

Human Erythrocyte Arginase Purification and Characterization

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Abstract: Arginase was purified from human erythrocytes. The purification steps included acetone precipitation, DEAE-cellulose chromatography and gel filtration on Sepharose 6B. The molecular weight of native arginase was found to be (94.406Kd), by gel filtration. The kinetic properties determined for the purified human erythrocyte arginase showed an optimum pH of 10 and an optimum temperature 45 °C. The Km for L-arginine monochloride and V_{max} values for human erythrocyte arginase found to be (200mM), and (47(Unit/ml)⁻¹) respectively

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I. Introduction

L-Arginase (L-arginine amidino hydrolase, EC 3.5.3.1.) is hydrolase and a metalloenzyme that response to hydrolysis of L-arginine to result urea and L-ornithine (1). There are at least two forms of arginase. Arginase cytosolic and is most abundant in the liver plays an important role in the ammonia detoxification in mammals, second isoenzyme, arginase II in extrahepatic mammalian tissues kidney, brain, intestine, mammary gland, erythrocytes, and skin(2,3), is involved in the production of ornithine as a precursor to proline, glutamate or polyamines, such as spermine and putresine, essential for cellular growth(4)

Increase the activity of arginase is associated with various diseases by reducing the supply of L-arginine needed by NOS to produce nitric oxide (plays an important role in homeostatic vasodilatation, regulation of blood flow (5), and also it is thought that it plays an important role in cancer growth as a preventative and therapeutic agent(6)), and by raising production of L-ornithine resulting in vascular structural problems (7) and development of carcinogenesis (8). Since this enzyme is important so it is important to study its properties which were not fully studied for that purified from erythrocyte.

II. Experimental

Materials and methods

The chemical reagents that are used: Sodium chloride, (Fluka, Switzerland), acetone, phosphate buffer, Coomassie Brilliant Blue, Bovine serum albumin, Acetic acid (BDH, England), Sepharose 6B, DEAE- Cellulose, (Pharmacia Fine Chemicals, Sweden), Tris-HCl, Blue dextran, (Sigma, USA),

Arginase assay

Arginase activity during purification was measured as Zofia and Maria (9) described. Briefly the reaction was done in a system containing 0.1 M Sodium barbitone (pH 9.5), 200 mM Substrate solution L-arginine monochloride (PH 7.5), 50 mM Manganese chloride solution, and the sample in a final volume of 1.2 ml; the incubation was carried out for 30 min at 37°C. The reaction was stopped with Trichloroacetic acid 20%, The ornithine concentration formed was determined by ninhydrin reaction (ninhydrin reagent and concentrated acetic acid added to the incubation medium), carried out in a boiling water bath for 60 minutes. Then the colored product was determined spectrophotometrically at 515 nm. Ornithine concentration was calculated from calibration standard curve, and the arginase activity expressed as μg of ornithine/min per 1000 ml.

Soluble protein was determined by the Bradford method (10).

Purification of arginase from human erythrocytes

The purification steps of erythrocyte arginase were adapted from Joseph et.al (11). Human blood was obtained from blood-bank of Al-Khalis hospital-Iraq with blood type of O+. The blood was washed with 0.9 % NaCl, and centrifuged off at (6000 rpm) for 10 min under -4°C. The precipitate washed with the

phosphate buffer (PH 7) twice and centrifuged off at (6000 rpm) for 10 min and the supernatant was kept as crude where the purification steps applied on.

The supernatant obtained above was precipitated by acetone where, cold acetone was mixed with the crude enzyme in a ratio (1:1) (v/v) in ice bath, the mixture is centrifuged at 9000 rpm for 10 min, the precipitate was dissolved in a small amount of 0.2M phosphate buffer pH 7 and dialyzed against the same buffer, then volume, an enzyme activity and protein concentration was measured.

DEAE-Cellulose Ion Exchange Chromatography:

Treated DEAE-Cellulose was packed in a column of (3×10 cm) and equilibration was done with (pH 7.4) buffer of 5 mM Tris-HCl. The dialyzed protein from the preceding step was then layered on the column. The column was first washed with 0.1 M Tris-HCl buffer, pH 7.4, followed by NaCl stepwise gradient ranging (0.05-0.5M) prepared in 5 mM Tris-HCl buffer is used to elute the bounded proteins, from the resin. The fractions of 5ml were collected, performed at a flow rate 1ml/min. Protein was measured spectrophotometrically at 280 nm, the fractions were also assayed for arginase activity. The active fractions were pooled together.

Gel Filtration Chromatography

Column of (1.5×65cm) was packed with Sepharose 6B Gel filtration, column equilibrated with Tris-HCl buffer (0.1M, pH 7.4). Above pooled fractions were concentrated and applied on the Column. 5ml of fractions were collected at 1ml/min. Protein was measured spectrophotometrically at 280 nm and for arginase activity was assayed. The active fractions were pooled together.

The native molecular weight was determined by gel filtration on Sepharose 6B. The standard proteins were Casein (31Kd), LPS for *Klesiella pneumoniae* (52.480 Kd), Bovine serum albumin (67 Kd), Superoxidase dismutase (89 Kd), Catalase (232 Kd).

Optimum pH

The pH dependence of the enzymatic activity of arginase from human erythrocytes was determined by using two buffer systems: sodium phosphate (pH 8.0 to 9.0) and barbiton buffer (pH 9.5 to 11.0).

Effect of temperature

Various temperature values ranged (30-70) °C are used to determine the preincubation effect of temperature on enzyme activity. The activity was determined after the incubation of the purified enzyme for one hour. at different temperature (30, 35, 40, 45, 50, 55, 60, 65, and 70) °C.

Kinetic Parameters:

The kinetic parameters of arginase (V_{max} and K_m) were determined by using various concentrations of the substrate L-arginine monochloride solution (100, 150, 200, 250, 300mM). The kinetic parameters were determined from the double reciprocal plot Lineweaver-Burk.

III. Results And Discussion

Enzyme Purification

Table (1) shows the purification results of human erythrocyte arginase. The specific activity of the purified human erythrocyte enzyme (51.85 units/mg) was 2.52-fold that of the crude erythrocyte extract. The treatment with acetone resulted in the increase of specific activity of the enzyme that also reported by Masaki *et al.* (12), and Joseph *et al.* (13). Erythrocyte arginase was not retained by DEAE-cellulose, that agree with finding of George *et al.* (14) That the kidney arginase was not retained by either DEAE- or CM-cellulose, and also agree with Okonji *et al.* (15) that reported the Tortoise (*Kinixys Erosa*) Liver Arginase get in washing on DEAE-cellulose, but it disagree with Joseph *et al.* (13) finding that the Erythrocyte arginase applied to a DEAE-cellulose column and eluted with a linear gradient of KCl. And this may be due to that the enzyme charge is weak and because of the high concentration of the buffer used (0.1M), too salty, that compete with enzyme to bind the resin; the enzyme is not retained by the resin. However, the specific activity in this step was increased to 34.92 units/mg and the enzyme was purified as 1.7-fold that of the crude erythrocyte extract so this step is success to riddance from some of proteins that get on the resin. Applying the enzyme finally to the sepharose 6-B gel filtration resulted in increase of specific activity and the fold of purifying with yield of 10.46.

Table (1): The purification of arginase from human erythrocyte

steps	Volume(ml)	Enzyme activity (units/ml)	Protein conc. (gm/ml)	Specific activity (unit/mg)	Total activity (units)	Fold	Yield (%)
crude	80	25.10	1.22	20.57	2008	1	100
acetone	45	28.00	0.98	28.57	1260	1.39	62.74
Ion exchange DEAE-cellulose	20	22	0.63	34.92	440	1.70	21.91
Gel filtration sepharose-6B	15	14	0.27	51.82	210	2.52	10.46

Molecular weight

The molecular weight for human erythrocyte arginase, estimated by gel filtration to give a result of (94.406Kd), and this result approaches to those recorded by Masaki et al (12) who found that arginase molecular weight to be (105 Kd) in human erythrocyte, Joseph Beruter et.al.(13) (107 Kd) for human liver arginase, Saleh A. et al (16) finding was (92Kd), and Shivraj et.al.(17) reported was (118Kd) for of buffalo liver arginase.

Optimum pH

The Effect of pH on the purified enzyme by using various buffers with pH rang of (8-11) shows that the activity of arginase increased with increasing pH until reach its maximum activity at pH 10 and, then the activity declined at higher pH Fig(4). this result in agreement with those reported with Snehal et al.(18), Nobuo et al.(19) who found the optimum pH of arginase purified from *Vignacatjangcotyledon* an, *Bacillus subtilis* respectively to be 10.0.

Those Saleh et al. (16) and Okonji et al.(16) found that the optimum pH of Fasciolagigantic Arginase, and Tortoise (*Kinixys Erosa*) Liver Arginase respectively to be 9.5, while R. E. Okonji et al.(20) reported that the optimum pH of 8 for Arginase purified from gut of grasshopper. in general these finding indicate that Arginase appears to show a basic optimum pH of 9.5–10.5, The variance in activity with pH propose that at the catalytic site an ionisable group may function (18).

Effect of Temperature on Enzyme Activity

Asit noticed in Fig.(5) that the preincubation of the purified enzyme in the range of temperature (40-70) for 1hr. detects the optimum temperature for arginase at 45 °C that approaches to those recorded by Saleh. et al(16), Shivraj et al (17), R. E. OKONJI et al(21), and M. Ferit (21) who found that the optimum temperature for arginase to be 40°C. Okonji et al(15) reported that arginase optimum temperature was 60°C.

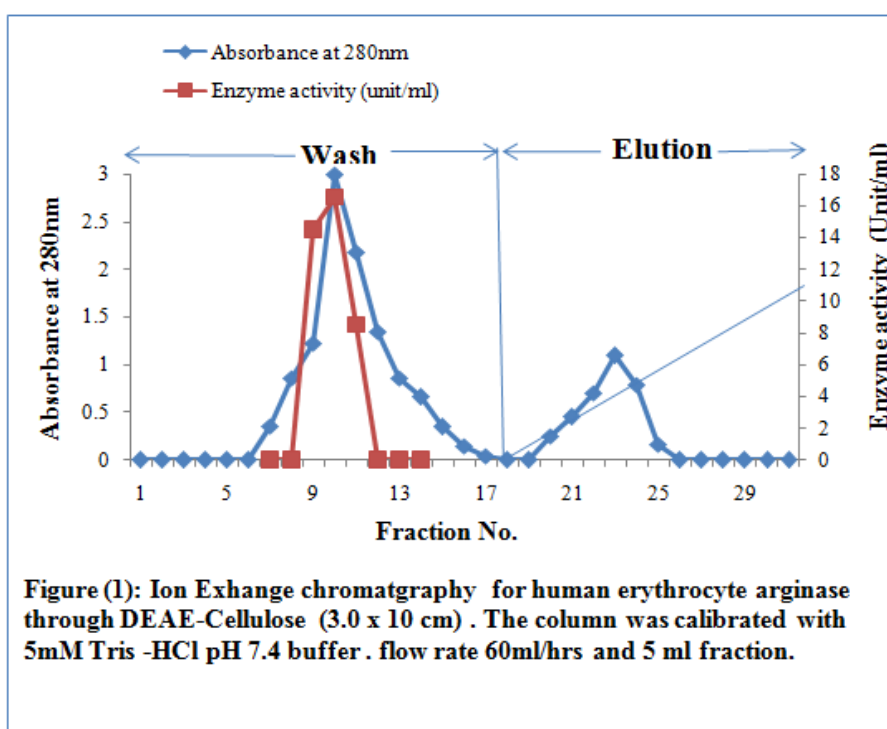
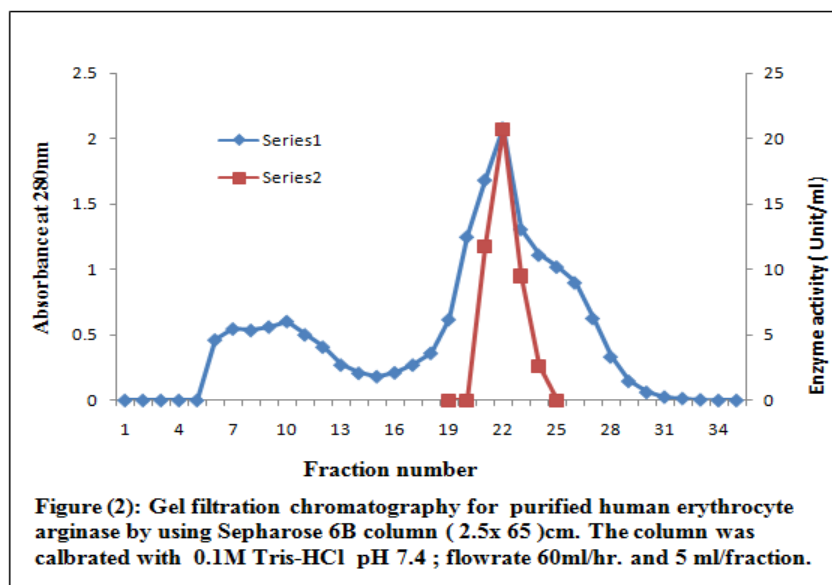
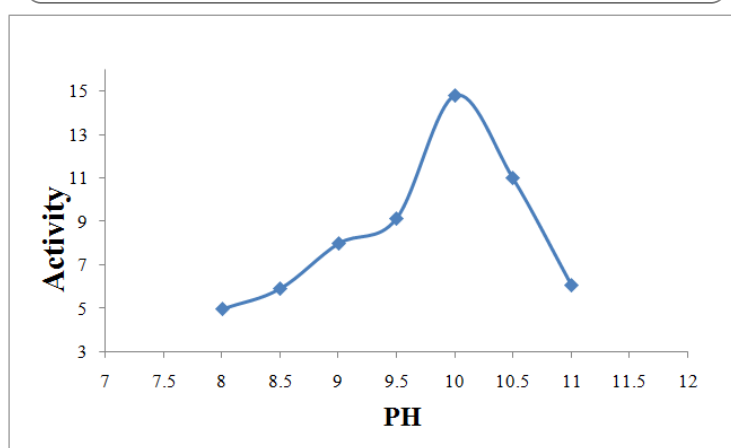
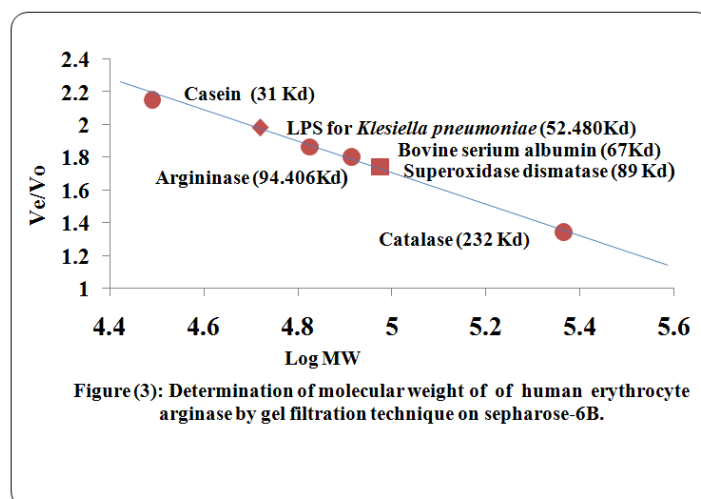


Figure (1): Ion Exchange chromatography for human erythrocyte arginase through DEAE-Cellulose (3.0 x 10 cm) . The column was calibrated with 5mM Tris -HCl pH 7.4 buffer . flow rate 60ml/hrs and 5 ml fraction.



Kinetic parameters

The substrate concentration effect on the activity of human erythrocyte arginase was clarified by Lineweaver-Burk plot Fig(6), the relation between the reciprocal of substrate concentration and the reciprocal of velocity, the K_m and V_{max} values for human erythrocyte arginase found to be (200mM), and $(47(\text{Unit/ml})^{-1})$ respectively. It can be noticed that the increasing in substrate concentration enhances the rate of the reaction until the further increasing in substrate



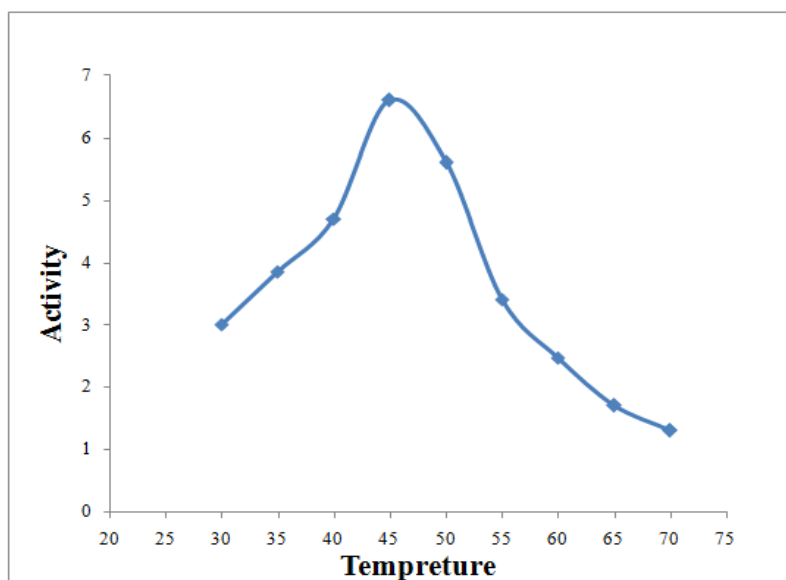


Figure (5): the effect of temperature on human erythrocyte arginase activity

Concentration become without effect on the reaction rate, because the enzyme becomes saturated, and the active site is occupied with the substrate. Ferit, (21) stated that K_m value for arginase purified from human Vitreous Humor were 6 mM to L-arginine, R. E. OKONJI et al (21), and Okonji et al (15) reported that K_m value to L-arginine was (40mM) for arginase purified from gut of grasshopper, and (66.7mM) for arginase purified from liver of tortoise, Ezima et al found that the K_m to be (17mM) for arginine and V_{max} value to be (1.39 $\mu\text{mole ml}^{-1} \text{min}^{-1}$) for arginase purified from fruit bat liver. The variance in millimolar concentration of arginine may be belong to the different methods that used and nonphysiological conditions generally used (1).

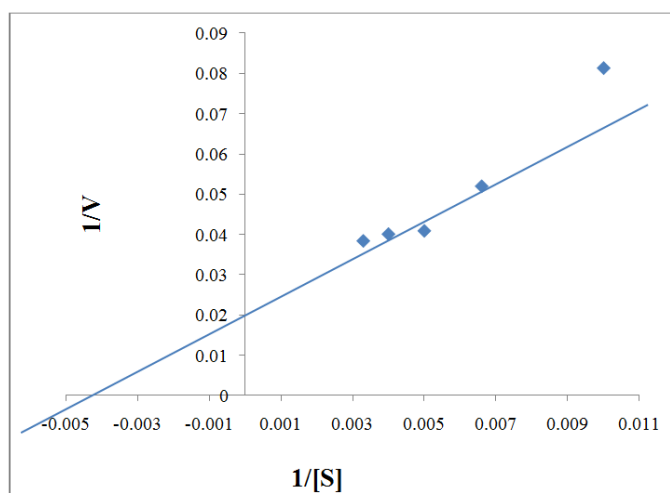


Figure (6): Kinetic constants of arginase purified from human erythrocytes according to Lineweaver-Burk

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