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Arginase from Human Full-Term Placenta

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Arginase was purified about 1800-fold from extracts of human full-term placenta; the enzyme appeared to be homogeneous by disc electrophoresis and molecular-sieve chromatography. The mol.wt. determination by gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis yielded a value of 70000 for the most pure and the partially purified enzyme. The human placenta arginase is a metalloenzyme with an optimum pH of 9.1. The K_m for L-arginine is 27mM. L-Ornithine and L-lysine show competitive inhibition with K_i values of 6.3 and 14mM respectively.

Arginase (EC 3.5.3.1) is a widely distributed enzyme involved in a variety of metabolic roles: the urea cycle in the liver of ureotelic animals, proline biosynthesis in mammary gland (Mepham & Linzell, 1967; Yip & Knox, 1972), polyamine biosynthesis in male genital tract (Della Pietra *et al.*, 1973), in lactating mammary gland (Oka & Perry, 1974) and in epidermis (Cotton & Mier, 1974).

Several lines of evidence have suggested a possible relationship between the functional role in cellular metabolism and the molecular characteristics of arginase from different tissues (Harrel & Sokolovsky, 1972; Vielle-Breitburd & Orth, 1972; Reyero & Dorner, 1975). Vertebrate hepatic arginases have mol.wts. of 120000 and 240000 (ureotelic and uricotelic animals respectively); the enzymes have a quaternary structure and each subunit has a mol.wt. of 30800 (Hirsh-Kolb & Greenberg, 1968). In mammary gland the enzyme activity has been separated by Glass & Knox (1973) into two fractions of mol.wt. 42000 and 94000. Tarrab *et al.* (1974) have reported evidence of three different arginase isoenzymes from rat liver and three isoenzymes have been also observed in human diploid fibroblasts (Van Elsen & Leroy, 1975).

The presence of arginase in human placenta was first demonstrated by Porta *et al.* (1975). In the present paper a procedure for the purification of the enzyme in homogeneous form and some of its properties are described.

Experimental

Materials

L-[*guanidino*-^{14}C]Arginine monohydrochloride and $Na_2{}^{14}CO_3$ were from The Radiochemical Centre, Amersham, Bucks., U.K.; urease, L-arginine, L-ornithine, L-lysine were from Sigma Chemical Co., St. Louis, MO, U.S.A. $MnCl_2,4H_2O$ and $(NH_4)_2SO_4$ were from Merck, Darmstadt, Germany. Acrylamide and methylenebisacrylamide were from Fluka, Buchs, Switzerland. *NNN'N'*-Tetramethylethylenediamine was from Eastman Organic Chemicals, Rochester, NY, U.S.A. Hyamine hydroxide was from Packard Instruments, Downers Grove, IL, U.S.A. Cytochrome *c*, chymotrypsinogen A, ovalbumin and bovine serum albumin were from Boehringer, Mannheim, W. Germany. Sephadex G-100 and Sephadex G-150 were from Pharmacia Fine Chemicals, Uppsala, Sweden. CM-cellulose (CM-52) was from Whatman, Maidstone, Kent, U.K. Diaflo PM-10 membranes were from Amicon, Lexington, MA, U.S.A. All other chemicals were the purest available grades from standard commercial sources.

Placentas from normal pregnancies, which terminated at 38–42 weeks in the birth of healthy infants of normal size, were investigated. Only the parts that were recognized as placental tissue by macroscopic examination were taken for assays. The placentas were frozen at −20°C and examined within a few days.

Arginase assay

The reactions were done in scintillation vials sealed with a rubber stopper and equipped with a polypropylene well (supplied by Kontes Glass Company, Vineland, NJ, U.S.A.). The well contained approx. 0.2ml of Hyamine hydroxide. The enzyme was activated by preincubation for 15min at 58°C in the presence of 2.5mM-$MnCl_2$ (pH7.5); the complete assay mixture (1.0ml) containing 0.1M-Tris/HCl buffer, pH9.1, 10mM-L-arginine and L-[*guanidino*-^{14}C]arginine (180000d.p.m.) was incubated for 30min at 37°C; the reaction was stopped by

addition of 0.25ml of 0.5M-H_2SO_4. Then the pH was adjusted to pH6 with phosphate buffer, and urease (sufficient to degrade 2μmol/min) was added to hydrolyse the [^{14}C]urea formed. After digestion with urease for 30min at 37°C, 0.5ml of 3M-H_2SO_4 was injected through the rubber cap in order to stop the reaction and to release $^{14}CO_2$ from the medium. Microdiffusion for 30min at 37°C ensured the complete trapping of $^{14}CO_2$ by the Hyamine hydroxide. The well was removed, dropped into a counting vial containing scintillation solution (Zappia & Ayala, 1972) and the radioactivity measured in a Tri-Carb liquid-scintillation spectrometer (Packard model 3380) equipped with an absolute activity analyser. Quantitative recovery of CO_2, in the reported experimental conditions, was demonstrated with $Na_2{}^{14}CO_3$. Corrections for quenching were made by external standardization. One enzyme unit is defined as the amount of enzyme that produces 1μmol of urea in 60min at 37°C. Specific activity is expressed in enzyme units/mg of protein. Protein was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Purification

The purification procedure was performed at 4°C. The protein solutions were concentrated by ultrafiltration through a Diaflo PM-10 membrane.

Analytical polyacrylamide-gel electrophoresis

All gels [7.5% (w/v) polyacrylamide] were prepared according to the method of Ornstein (1964) or Davis (1964). Electrophoresis was performed in Tris/glycine buffer (6g of Tris, 28.8g of glycine made up in 1 litre of water, pH8.3) with a current of 3mA/tube. Protein samples (50μg) were applied to the gel. After removal from the tubes, gels were stained with Coomassie Brilliant Blue [1.25g in 454ml of aq. 50% (v/v) methanol and 46ml of acetic acid] for 15h at 20°C. Gels were then destained with frequent changes of acetic acid/methanol/water (3:2:35, by vol.).

Molecular-weight determination

The molecular weight of human placenta arginase was determined by Sephadex G-100 chromatography at 15°C as described by Andrews (1964). The column (1.8cm×90cm) was previously equilibrated in 0.01M-Tris/HCl buffer, pH7.5, containing 0.01M-$MnCl_2$.

Horse heart cytochrome *c* (mol.wt. 12400), chymotrypsinogen A (mol.wt. 25700), ovalbumin (mol.wt. 46000) and bovine serum albumin (mol.wt 67000) were used as molecular weight standards (5mg each). Fractions (2ml) were checked for arginase activity and protein content was determined by measuring E_{280} and E_{412} (cytochrome *c*).

The molecular weight of homogeneous preparations of arginase was also determined by the procedure of Shapiro *et al.* (1967) by electrophoresis in 10% polyacrylamide gels (pH8.3) containing 0.1% sodium dodecyl sulphate and with cytochrome *c*, chymotrypsinogen A, ovalbumin and bovine serum albumin as molecular-weight standards.

Results

Purification procedure for human placenta arginase

For the purification, some of the steps previously described by Schimke (1962) and Tarrab *et al.* (1974) for rat liver arginase were adapted to human placenta arginase. The purification procedure is summarized in Table 1. Unless otherwise stated all procedures were carried out at 4°C.

Step 1: *extraction.* The placentas were exsanguinated, minced and homogenized in a Waring Blendor with 3vol. of 0.01M-Tris/HCl, pH7.5, containing 0.1M-KCl and 0.05M-$MnCl_2$. The mixture was centrifuged at 13000*g* for 30min and the pellet was discarded.

Step 2: *acetone fractionation.* To supernatant from step 1 acetone (1.5vol./vol. of supernatant) at −10°C was added slowly while the mixture was stirred. The resulting suspension was centrifuged at 13000*g* for 15min at −10°C and the supernatant was discarded. The precipitate was air-dried at room temperature and then homogenized in a Waring Blendor with 0.7vol. (of the original placenta weight) of 0.01M-Tris/HCl, pH7.5, containing 0.05M-$MnCl_2$. The homogenate was dialysed overnight against the same buffer and centrifuged at 30000*g* for 1h.

Step 3: *heat treatment.* The protein solution

Table 1. *Purification of human placenta arginase*

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Homogenate supernatant	1350	43200	2126	0.05	100	1
2. Acetone fractionation	172	1995	593	0.3	27.9	6
3. Heat treatment	150	1050	907	0.86	42.7	17.2
4. Ethanol precipitation	13	63.7	1404	22.04	66	441
5. CM-cellulose chromatography	4	5.5	271	49.2	12.75	984
6. Sephadex G-150 filtration	2	2.5	225	90	10.6	1800

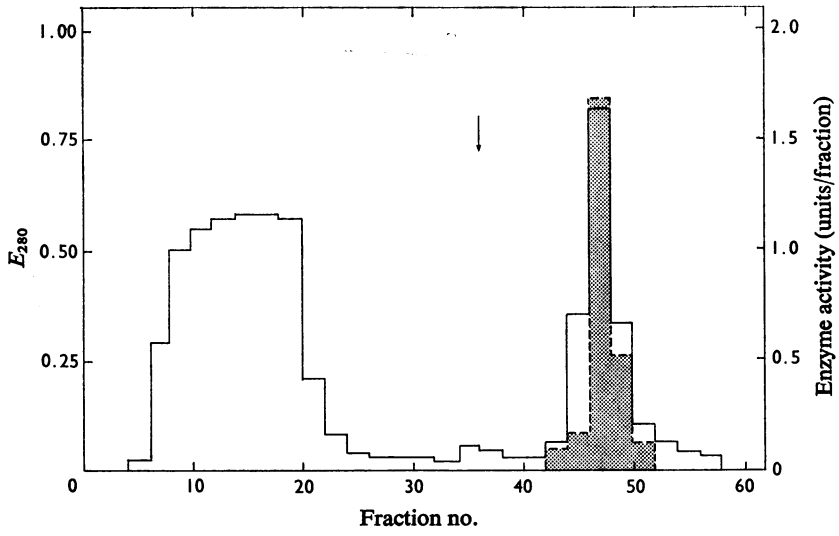

Fig. 1. *Elution pattern of human placenta arginase from CM-cellulose chromatography*

The enzyme from step 4 (Table 1) (63.7mg, 13ml) was applied to a CM-cellulose column (2.5cm×10cm). Chromatography was performed as described in the text and the active fractions (20ml) were pooled and concentrated by ultrafiltration. ——, Protein absorbance; ----, arginase activity. The arrow shows the addition of 1M-KCl.

obtained in step 2 was heated in a water bath for 20min at 60°C, cooled in an ice bath and centrifuged at 13000*g* for 15min. The sediment was discarded.

Step 4: *ethanol precipitation.* Ethanol (3vol.) containing 0.05M-$MnCl_2$ at −10°C was added to the supernatant (step 3). After centrifugation at −10°C (30000*g*) for 15min, the supernatant was discarded. The precipitate was completely re-dissolved by stirring slowly in a small volume of 0.01M-Tris/HCl, pH7.5, containing 0.05M-$MnCl_2$ and then freeze-dried. The dry powder was re-dissolved with a small volume of 0.01M-Tris/HCl, pH7.5, and dialysed against three changes of the same buffer for 4h.

Step 5: *CM-cellulose chromatography.* The dialysed solution was applied to a column (2.5cm×10cm) of CM-cellulose previously equilibrated with 0.01M-Tris/HCl, pH7.5. The column was washed with this buffer until no absorbance at 280nm was detected. Arginase was then eluted as a single symmetrical peak with 0.01M-Tris/HCl, pH7.5, containing 1.0M-KCl. Fig. 1 shows a typical elution pattern. The active fractions were pooled and concentrated by ultrafiltration through a Diaflo PM-10 membrane. Out of several attempts to purify the enzyme by CM-cellulose chromatography at various ionic strengths, the procedure outlined above proved the most effective.

Step 6: *Sephadex G-150 chromatography.* The enzyme was further purified by filtration on a Plexiglass column (1.5cm×90cm) of Sephadex G-150 (Pharmacia) equilibrated with 0.01M-Tris/HCl, pH7.5, containing 0.01M-$MnCl_2$. The activity appeared largely in a single peak (Fig. 2). The fractions having the highest specific activity (65–71) were pooled, concentrated by ultrafiltration and used as the enzyme source in subsequent experiments. The arginase preparation thus obtained gave a single protein band in polyacrylamide-gel electrophoresis, and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis confirmed the protein homogeneity. The specific activity of the enzyme was about 1800-fold greater than the specific activity of the crude extract (Table 1).

Determination of molecular weight

The molecular weight determined by gel filtration on a Sephadex G-100 column (Fig. 3) and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by a sample from step 6 was 70000 (Porta *et al.*, 1975). The same molecular weight was found when the enzyme was partially purified (about 1000-fold) by 40–70%-satd.-$(NH_4)_2SO_4$ precipitation, heat treatment, and CM-cellulose and Sephadex G-150 chromatography without acetone or ethanol precipitation.

Activation by Mn^{2+} ions

Human placenta arginase, like other mammalian arginases, depends upon a temperature-dependent

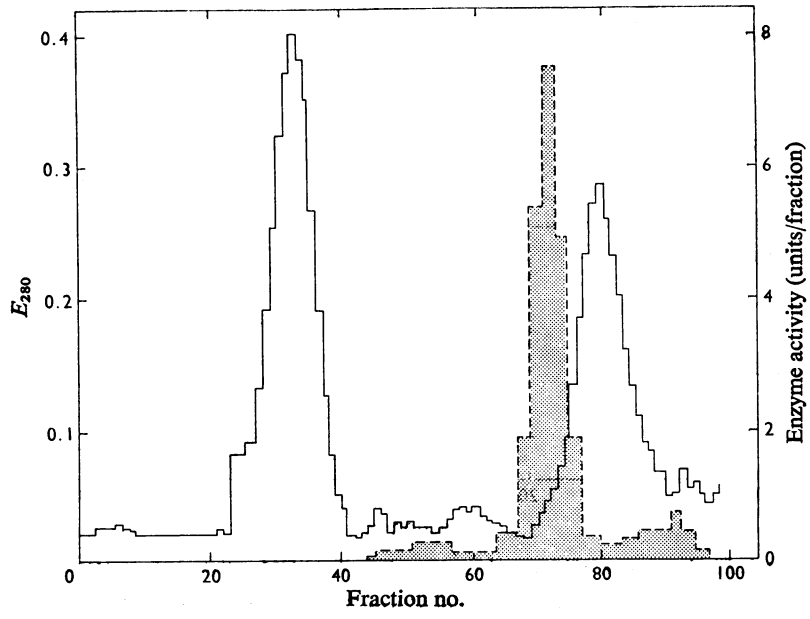

Fig. 2. *Sephadex G-150 chromatography of human placenta arginase*

The column was loaded with a 4ml sample containing 5.5mg of protein (from step 5, Table 1). The filtration was performed as described in the text. Fractions (2ml) were collected. The most active fractions were pooled and concentrated by ultrafiltration. ——, Protein absorbance; ----, arginase activity.

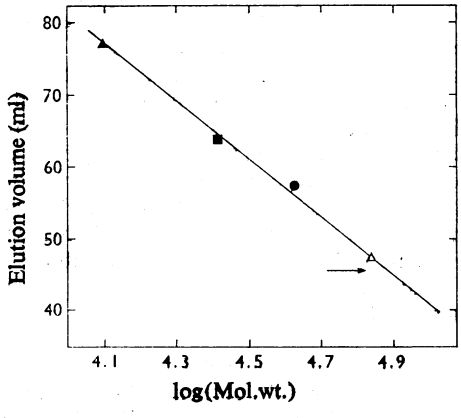

Fig. 3. *Determination of the molecular weight of human placenta arginase on Sephadex G-100*

The column (1.8cm×90cm) was equilibrated with 0.01M-Tris/HCl, pH7.5, containing 0.01M-$MnCl_2$. The proteins used as standards were: ▲, cytochrome *c*; ■, chymotrypsinogen A; ●, ovalbumin; △, bovine serum albumin. The arrow marks the elution position of arginase.

binding with Mn^{2+} ions for complete activation. A preincubation of the purified enzyme at 58°C for 15min in the presence of 2.5mM-$MnCl_2$ increases enzyme activity about 3-fold, whereas after preincubation at 37°C the enzyme activity reached only 50% of the maximum.

pH optimum

After enzyme assays in 0.1M-Tris/HCl between pH values 7 and 10, a pH optimum of 9.1 was observed.

Substrate dependence

From a double-reciprocal plot of initial rate and substrate concentration the Michaelis constant of the step-6 enzyme for L-arginine was found to be 27mM in Tris/HCl, pH9.1, in the presence of 2.5mM-$MnCl_2$. L-Ornithine (K_i 6.3mM) and L-lysine (K_i 14mM) appeared to be competitive inhibitors (Fig. 4).

Discussion

The purification procedure for human placenta arginase reported in the present paper yields a protein that appears to be homogeneous. At least, both molecular-sieve chromatography of the pure enzyme on Sephadex G-100 and disc electrophoresis at pH8.3 fail to reveal the presence of contaminating material. No evidence for the presence of isoenzymes was detected. The mol.wt. determined by gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was 70000. Since this value does not

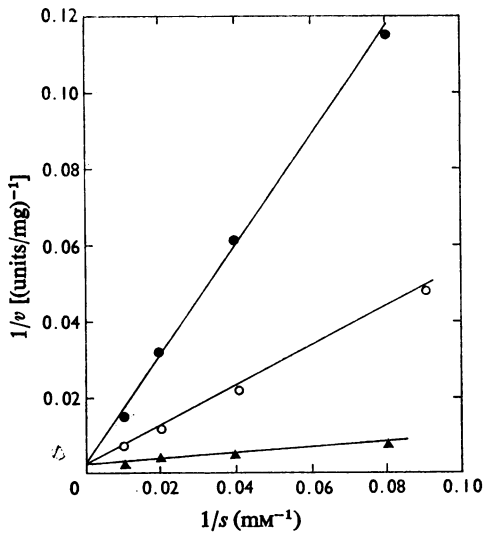

Fig. 4. *Double-reciprocal plot of the arginase activity of human placenta versus* L-*arginine concentration*

Assays were performed as described in the text by using 10μg of purified enzyme per assay. L-Arginine was used as substrate for K_m determination (▲), and also with 100mM-L-ornithine (●) and 100mM-L-lysine (○) for K_i determinations.

closely resemble the molecular weights of other arginases, it was of interest to inquire whether this molecular weight was the real one or a consequence of purification methodology. Accordingly a partial purification (about 1000-fold) of the enzyme was used involving steps unlikely to produce changes in the quaternary structure: the same molecular weight was found, and it is therefore not likely that the pure enzyme is an artifact of disaggregation. The question of dissociation into subunits and the possible association into aggregates must await more detailed studies.

The enzyme appears to be a metalloenzyme, since the catalytic activity seems to be correlated with the amount of bound Mn^{2+} ions. The K_m decreases with enzyme purification from 170mM in the crude placental extract (Porta *et al.*, 1975) to 27mM in the 1800-fold purified preparation; this value is in the range (<50mM) found generally for ureotelic vertebrate arginases (Mora *et al.*, 1965).

Competitive inhibition by L-ornithine is a characteristic of arginase from human placenta, and this has been observed with arginase from chicken liver, *Neurospora crassa* (Mora *et al.*, 1966) and rat mammary gland and liver (Glass & Knox, 1973). The human placental enzyme is also competitively inhibited by L-lysine, as found with arginase of the insect fat-body (Reddy & Campbell, 1969) and human liver (Bascur *et al.*, 1966).

Our results support the view that arginases present in different organs are different enzyme proteins. Concerning the role of arginase in human placenta, we suggest the possibility that the function of the enzyme is to provide a reservoir of ornithine for the production of spermidine.

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