

# Expression of human liver arginase in *Escherichia coli*

## Purification and properties of the product

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Arginase is an enzyme that catalyses the hydrolysis of arginine to urea and ornithine. It is abundantly present in the liver of ureotelic animals (i.e. those whose excretion is characterized by the excretion of uric acid as the chief end-product of nitrogen metabolism), but its purification has hitherto not been simple, and the yield not high. Starting with a partially truncated cDNA for human liver arginase recently made available, we constructed an expression plasmid that had tandemly linked *tac* promoters placed upstream of a full-length cDNA. By selecting *Escherichia coli* strain KY1436 as the host micro-organism, we established an efficient system for the production of human liver arginase protein. Chromatographies on CM-Sephadex G-150, DEAE-cellulose and Sephadex G-150, followed by preparative agar-gel electrophoresis, yielded 10 mg of apparently homogeneous enzyme protein from 1 g (wet wt.) of *E. coli* cells. *E. coli*-expressed human liver arginase had chemical, immunological and most catalytic properties indistinguishable from those of purified human erythrocyte arginase. However, *E. coli*-expressed arginase was a monomer of  $M_r$  35000, whereas the purified erythrocyte arginase was trimer of  $M_r$  105000. They differed also in pH- and temperature-stabilities. Gel-filtration experiments with these two purified arginases under various conditions, as well as with unfractionated human liver and erythrocyte cytosol preparations, indicated that the native form of human arginase should be of  $M_r$  35000, and that the trimeric appearance of human erythrocyte arginase after purification was an artifact of the purification procedures. It was thus concluded that, in Nature, the liver and erythrocyte arginases are identical proteins.

## INTRODUCTION

Arginase (EC 3.5.3.1; L-arginine amidinohydrolase) is a key enzyme of the urea cycle that catalyses the final step of urea formation in the mammalian liver. Despite its abundance in the liver, purification of human liver arginase has been reported only with rather poor yield and purity (Bascur *et al.*, 1966; Berüter *et al.*, 1978) or with complicated methods (Greenberg *et al.*, 1956; Berüter *et al.*, 1978). Human erythrocytes have been used as a better starting material for the purification of human arginase, yielding an apparently homogeneous preparation of arginase, though again by rather tedious procedures (Nishibe, 1973). Moreover, no conclusive evidence has been obtained for the identity of this purified erythrocyte arginase with liver arginase. In addition, a fairly large amount of human liver arginase as a pure protein is needed, not only for biochemical studies, but also for more practical purposes. For example, the release of liver arginase into plasma has been reported to be useful as a diagnostic index of liver damage, such as that caused by carbon tetrachloride (Kaiju, 1938; Utzino & Ogawa, 1957; Ogawa, 1958). For developing an appropriate immunoassay system for laboratory diagnosis, pure antigen arginase is a prerequisite. Also, several cases of hyperargininaemia have been reported to reflect a hereditary deficiency of liver arginase (Cederbaum & Shaw, 1977; Spector *et al.*, 1983; Bernar *et al.*, 1986); in such cases human liver arginase may find a potential therapeutic application.

Molecular cloning and nucleotide sequence of cDNA for human liver arginase has been reported, but the longest cDNA used for sequencing still lacked 24 nucleotides at its 5' end, thus yielding, when expressed in *Escherichia coli*, an arginase-like

product that had only 0.018% of the enzymic activity of the native enzyme (Haraguchi *et al.*, 1987). We therefore attempted to construct an expression plasmid housing a full-length cDNA for human liver arginase, and established a system for producing arginase protein that yielded as much as 10 mg of purified arginase per 1 g wet weight *E. coli* cells. The product, after purification by relatively simple procedures, was characterized in comparison with the human erythrocyte arginase that we had purified earlier (Ikemoto *et al.*, 1989). Such a comparison led us to conclude that human liver and erythrocyte arginases are identical proteins, both having  $M_r$  values of 35000 in their native states.

## MATERIALS AND METHODS

### Materials

CM Sephadex C-50, Sephadex G-150, Sephadex G-50 (fine grade), and CNBr-activated Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose (DE52) was obtained from Whatman.  $M_r$  standards and BSA were Sigma products. Analytical reagents were obtained from the following commercial sources: urease (type II), peroxidase (type II; RZ, 3.13), and L-glutamate dehydrogenase (type II) from Toyobo Co., Osaka, Japan; NADPH from Kohjin Co., Tokyo, Japan; restriction endonucleases and other enzymes from Takara Shuzo, Kyoto, or Toyobo, Osaka, Japan. SDS, isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG), disuccinimidyl suberate, dimethyl sulphoxide and other chemical reagents were products of Nacalai Tesque Co., Kyoto, Japan. Oligonucleotides were synthesized

Abbreviation used: IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside.

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with a DNA synthesizer (model 381A; Applied Biosystems) and purified by electrophoresis. Plasmid PTTQ18 (Stark, 1987) was purchased from Amersham International. *E. coli* K-12, strain HB101, which was obtained from Takara Shuzo Co., was used for bacterial transformation to construct plasmids. *E. coli* K-12, strain KY1436 (Yano *et al.*, 1987), was used for the expression of cloned cDNA for human liver arginase.

#### Construction of expression plasmids

cDNA phARG6 for human liver arginase (Haraguchi *et al.*, 1987) was used. Its 1.2 kb *SacI*-*HindIII* fragment, a synthetic double-strand oligonucleotide, and PTTQ18 (Stark, 1987) digested by *EcoRI* and *HindIII* were ligated as shown in Fig. 1. The resulting plasmid, pTAA11, was used for further construction. The 200 bp *BamHI* fragment of pKK223-3 (Brosius,

1984) was cloned into the *BamHI* site of pUC9 (Vieira & Messing, 1982). The resulting plasmid, pUCtacP, was digested by *EcoRI*, and the 200 bp fragment, containing a *tac* promoter, was ligated with pTAA11 digested by *EcoRI*. Finally plasmid pTAA12, containing tandem *tac* promoters, was obtained as shown at the bottom of Fig. 1.

#### Expression of the arginase gene in *E. coli*

*E. coli* K-12 strain KY1436 cells containing pTAA12 were grown in 10 litres of L broth containing ampicillin (50 µg/ml) at 30 °C. At an  $A_{600}$  of 1, 1 mM-IPTG was added. The bacteria were collected when the  $A_{600}$  was 3.5. A portion of the cells was resuspended in an adequate volume of 10 mM-Tris/HCl buffer, pH 7.4. The suspended cells were sonicated and centrifuged. The supernatant was used for the purification of arginase.

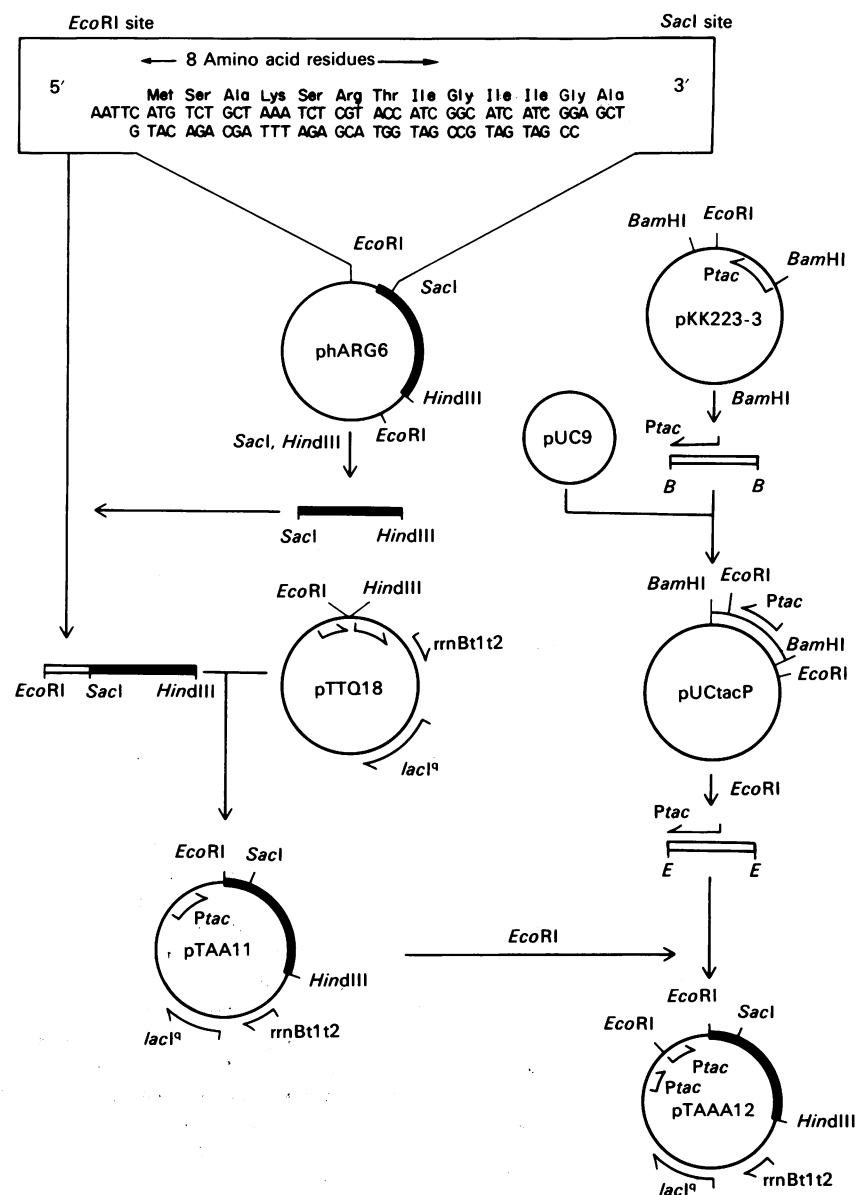


Fig. 1. Strategy of construction of plasmid pTAA12.

The 1.2 kb *SacI*-*HindIII* fragment of phARG6 and synthetic double-strand oligonucleotide were ligated, and the ligated fragment was subcloned using pUC9. The cloned fragment and pTTQ18 digested by *EcoRI* and *HinIII* were consecutively ligated. The resulting plasmid, pTAA12, was obtained as described in the text.

### Assay of arginase activity

The rate of the release of urea from L-arginine by arginase was monitored in a system containing urease, L-glutamate dehydrogenase and NADPH (Özer, 1985). The incubation mixture (2.0 ml) contained 0.1 M-Tris/HCl buffer, pH 8.3, without Mn(II), 100 µg of L-glutamate dehydrogenase, 0.5 mg of urease, 0.5 µM-NADPH, and 50 mM-L-arginine. After a preincubation for 1 min at 30 °C, a linear decrease in  $A_{340}$  was recorded for 1 min at the same temperature. One unit of arginase was defined as the amount of enzyme that released 1.0 µmol of urea for 1 min under the given conditions. For the pH-profile study the arginase reaction was terminated by adding 0.1 ml of 6 M-HCl; the urea released was then determined with urease, and an indophenol method was used to assay  $\text{NH}_3$  (Chaney & Marbach, 1962).

### Immunological methods

Antisera were raised in rabbits against human erythrocyte arginase (Ikemoto *et al.*, 1989) and *E. coli*-expressed human liver arginase respectively, and IgGs were prepared by  $(\text{NH}_4)_2\text{SO}_4$  fractionation and DEAE-cellulose chromatography.

An e.i.s.a. system was developed using anti-[human liver arginase (*E. coli*-expressed)] IgG as the first antibody for coating 96-well microtitre plates and horseradish-peroxidase-conjugated Fab' fragment (Ishikawa *et al.*, 1983) of the aforementioned IgG as the second antibody. The peroxidase activity was determined colorimetrically at 492 nm with *o*-phenylenediamine in 0.1 M-citrate buffer, pH 5.0, as the substrate (Joyce *et al.*, 1977). The e.i.s.a. system was found to be applicable to quantifying arginase antigen over the range 3–800 ng of protein.

For Western-blot analysis, IgGs were further purified by affinity chromatography using an arginase-Sepharose 4B column with the corresponding antigen arginase as the ligand (Brusdeilins *et al.*, 1985). Subclasses of the affinity-purified IgGs were found to be IgG<sub>1</sub> for anti-(erythrocyte arginase) antibody and IgG<sub>2</sub> for anti-(liver arginase) antibody. Western-blot analysis was carried out as described by Towbin *et al.* (1979), using nitrocellulose membrane and commercially available (Capell) peroxidase-conjugated goat anti-rabbit IgG as the second antibody.

### Other methods

SDS/PAGE was carried out by the method of Laemmli (1970), with 12.5% acrylamide and 0.1% SDS. Gels were stained with Coomassie Brilliant Blue (Burgess, 1969).

Preparative agar-gel electrophoresis was carried out using 8 cm × 10 cm gels of 10 mm thickness containing 1% agar in 50 mM-Tris/HCl buffer, pH 7.4. The gel was placed in a submerged electrophoretic tank of the type commonly used for analysis of DNA. The sample (50 mg of protein) was loaded into the centre slot of the gel, and electrophoresis was carried out for 1 h at a constant current of 30 mA with cooling. A piece of the gel was used for the location of the migrated arginase, and the arginase band thus found was sliced and then homogenized with 5 vol. of 50 mM-Tris/HCl buffer, pH 7.4, using a Teflon/glass homogenizer. The homogenate was centrifuged for 5 min at 4 °C, and the supernatant was dialysed overnight against 2 mM-Tris/HCl, pH 7.4, and freeze-dried.

Chemical cross-linking of arginase protein was carried out with glutaraldehyde (Hatanaka *et al.*, 1985). A portion (0.1 ml) of 0.25% glutaraldehyde diluted with distilled water was added to 1.0 ml of 50 mM-sodium phosphate buffer, pH 7.2, containing purified arginase (approx. 1 mg), mixed well by stirring for 1 h at room temperature, and then quenched by adding 0.1 ml of 1 M-Tris. The mixture was subjected to gel filtration at a flow rate of 6 ml/h with the same buffer. With each fraction, protein was

determined by the method of Lowry *et al.* (1951), with BSA as the standard, and arginase activity was determined by the coupled enzymic method described above. The amino acid composition of purified arginase (approx. 0.4 mg) was determined by using a JEOL (Tokyo, Japan) JLC-300 h.p.l.c. apparatus equipped with a 6.0 mm × 90 mm column containing LC-R-6 gel.

## RESULTS

### Selection of expression vectors and strains of *E. coli*

As shown in Fig. 1 (see above), plasmid pTAA11 has a full-length DNA for human liver arginase placed downstream of the *tac* promoter. Several *E. coli* strains transformed by pTAA11 were found, however, to express poorly only arginase, probably owing to the unfavourable distance (15 bp) between the Shine-Dalgarno sequence (SD) and the initiation codon ATG. We therefore constructed another plasmid, pTAA12, in which *tac* promoters were tandemly placed with a distance of 10 bp between the Shine-Dalgarno sequence and ATG (Fig. 1). Among *E. coli* strains tested, strain KY1436, when transformed by pTAA12 and induced by IPTG, was found to produce large amounts of human arginase, as can be seen by Coomassie Blue protein staining of SDS/PAGE gels of the crude extract from cultured cells (Fig. 2, lanes 1–6).

### Purification of *E. coli*-expressed human liver arginase

pTAA12-transformed KY1436 cells (10 g wet wt.) were suspended in 100 ml of 10 mM-Tris/HCl buffer, pH 7.4, and the sonicated and centrifuged supernatant was used for the starting material. The purification procedures were generally similar to, but simpler than, those used for human erythrocyte arginase (Ikemoto *et al.*, 1989). In short, the supernatant was applied to a CM-Sephadex C-50 column (19 mm × 120 mm) previously equilibrated with 10 mM-Tris/HCl buffer, pH 7.4. Proteins were eluted with a linear gradient of NaCl from 0 to 0.6 M, and the arginase activity of the eluate in each fraction was assayed as described in the Materials and methods section. Fractions

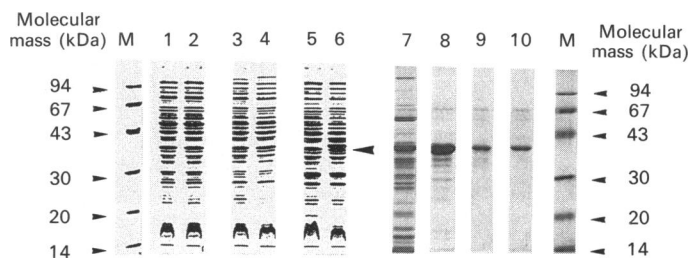


Fig. 2. Expression of human liver arginase in several strains of *E. coli*

SDS-PAGE was carried out with the sonicated supernatants from *E. coli* cells (lanes 1–6) and with the protein samples obtained in several steps of the purification (lanes 7–10). Lanes 1 and 2, *E. coli* strain RB791; lanes 3 and 4, W3110; lanes 5 and 6, KY1436. The amount of proteins applied to each lane was approx. 500 µg. Lanes 1, 3 and 5, without IPTG induction; lanes 2, 4 and 6, with IPTG induction. The purification was started with the sonicated supernatant from strain-KY1436 cells; lane 7, after Sephadex C-50 chromatography; lane 8, after DEAE-cellulose (DE52) chromatography; lane 9, after gel filtration on Sephadex G-150; lane 10, after agar-gel electrophoresis. The amounts of proteins applied were: lane 7, approx. 100 µg; lane 8, 50 µg; lane 9, 25 µg; lane 10, 20 µg. Lane M contained marker proteins: phosphorylase *b* ( $M_r$  94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), and  $\alpha$ -lactalbumin (14400). An arrowhead to the right of lane 6 indicates the appearance of an extra protein band with  $M_r$  35000, which corresponds to the major band on lanes 7–10.

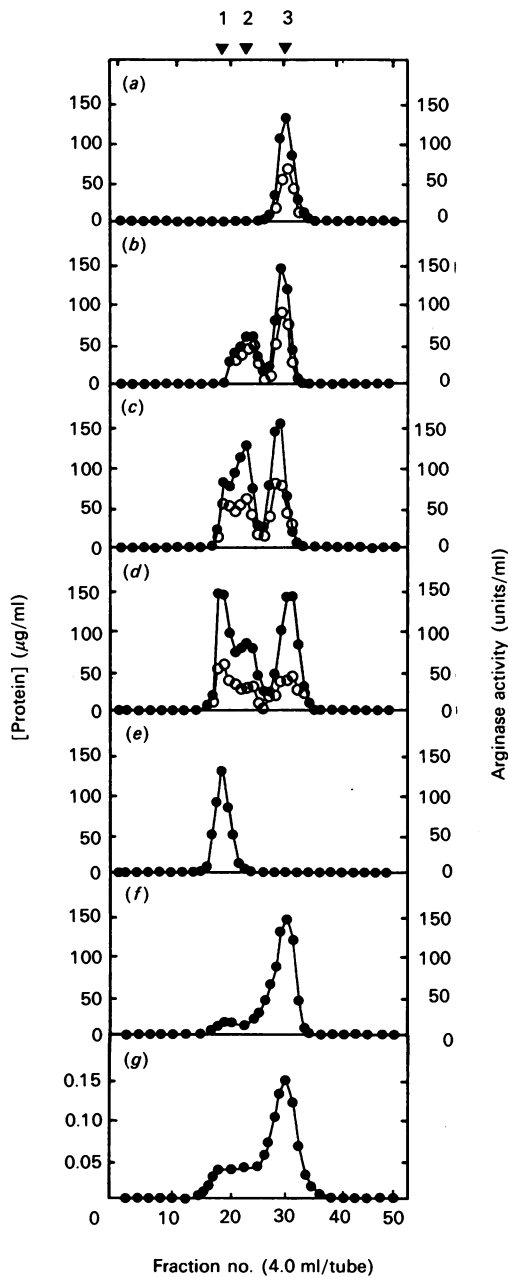


Fig. 3. Sephadex G-150 chromatography of various preparations of human arginase

A Sephadex G-150 column (19 mm  $\times$  1000 mm) was equilibrated with the respective buffer solution, and the gel filtration was carried out at 4 °C at a flow rate of 6 ml/h; 4.0 ml fractions were collected. Protein concentrations (O) of each fraction was determined by the Lowry *et al.* (1951) method, and the elution position from the column of arginase (●) was determined either from the enzymic activity of each fraction, in the case of (a)–(f), or from the result of e.l.i.s.a. for arginase antigen (see the Materials and methods section) in the case of (g). (a)–(d), *E. coli*-expressed human liver arginase (approx. 2 mg of protein each); (e) purified human erythrocyte arginase (approx. 2 mg of protein); (f) unfractionated homogenate of human liver tissue (approx. 50 mg of protein); (g) unfractionated human erythrocyte haemolysate (approx. 100 mg of protein). Arrowheads above the Figure indicate the elution position corresponding to: 1,  $M_r$  105000; 2,  $M_r$  70000; 3,  $M_r$  35000. (a) In 10 mM-Tris/HCl buffer, pH 7.4; (b) in 10 mM-glycine/NaOH buffer, pH 10.0; (c) in glycine/NaOH buffer, pH 11.0; (d) after cross-linking with glutaraldehyde and in 0.1 M-sodium phosphate buffer, pH 7.4; (e)–(g) in 10 mM-Tris/HCl buffer, pH 7.4.

containing arginase were collected and dialysed against 10 mM-Tris/HCl buffer, pH 7.4, at 4 °C overnight. The sample was applied to a DEAE-cellulose column (19 mm  $\times$  100 mm) equilibrated with the same buffer, and pass-through fractions were pooled and freeze-dried. The dried material was dissolved in 2.0 ml of distilled water, then gel filtration on a Sephadex G-150 column (19 mm  $\times$  1000 mm) was carried out at 4 °C, the arginase activities of the eluted fractions being monitored. The eluted fractions containing arginase were collected and freeze-dried. The partially purified enzyme thus obtained was finally subjected to a preparative agar-gel electrophoresis as described in the text. The yield of the purified enzyme was 10 mg or 2000 units of arginase per 1 g (wet weight) of *E. coli* cells. The SDS/PAGE data (Fig. 2, lanes 7–10) illustrate the relative ease of the purification, apparent homogeneity being obtained after the last step.

#### Characterization of *E. coli*-expressed human liver arginase

**$M_r$  determination.** SDS/PAGE of *E. coli*-expressed arginase gave an  $M_r$  value of 35000 (Fig. 2, lane 10). The gel filtration on a Sephadex G-150 column at pH 7.4 also gave an  $M_r$  of 35000 (Fig. 3a), indicating that the bacterial product behaved as a monomer. The elution position of purified human erythrocyte arginase corresponded to  $M_r$  105000 (Fig. 3e), as had been reported previously (Ikemoto *et al.*, 1989). However, Figs. 3(b) and 3(c) show the occurrence at alkaline pH values of additional molecular species of liver arginase having  $M_r$  values of 70000 and 105000, which may correspond to a dimer and a trimer respectively of the monomeric ( $M_r$  35000) species. Even at pH 7.2, when *E. coli*-expressed arginase was subjected to chemical cross-linking with glutaraldehyde before its dilution on gel filtration, the presence of dimeric and trimeric forms could be demonstrated (Fig. 3d). Co-existence of monomer, dimer and trimer was also shown by Western-blot analysis of the *E. coli*-expressed liver arginase sample that had been treated with another bifunctional reagent, disuccinimidyl suberate. Thus, in addition to a major band of  $M_r$  35000, immunoreactive bands with lesser staining intensities of  $M_r$  70000 and 105000 appeared (results not shown). These results, taken together, indicate (i) that *E. coli*-expressed human liver arginase has an  $M_r$  of 35000, (ii) that it exists as a monomer in its highly diluted solution, and (iii) that, when concentrated, it tends to self-associate only partially into its dimer and trimer. Fig. 3(f) shows the behaviour of human liver arginase present in the unfractionated homogenate. Most of the arginase activity emerges at the monomeric position, though the occurrence, in some fractions, of dimeric and trimeric species, can also be seen. Essentially the same pattern of elution was obtained with untreated human erythrocyte lysate (Fig. 3g), in sharp contrast with the exclusively trimeric nature of the purified preparation of human erythrocyte arginase (Fig. 3e).

**Amino acid composition and pI values.** The amino acid composition of *E. coli*-expressed human liver arginase is shown in Table 1, where it is compared with that of human erythrocyte arginase (Ikemoto *et al.*, 1989). The pI value for *E. coli*-expressed arginase was determined by isoelectric focusing (LKB Ampholine kit) to be 10.0, which was identical with the value for human erythrocyte arginase (Nishibe, 1973). These data may support chemical identity of the two enzyme species. The amino acid compositions of liver arginase of several other sources are also shown in Table 1, indicating close similarities among mammals, with only that for the chicken showing a distinct difference.

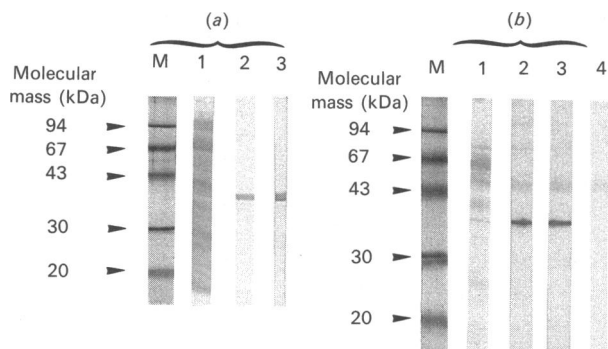
**Immunological cross-reactivity.** Fig. 4 shows the Western-blot data, which indicate that affinity-purified IgGs cross-react non-

**Table 1. Amino acid composition of arginases from several sources**

References: <sup>1</sup>Ikemoto *et al.* (1989). <sup>2</sup>Totani (1973); <sup>3</sup>Harell & Sokolovsky (1972); <sup>4</sup>Hirsch-Kolb & Greenberg (1968); <sup>5</sup>Grazi & Magri (1972).

Amino acid	Composition (mol/100 mol)					
	Human liver, <i>E. coli</i> -expressed	Human erythrocyte <sup>1</sup>	Pig liver <sup>2</sup>	Bovine liver <sup>3</sup>	Rat liver <sup>4</sup>	Chicken liver <sup>5</sup>
Lysine	7.4	7.5	9.0	8.2	9.0	5.5
Histidine	2.4	2.8	3.3	2.8	2.4	1.7
Arginine	4.0	4.0	4.4	3.8	3.4	5.2
Aspartic acid	9.2	9.2	9.4	8.7	9.0	10.5
Threonine	6.5	6.6	5.9	5.6	7.1	4.6
Serine	6.3	6.0	5.3	4.5	6.2	5.6
Glutamic acid	8.1	8.1	8.0	8.9	9.0	13.7
Proline	7.4	7.5	6.7	6.7	7.4	5.2
Glycine	12.5	11.9	11.5	10.1	9.3	8.2
Alanine	5.5	5.5	5.5	5.6	6.7	8.2
Half-cystine	ND*	ND*	1.7	0.7	2.0	2.0
Valine	7.2	7.4	8.6	8.4	8.9	7.4
Methionine	0.8	0.9	1.7	1.4	1.3	1.7
Isoleucine	6.2	6.4	4.7	6.0	5.3	4.3
Leucine	10.2	10.5	8.5	10.0	7.4	8.9
Tyrosine	2.6	2.7	3.3	3.2	2.5	2.6
Phenylalanine	2.9	2.9	4.4	3.4	3.2	3.3
Tryptophan	ND*	ND*	ND*	0.9	1.3	1.2

\* Not determined and omitted from the calculation.

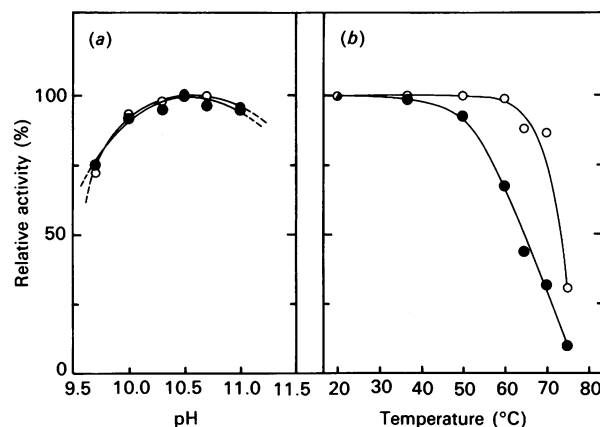


**Fig. 4. Immunological cross-reactivity between human liver and erythrocyte arginase**

In (a): lanes 1 and 2, sonicated supernatant (approx. 200 µg protein) from *E. coli* KY1436 cells; lane 3, purified liver arginase (approx. 10 µg of protein). For lane 1, proteins were stained with Amido Black 10B. Lanes 2 and 3 are the results of Western-blot analysis with anti-(human erythrocyte arginase) antibody. In (b): lanes 1 and 2, crude extract from acetone-dried powder of human erythrocyte haemolysate (approx. 100 µg of protein); lane 3, purified erythrocyte arginase (approx. 10 µg of protein); lane 4, blank control. For lane 1, proteins were stained with Amido Black 10B. Lanes 2, 3 and 4 are the results of Western-blot analysis with purified anti-(human liver arginase) antibody. Note that a minor broad band of 45 kDa is seen in lane 4 (blank lane) as it is on lanes 2 and 3, suggesting non-specific staining with this antibody preparation.

discriminately with liver and erythrocyte arginases. The identity of the  $M_r$  values on SDS/PAGE between liver and erythrocyte arginases can also be seen.

**Catalytic properties.** *E. coli*-expressed human liver arginase was found to be almost equally active between pH 9.7 and 11.0, with a broad peak at pH 10.5 (Fig. 5a), a pH-dependence which is essentially the same as that of purified erythrocyte arginase (Ikemoto *et al.*, 1989). When the enzyme solution at pH 10 was maintained at a given temperature between 20 and 75 °C for



**Fig. 5. Effect of pH and temperature on *E. coli*-expressed human liver arginase and purified human erythrocyte arginase**

●, *E. coli*-expressed human liver arginase; ○, purified human erythrocyte arginase. In (a) the purified enzyme in 0.1 M-Tris-HCl buffer, pH 7.4, containing 5 mM-Mn(II), was preincubated for 10 min at 55 °C and dialysed against distilled water for 2 h. A 0.1 ml portion of the sample (approx. 10 µg of protein) was added to 2.0 ml of the assay mixture, which contained 50 mM-L-arginine as substrate in 0.1 M-glycine/NaOH buffer at various pH values from 9.7 to 11.0, and mixed well. After incubation for 1.0 min under the given conditions, the enzymic reaction was stopped by adding 0.1 ml of 6 M-HCl. A 0.1 ml portion of the mixture was used for the determination of urea produced. In (b) the purified enzymes (approx. 20 µg of each) were left in 0.1 M-glycine/NaOH buffer, pH 10.0, at various temperatures from 20 to 75 °C. After the incubation for 10 min at a given temperature, an aliquot was withdrawn and the arginase activity was determined.

10 min, *E. coli*-expressed arginase lost 50% of its activity at 66 °C, whereas purified erythrocyte arginase did so at 72 °C (Fig. 5b). As shown in Fig. 6, the activity of *E. coli*-expressed arginase was almost unchanged for longer incubation periods of incubation at 25 °C at pH 8.3, but it decreased at lower pH values.

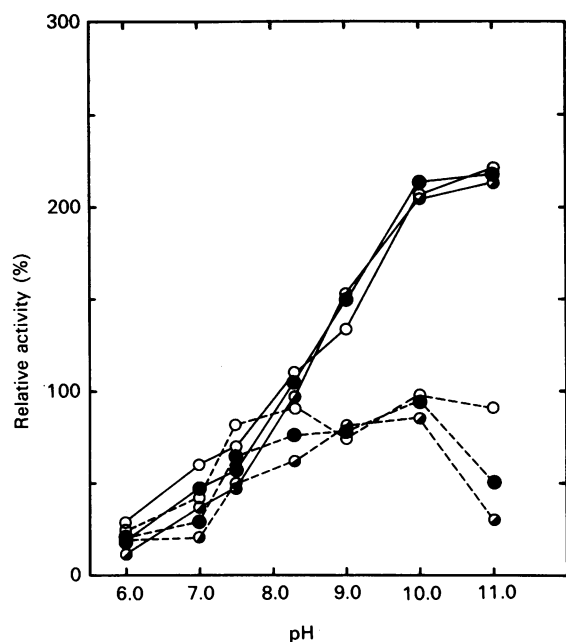


Fig. 6. pH-stability of *E. coli*-expressed human liver arginase and purified human erythrocyte arginase

Purified enzymes from the *E. coli*-expressed product (—) and from human erythrocyte arginase (----) (approx. 10 µg each) were left for 5 (○), 10 (●) or 20 h (◐) at 25 °C in buffer solutions without Mn(II). The buffers used were: 0.1 M-sodium phosphate buffers at pH 6.0–7.5; 0.1 M-Tris/HCl buffers at pH 8.3 and 9.0; 0.1 M-glycine/NaOH buffers at pH 10.0 and 11.0. The arginase activities that remained or were enhanced were determined at pH 8.3 and without Mn(II), and were expressed in percentage of the respective controls taken at 0 h and at the respective pH values.

Purified erythrocyte enzyme was more unstable, even at pH 8.3, and underwent extensive loss of the activity at pH 6.0 and 11.0. By contrast, *E. coli*-expressed enzyme was markedly activated by 5 h preincubation at alkaline pH values, which persisted with incubation periods of up to 20 h. Table 2 summarizes the kinetic data obtained when several arginine analogues were used as the inhibitors. Practically no difference was found between *E. coli*-expressed arginase and purified erythrocyte arginase (Ikemoto *et al.*, 1989).

## DISCUSSION

An efficient method for a large-scale production of human liver arginase in *E. coli* cells using cloned cDNA is described. The use of an expression plasmid having a tandemly linked *tac*

promotor placed upstream of a full-length cDNA for the enzyme (Fig. 1) and the selection of strain KY1436 were the two keys to successful outcome. The product protein accumulated within *E. coli* cells, which was readily purified to apparent homogeneity with a high yield (Table 1 and Fig. 2).

The gel filtration and SDS/PAGE of the *E. coli*-expressed human liver arginase clearly indicate the monomeric nature of the product in a diluted solution at a neutral pH value (Fig. 3a). The calculated  $M_r$  was 35000. Both the native form of human liver arginase and the human erythrocyte arginase were found to be essentially monomeric, although a portion tended to self-associate to form dimers and/or trimers (Figs. 3f and 3g). Such associability was found also with the *E. coli*-expressed product, particularly when its solution was adjusted to alkaline pH values and to a less diluted state (Figs. 3b, 3c and 3d). By contrast, the purified preparation of human erythrocyte arginase behaved on gel filtration as a single entity of  $M_r$  105000 (Fig. 3e), although it had been shown to be composed of three identical subunits of  $M_r$  35000 (Ikemoto *et al.*, 1989). In fact the purification procedures for human erythrocyte arginase that resulted in yielding exclusively trimeric enzyme included extensive concentration and a heat treatment (60 °C) at an alkaline pH value (pH 8.8) (Ikemoto *et al.*, 1989), which, as is shown in Figs. 3(b), 3(c) and 3(d), might have provided conditions favourable to self-association of monomers to dimers/trimers. Thus the present findings lead to the conclusion that the native form of human arginase, either in the liver or in the erythrocyte, is a monomer, and the dimeric and trimeric forms that are observable under various conditions or found after purification are artefacts. This may not hold, however, in the case of other animal species, because a molecular species of  $M_r$  120000 is known to prevail in the untreated homogenate of pig liver (Sakai & Murachi, 1969). Self-associability of arginase molecules can be quite different from one animal species to another. Even in humans, arginase molecules can readily self-associate. Whether the self-associability inherent to arginase molecules relates to metabolic regulation of, and/or by this enzyme, remains to be answered.

Chemical, immunological, and most, if not all, catalytic properties of the *E. coli*-expressed human liver arginase were indistinguishable from those of human erythrocyte arginase which had been purified from the haemolysate by conventional methods (Nishibe, 1973; Ikemoto *et al.*, 1989) (Tables 1 and 2; Figs. 3, 4, and 5). The specific activity of the *E. coli*-expressed arginase was calculated to be 389 units/mg of protein, which was in the same order of magnitude as the reported value (204 units/mg of protein) for purified human erythrocyte arginase (Ikemoto *et al.*, 1989). The distinct difference observable between monomeric (*E. coli*-expressed) and trimeric (purified from erythrocyte) arginase species lies in pH- and temperature-stability (Figs. 5 and 6). The monomer was less heat-stable than the trimer

Table 2. Inhibition of human liver and erythrocyte arginase by arginine analogues

Arginine analogue	Concentration (mM)	Mode of inhibition*	$K_i$ (mM)	
			<i>E. coli</i> -expressed	Erythrocyte†
Lysine	10	Competitive	2.5	2.7
Homoarginine	5	Competitive	5.2	5.0
Argininosuccinic acid	5	Non-competitive	23.7	22.5
Canavanine	5	Non-competitive	33.0	34.1
Octopine	5	Non-competitive	35.8	36.4

\* From the Lineweaver-Burk plots.

† Taken from Ikemoto *et al.* (1989).

when the activity was determined at pH 10 for an incubation period of 10 min; activity was markedly enhanced, however, by 5–20 h incubation at 25 °C and at alkaline pH values. The trimer showed a decrease of its activity under the latter conditions. These findings, taken together, suggest that the 'artefactual' trimer formation causes a change in conformational stability of arginase molecules, with very little change in general catalytic properties. The conclusion that a trimer erythrocyte arginase is an artefact stemming from the native monomer, together with the aforementioned indistinguishability in chemical and immunological properties between *E. coli*-expressed liver arginase and purified erythrocyte arginase, fully supports the notion that the arginase molecule present in the human erythrocyte is identical with that in human liver.

We thank Dr. Y. Haraguchi and Dr. M. Takiguchi (Kumamoto University, Kumamoto, Japan) for providing us with plasmid phARG6. We also thank Professor T. Yura (Kyoto University, Kyoto, Japan) for the gift of *E. coli* KY1436.

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Received 16 March 1990/17 April 1990; accepted 23 April 1990