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Apocarbonic Anhydrase-Enzymatic Determination of Zinc in Fruit Juices

Ayşe KARAGÖLGE, Nazan DEMİR, Yaşar DEMİR & İrfan KÜFREVİOĞLU
Faculty of Sciences, Department of Biochemistry
Atatürk University, Erzurum-TURKEY

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In this study, a new enzymatic method for determining trace amount of zinc was employed on ten different fruit juices. The basis of the enzymatic method is that apocarbonic anhydrase regains its activity in proportion to the Zn^{2+} existing in a solution. Thus, the more activated the enzyme is in the medium, the higher the Zn^{2+} concentration in the solution will be.

For this purpose, bovine carbonic anhydrase was purified from erythrocyte hemolisate by affinity chromatography. The Zn^{2+} bound to the enzyme was removed by of the purified enzyme against the solution of pyridine-2-6-dicarboxylic acid. Thus 100% purified enzyme was obtained.

First, the presence of Zn^{2+} in the fruit juices sample was determined by the enzymatic method. Then the content of Zn^{2+} in the same sample was measured by atomic absorption spectroscopy.

According to the results obtained, the enzymatic method that we have developed is a variable means of determining

Key words: Fruit juices, Zinc determination, Apocarbonic anhydrase.

Introduction

Some enzymes have had applications in analytical chemistry because of their substrate specificity[1,2]. Metallo-enzymes function by means of the metal ions located in their active sites. These ions are bound to the enzymes specifically, giving rise to an analytical basis for the determination of the metal ion in question. Some studies have been done using amino peptidase, alkaline phosphatase and polyphenol oxidase for the determination of trace elements such as zinc and copper[3,4].

Carbonic anhydrase ((E.C.4,2,1,1), CA: Carbonate Hydrolyse) is a zinc-containing metallo-enzyme, which catalyses the hydration of CO_2 and the dehydration of H_2CO_3. When the zinc covalently bound to the active site is removed, apocarbonic acid (apoCA) is obtained, resulting in a deactivated enzyme[5]. The apoCA may display activity proportional to the amount of Zn^{2+} added to the reaction medium, and this is the basic principle of the method. This was first attempted by Kobayashi et al.[6]. They used the Zn^{2+} in fruit juices and water for the reactivation of apoCA and determined the activity by means of esterase action. However, they used 1,10-Phenanthroline as chelating agent and were not able to obtain the apoenzyme at a ratio of 100%, nor were they able to remove entirely the native Zn^{2+} present in the enzyme. Küfrevioğlu and Keha[7] tried a different chelating agent, dipicolinic acid (pyridine-1,6-dicarboxylic acid), and achieved a more purified enzyme (97%) in a short time (3h). They used urine cerebrospinal fluid and serum as samples

and inactivated the CA present in the serum through boiling. Recently Demir et al.[8] obtained the enzyme at a high purity (100%) by extending the dialysis time to up to 5 h. They tested the method in different sample obtained from patients with diabetes mellitus and cirrhosis.

Materials and Methods

Preparation of apoCA from Bovine Erythrocytes

Bovine blood was anticoagulated with 1.5 ml of ACD solution (22 g of sodium citrate dihydrate 8 g of citric acid and 24 g of dextrose per liter) per 100 ml blood, and red blood cells were separation and washed with saline. The cells were lysed with distilled water and the ghosts were removed by centrifuge. CA was purified (400-fold) by affinity chromatography[9]. The purified CA was concentrated on a Sephadex G-25 in a batchwise process and was dialyzed first against distilled water and then against Tris-H_2SO_4 (0.05 M, pH 7.4). The resultant enzyme ($\sim$100 mg) was dialyzed against 0.075 M pyridine-2-6-dicarboxylic acid in 0.2 M phosphate buffer (pH 7.4) for 5 h in order to remove the Zn^{2+} from the enzyme[10].

SDS-PAGE Electrophoresis

The purification was checked with 3-10% SDS-PAGE. Electrophoresis was performed according the method described by to Laemmli[11].

Enzyme Activity Determination

CA activity in an eluate obtained during purification was determined by the method described by Wilbur and Anderson[12], as modified by Rickly et al.[13]. The esterase action of CA was used[14] for the preparation of a standard Zn^{2+} curve. In this method, 4-nitrophenyle acetate is hydrolysed to 4-nitrophenol by CA and the absorbance of the product is measured at 348 nm. Reaction mixtures in 3 ml cuvettes contained 0.1 ml apoenzyme solution, 1.0 ml Tris-H_2SO_4 (0.05 M, pH 7.4), 0.4 ml fruit juices (or standard) and 1.5 ml substrate. Three minutes later, the absorbances of the sample and blank cuvettes (distilled water rather than the sample) were measured at 348 nm and 25 °C. The 4-nitrophenyl acetate solution was prepared by dissolving 27.2 mg of the ester in 1 ml of acetone and then adding this to 49.0 ml of distilled water dropwise while stirring.

Protein Determination

The protein content of chromatographic eluates was measured spectrophotometrically at 280 nm by the coomassie brillant blue method[15].

Preparation of Fruit Juices Extract

Fruit were chopped with knives and blended in 200 ml of buffer (0.1 M Na_3PO_4, % PVPP, 10 mM Ascorbic acid, pH 7) per 100 g of fruits. The suspension was filtered twice through a fiberglass window screen. The pulp was squeezed dry and discharged. The filtrate was centrifuged (30 min. at 20000 rpm in a Suprafuge centrifuge) in a continuous flow.

Zinc Determination in Fruit Juices

In order to show the applicability of this method to Zn^{2+} determination in fruit juices, measurements were made in fruit samples. Since low esterase activity, which interferes with Zn^{2+} determination, is observed in fruit juices, water bath for 1 h in capped tubes. Thus the esterase activity of enzyme was totally inhibited. Finally, the Zn^{2+} content in samples of fruit juices was determined as part of enzyme activity determination, as described. Zn^{2+} content in samples of fruit juices was determined by a Shimadzu AA-680/G V-3 atomic absorption spectrometers, as described previously[16].

Results and Discussion

The purification level of bovine carbonic anhydrase isolated from bovine erythrocyetes is given Table 1, the activity-absorbance graph is given Figure 1, and the SDS-PAGE electrophoresis of the enzyme is shown in Figure 2. Table 2 shows the statistical analyses of the results of ten fruit juice samples. The results show that there is a significant correlation between the enzymatic method and the atomic absorption method (Table 2). These values are compatible with the literature[17].

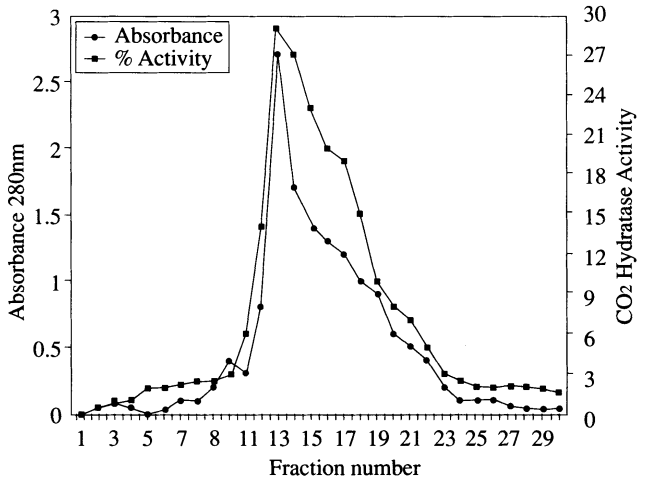

Figure 1. Affinity chromatography of bovine erythrocyte hemolysate carbonic anhydrase in the presence of 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$, pH: 5.6. Absorbance at 280 nm; Carbonic anhydrase activity.

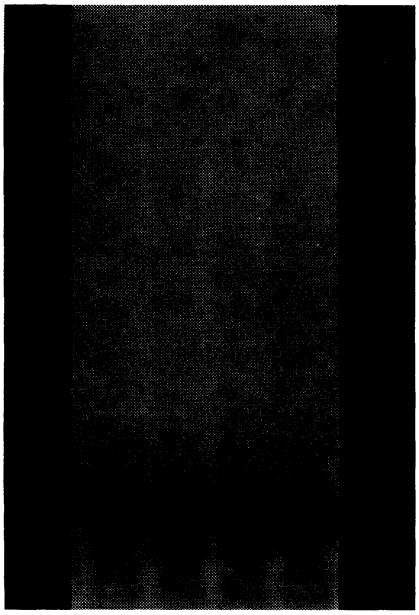

Figure 2. Electrophoretic pattern of purified bovine erythrocyte carbonic anhydrase.

Table 1. Carbonic Anhydrase from Bovine Erythrocyte.

	Volume ml	Total activity	Protein (mg/ml)	Specific activity	Yield %
Erythrocyte hemolysate	300	2040	160	0.043	100
Purified enzyme	125	1335	0.62	400	65

Table 2. Statistical evalution of the results of the enzymatic and absorption

Fruit	Zn^{2+} concentration (mg/100 ml) with enzymatic method N = 10	Zn^{2+} concentration (mg/100 ml) with atomic absorption method N = 10	Sdutent's t-test			Correlation		
			t		P	t		P
Kiwi	24.5 ± 0.04	25.33 ± 0.55	261	>	0.05	0.984	<	0.001
Plumb	27.58 ± 0.34	27.89 ± 0.72	0.67	>	0.05	1.000	<	0.001
Peach	34.09 ± 0.46	31.28 ± 1.25	3.65	>	0.05	0.981	<	0.001
Mulberry	11.0 ± 1.72	10.3 ± 0.70	0.67	>	0.05	0.900	<	0.001
Pear	164.84 ± 0.87	162.35 ± 0.17	0.67	>	0.05	0.900	<	0.001
Strawberry	19.77 ± 0.06	20.57 ± 0.95	2.18	>	0.05	1.000	<	0.001
Apple	110.21 ± 0.92	105.52 ± 1.75	2.22	>	0.05	1.000	<	0.001
Apricot	46.48 ± 0.35	46.27 ± 0.87	0.60	>	0.05	0.987	<	0.001
Cherry	190.5 ± 0.57	190.31 ± 0.66	3.87	>	0.05	1.000	<	0.001
Orange	180.72 ± 0.92	180.37 ± 0.78	1.94	>	0.05	1.000	<	0.0001

Previous reports[6] have mentioned a removal of Zn^{2+} of up to 97%. The remainder (3%) may have important disadvantages since the enzyme, having bound to Zn^{2+}, is active, and thus there is baseline to substract. As can be seen in Figure 3, while the enzyme content is 3.6 nmol, the saturation amount of Zn^{2+} is 13.8 nmol. In the presence of 3% active enzyme, however, larger amounts of apoenzyme are needed for accurate measurement. We achieved a removal of 100% Zn^{2+} from the enzyme by prolonging the duration of dialyses.

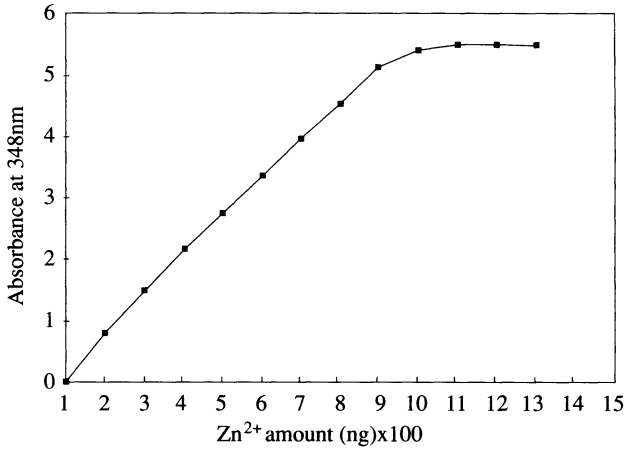

Figure 3. Standard curve obtained with constant apoCA (3.6 $\times 10^{-5}$ M) and changing Zn^{2+} concentrations.

The present study provides a method that may be easily applied in many laboratories having a spectrophotometer. It is our opinion that for this purpose, apoCA may be produced as a commercial material. Furthermore, the stability of apoCA as a protein and the high esterase activity of bovine CA are important advantages of this method. This method is very quick as well, each analysis taking only 3 minutes, and can be performed easily in any laboratory.

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