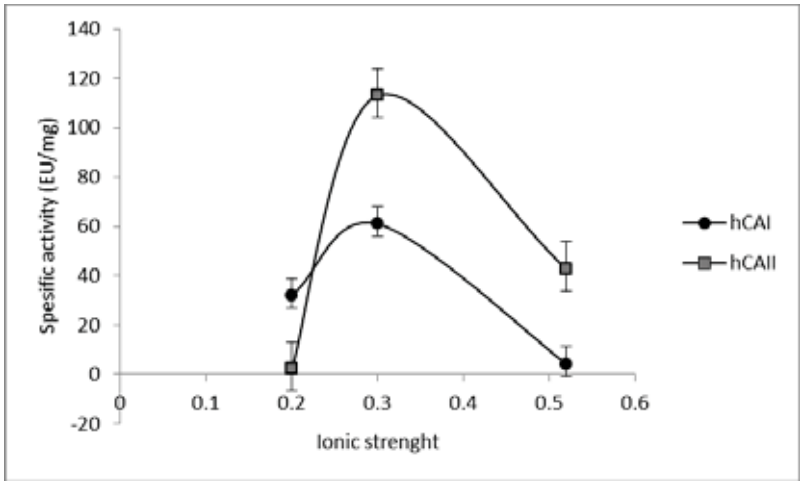


FIGURE 3
Effect of ionic strength

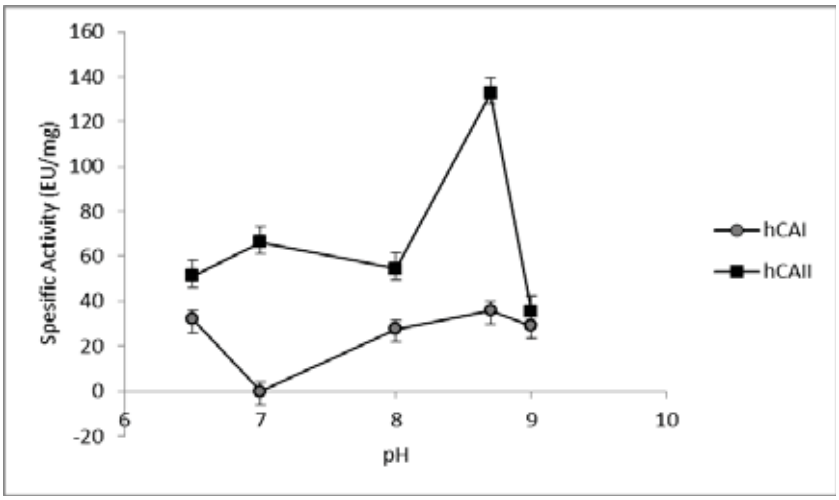


FIGURE 4
Effect of pH

addition, the binding capacities of the affinity gel for the CA I and CA I were best values another studies [9-14]. Thus, Sepharose 4B-L tyrosine - sulfathiazole affinity gel is displayed to be favourable for the purification of CA I and CA II in active form. These results show that the procedure used in the purification is good enough to be used in further studies. It is interesting, when we changed the compounds of the buffer, pH and ionic strengths, purification fold of CA II was better than CA I. By changing the elution conditions is advantage of purification of CA isoenzymes.

We focussed in this study to develop an original and easy chromatographic method for the purification of CA isoenzymes. Affinity chromatography is a suitable technique which is easily applicable for the rapid purification. For this purpose, in our study describes an original affinity gel synthesis was prepared using Sepharose 4B with sulfathiazole, an inhibitor of carbonic anhydrase, which is also commercially purchasing as well. In this reaction, L-tyrosine was used as a spacer arm for controlling the steric effects between the matrix and ligand. Hydroxyl groups that Sepharose contains, provide the binding of the ligand to the matrix in mild conditions. The purification of an each isozyme has been controlled by SDS-polyacrylamide gel electrophoresis.

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Conflicts of Interest. All authors declared that they have no conflicts of interest.

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STUDY ON THE INFLUENCE OF ECOLOGICAL GARDEN LANDSCAPE DESIGN ON WATER ENVIRONMENT

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ABSTRACT

The objective of this study was to focus on influence of ecological garden landscape design on water environment. The effects of hydraulic loading rate (HLR), pH, initial total phosphorus concentration and pollutant loading rate (PLR) on pollutants removal were investigated. This study finds that although the influent loading rates were highly variable, the subsurface wastewater infiltration (SWI) system performed well in treating the sewage effectively. Hydraulic and pollutant loading rates had negative influence on the pollutants removal. Pollutant removal efficiencies decreased with loading rates increasing. The performance of the underground infiltration system is very stable, and various pollution indicators also have certain removal efficiency. After the underground infiltration rate system treatment of raw water, TP and so on have all declined, and the quality of landscape water has been significantly improved.

KEYWORDS:
Ecological landscape, Design, Water environment, SWI

INTRODUCTION

In recent years, the original form of the earth's surface has been changed drastically by land development and urban expansion. A large number of human behaviors, such as deforestation, river diversion and underlying surface hardening, lead to the loss or occupation of a tremendous amount of habitat, which is closely related to the lives of animals and plants and results in the deterioration of the atmosphere and the water environment. In the process of urbanization, the natural landscape's patch fragmentation and poor patch connectivity have become a more serious issue, and the ecological environment is faced with the problem of species extinction and serious pollution [1].

Landscape has important practical significance for protecting species diversity, preventing soil erosion, filtering pollutants, resource management and global change [2]. It improves the ability of bio-circulation and exchange, and improves the stability of ecosystem. With increasing emphasis on urban ecological environment, more and more attention has been paid to urban ecological landscape.

Subsurface wastewater infiltration (SWI) system has proved to be a good alternative for wastewater advanced treatment with the consideration of efficiency and cost [3-6]. In China, more attention to the SWI system has been obtained since the late 1990s when non-point source pollution control was introduced [7]. Since then, large scale SWI systems have been applied in many areas around the world to treat municipal wastewater. Some demonstration treatment systems were also established in American, Australia, Russia, Japan, China and other countries, gained popularity as a cost effective wastewater management option in both developed and developing countries [8-12]. Nowadays, the major research focus about the SWI system is the design, performance and mechanisms of pollutant removal [13-18]. In SWI treatment, wastewater is firstly treated by conventional physico-chemical or biological treatment and then allowed to infiltrate through aerated unsaturated zone wherein it gets purified through processes such as filtration, adsorption, chemical reaction and biodegradation. SWI system has demonstrated a consistent capacity to decompose organic matte [19].

Therefore, the objective of this study was to focus on influence of ecological garden landscape design on water environment. The effects of hydraulic loading rate (HLR), pH, initial total phosphorus concentration and pollutant loading rate (PLR) on pollutants removal were investigated.

MATERIALS AND METHODS

Ecological garden landscape design. It should follow the principles of regional natural conditions, follow the principles of environmental protection, follow the principles of plant ecology, and follow applicable and economic principles.

System description. The SWI systems were 1.6 m long and 1.3 m wide with a depth of 1.1 m. The matrix was 7% activated sludge, 62% brown