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Purification and Characterization of Arginase from *Schizosaccharomyces pombe*

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Abstract: Arginase was purified to homogeneity from *Schizosaccharomyces pombe*. The purified enzyme is a tetramer with a subunit molecular weight of 42,000. Activity is optimal at pH 10.0 and at 60℃. The enzyme migrated during isoelectric focusing showing a pI=5.4. The enzyme exhibited hyperbolic kinetics at pH 10.0 with an apparent K_m for L-arginine of 18 mM. Arginase activity was strongly inhibited by L-glutamate.

Key words: antibody, arginase, purification, ureotelic type.

Arginase (EC. 3.5.3.1.) catalyzes the hydrolysis of arginine to ornithine and urea. In ureotelic organisms, arginase participates in the urea cycle (Jones *et al.*, 1961). Uricotelic organisms do not contain an active urea cycle (Jones *et al.*, 1961; Ratner *et al.*, 1953). The uricotelic arginase catalyzes the hydrolysis of arginine, but its role in metabolism has not been ascertained (Mora *et al.*, 1966). The ureotelic and uricotelic arginases differ in properties such as native molecular weight, electrophoretic mobility, and immunologic relatedness (Baranczyk-Kuzma *et al.*, 1976; Beruter *et al.*, 1978; Borkovich and Weiss, 1987; Herzfeld and Raper, 1976; Skrzypek-Osiecka and Poremaka, 1983). On the basis of the quaternary structure and kinetic properties of the enzyme, two classes of arginases have been proposed: arginase of ureotelic type which has a low molecular mass (140 kDa) and a low apparent K_m for L-arginine (10 mM), and arginase of uricotelic type which has a high molecular mass (260 kDa) and a high apparent K_m for L-arginine (100 mM) (Borkovick and Weiss, 1987). L-Arginine can serve as a nitrogen source for the growth of many microorganisms including fungi which couple arginine catabolism to synthesis of proline and bacteria which degrade this aminoacid through several metabolic pathways (Davis, 1986). The arginase pathway is the best known enzymatic process of arginine catabolism in living organisms (Davis, 1986; Abdelal, 1979; Jackson *et al.*, 1986). Nevertheless, biochemical studies on bacterial arginase are scarce. Although the ureotelic type arginase has been purified and characterized from budding yeast, *Saccharomyces cerevisiae* (Pennickx *et al.*, 1974), no properties of the purified enzyme from fission yeast, *Schizosaccharomyces pombe*, have been reported. The cell cycle of fission yeast compared to the budding yeast is basically similar to that of higher eucaryotes (Mitchison and Creanor, 1971). Therefore, the budding and fission yeast arginases may differ in some properties.

The present paper describes the purification to apparent homogeneity of arginase from *S. pombe* and reports some of the properties of the purified enzyme. The influence of the arginine metabolites on the enzyme activity has also been studied.

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Materials and Methods

Strain and growth condition

Schizosaccharomyces pombe strain 972(h^-) was used for the purification of arginase. *S. pombe* was grown in a YE medium (Gutz *et al.*, 1974) containing 1 mM L-arginine. The 3-liter cultures were grown at 30℃ and collected at mid-log phase by centrifugation at 4,000×*g* for 15 min. The cell was able to be frozen at −20℃ without loss of arginase activity.

Enzyme assay

The assay for arginase was based on the formation of urea from arginine. The reaction mixture consisted of 50 mM glycine-NaOH (pH 10.0), 50 mM L-arginine (pH 10.0), 0.1 mM $MnCl_2$ and enzyme solution in a final volume of 1.0 ml. After 30 min incubation at 60℃, the reaction was stopped by adding 0.5 ml of 0.5 M $HClO_4$. The urea content was measured colori-

metrically by the modified method of Robert (Robert, 1970). One unit is defined as the amount of enzyme producing 1 μmol urea per min.

Protein determination

Protein was estimated following the procedure of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Enzyme purification

All procedures were carried out at 0~4℃. Approximately 20 g of frozen cells were homogenized in a "Bead-beater" (Biospec Products) apparatus using 50 mM Tris, 20 mM L-arginine, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 8.0). The supernatant fluid was decanted from the beads and cell debris was removed by centrifugation (60,000×*g*, 60 min). 1 M L-arginine solution (pH 8.0) was added to the supernatant fluid to produce a final concentration of 100 mM. The solution was then heated at 60℃ for 10 min while stirring. The solution was cooled quickly to 4℃ and denatured proteins were removed by centrifugation (60,000 ×*g*, 60 min). The resulting supernatant fluid was fractionationed with 30~60% $(NH_4)_2SO_4$. After centrifugation (15,000×*g*, 30 min), the pellets were dissolved in 10 mM Tris, and 0.1 mM PMSF (pH 8.0) (buffer A). This solution was dialyzed in 2×2 liters of buffer A containing 100 mM KCl (buffer B). The dialyzed material was applied to DEAE-Sepharose CL-6B column (2.5×15 cm) equilibrium with buffer B. The column was washed with the same buffer. The enzyme was eluted by using a linear gradient of 100~400 mM KCl. Active fractions were pooled and concentrated using an Amicon concentrator (YM 10 membrane). This solution was applied to a Bio-Gel A-1.5 m (1.2× 120 cm) equilibrated with buffer B. Protein was eluted with the same buffer. Active fractions were pooled and then concentrated. The enzyme fractions were applied to a FPLC Mono-Q column (0.7×5 cm) equilibrated buffer B. The column was washed with the same buffer and the enzyme was eluted by using a linear gradient of 100~300 mM. Active fractions were combined and a 3 M $(NH_4)_2SO_4$ solution added to give a final concentration of 1.2 M. This solution was applied to a FPLC Phenyl-Superose column (0.7×5 cm) equilibrated with buffer B containing 1.2 M $(NH_4)_2SO_4$ and eluted with a salt gradient to buffer B. Active fractions were pooled and concentrated. The concentrated material was applied to a FPLC Superose 6 (1×30 cm) equilibrated with 20 mM Tris (pH 8.0) buffer and eluted with the same buffer. The purified enzyme solution was collected and used for all other studies.

Homogeneity and molecular weight

Molecular weight of the native enzyme was measured by a FPLC Superose 6 gel filtration. The molecular weight of subunits was obtained by Laemmli's method (Laemmli, 1970) employing SDS polyacrylamide gel.

Isoelectric focusing

Isoelectric focusing was performed in Phast Gel IEF (pH range 3 to 9) on a Pharmacia LKB Phast system. Isoelectric focusing was performed together with pI stanard markers. Proteins in the gel were stained with Coomasie Brilliant Blue R-250.

Preparation of antibody

One half mililiter of purified arginase (1 mg/ml) was emulsified with an equal volume of Freund's Complete Adjuvant and injected intramuscularly in the hip of a white rabbit. After 2 weeks, the rabbit was given a booster injection of the same dose of antigen in Freund's Incomplete Adjuvant. Two weeks after this booster injection the rabbit was bled and the antiserum collected.

Western blotting

After SDS-polyacrylamide gel electrophoresis (15%), proteins were transfered onto nitrocellulose membrane which was blocked with 1% bovine serum albumin in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0). After washing, the membrane was incubated with TBST containing the dilution of primary antibody for 30 min at room temperature. The membrane was washed in TBST three times to remove unbound antibody. The membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody and signals were developed using the alkaline phosphatase substrate solution containing BCIP and NBT.

Results and Discussion

Purification of arginase

Cells were harvested during logarithmic growth to decrease the exposure of arginase to protease produced in increased amounts at the onset of the stationary phase. Partial proteolysis of arginase *in vivo* or during purification may be responsible for the subunit size or charge heterogeneity of some purified arginase (Borkovich and Weiss, 1987; Pennickx *et al.*, 1974; Spolarics and Bond, 1989). Inclusion of PMSF in buffers used for the purification of the enzyme nearly abolished the proteolysis of arginase. The enzyme in extracts obtained from log phase cells was heat stable (data not shown). The same case of heat stability has been observed for the *Neurospora crassa* and *Saccharomyces*

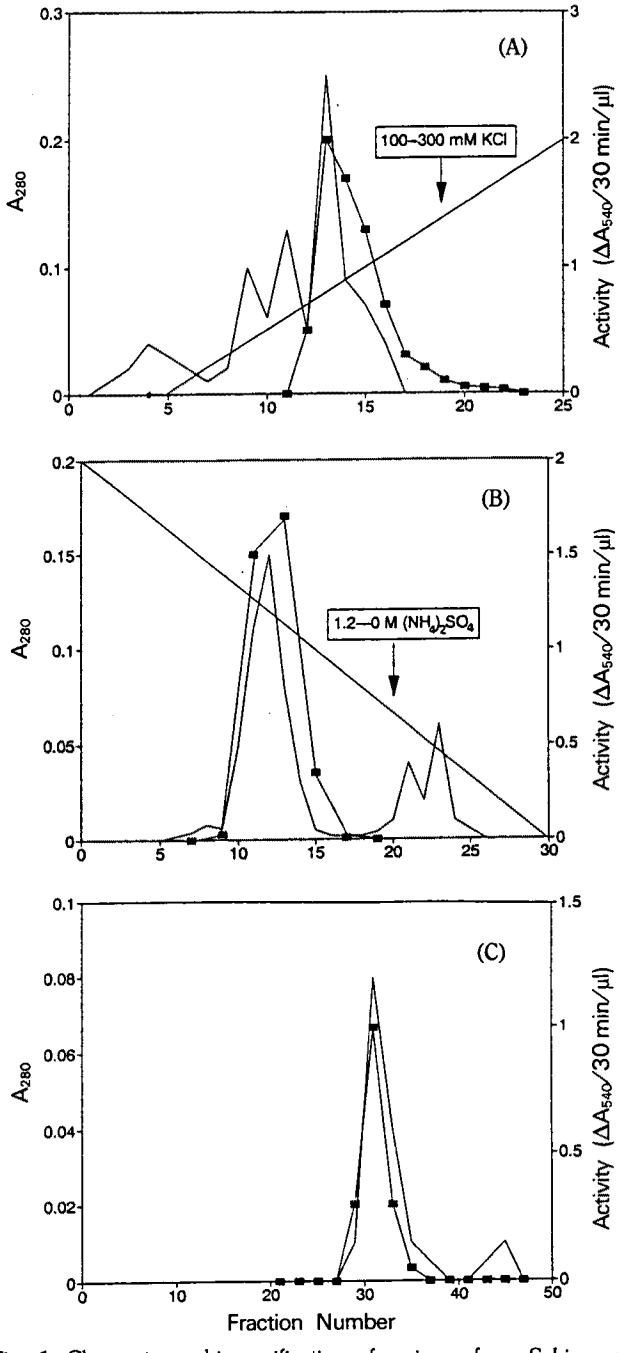

Fig. 1. Chromatographic purification of arginase from *Schizosaccharomyces pombe*. (A) Mono Q column chromatography. (B) Phenyl Superose column chromatography. (C) Superose 6 column chromatography. OD_{280} (—); Activity (■—■).

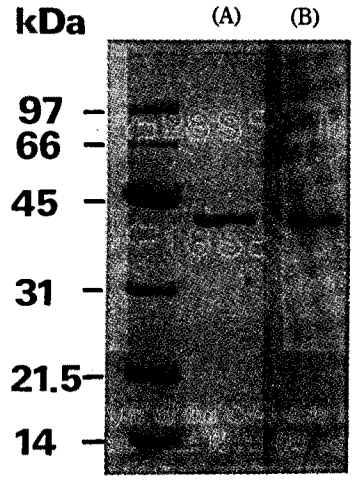

Fig. 2. SDS-polyacrylamide gel electrophoresis and Western blot analysis of arginase. Lane A: active fractions of Superose 6 gel filtration was electrophoresed on a 15% SDS-polyacrylamide gel. Lane B: Western blot analysis of the purified arginase using anti-arginase antibody. Molecular markers (97, 66, 45, 31, 21.5 kDa) are shown at the left.

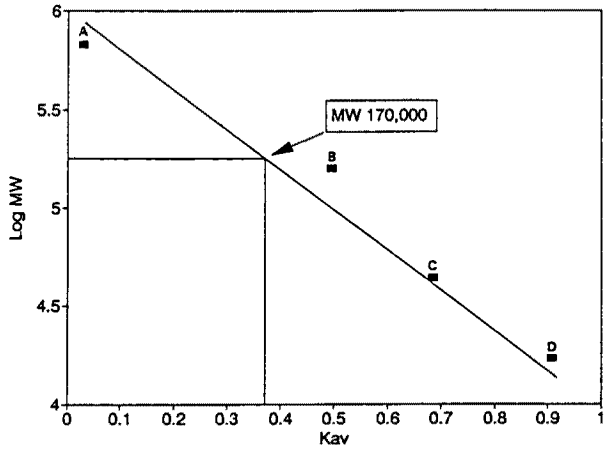

Fig. 3. Native molecular weight determination of arginase by molecular sieve FPLC on Superose 6 column. A, tyroglobulin (670 kDa); B, gamma globulin (158 kDa); C, ovalbumin (44 kDa); D, myoglobin (17 kDa).

cerevisiae arginases (Borkovich and Weiss, 1987; Pennickx *et al.*, 1974). Arginase was absorbed into the Mono Q column and eluted with 140 mM KCl (Fig. 1, A). The enzyme eluted at 0.8 M $(NH_4)_2SO_4$ on the Phenyl Superose column (Fig. 1, B). Finally, the enzyme was purified through use of Superose 6 gel filtration (Fig. 1, C). The purification of arginase is summarized in Table 1. The 6.3% yield is similar to other arginase purification protocols (Borkovich and Weiss, 1987; Harell and Sokolovsky, 1972; Herzfeld and Raper, 1976; Moreno-Vivian *et al.*, 1992; Pennickx *et al.*, 1974).

Determination of molecular weight and Western blot analysis

Analysis of active fractions from Superose 6 on SDS-polyacrylamide gel electrophoresis indicated the presence of a single band and Western blot analysis using rabbit polyclonal antibody against the purified enzyme also showed one band (Fig. 2). The molecular weight of the purified arginase subunit from *S. pombe* was 42,000 as determined by SDS-polyacrylamide gel electrophoresis. The molecular weight of purified native en-

Table 1. Purification of arginase from *Schizosaccharomyces pombe*

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude	1072.3	91306	85	100	1
Heat treatment	653.2	101403	155	111	1.8
$(NH_4)_2SO_4$ (30~60%)	176.4	78706	446	86.2	5.2
DEAE-Sepharose	9.2	45697	4967	50.0	55.4
Bio-Gel-A-1.5 m	3.8	36551	9619	40.0	113.1
Mono Q	0.74	28113	38099	30.9	448.2
Penyl superose	0.12	6061	50508	6.6	594.2
Superose 6	0.1	5704	57400	6.3	675.3

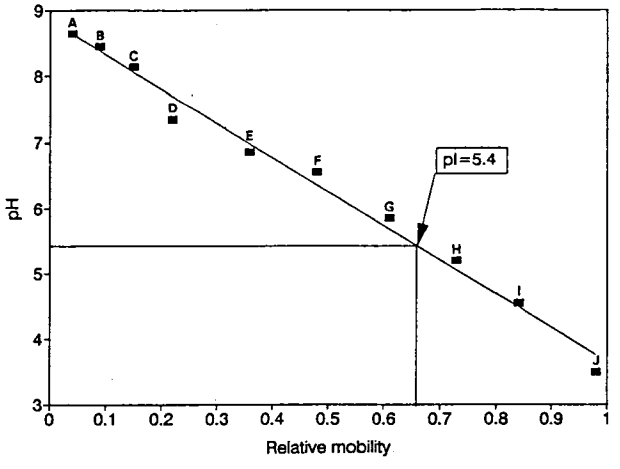

Fig. 4. Determination of pI value by isoelectric focusing. pI standard markers were as follows. A, lentil lectin basic band (9.65); B, lentil lectin middle band (8.45); C, lentil lectin acidic band (8.15); D, myoglobin basic band (7.35); E, myoglobin acidic band (6.85); F, human carbonic anhydrase B (6.55); G, bovine carbonic anhydrase B (5.85); H,β-lactoglobin (5.20); I, soybean trypsin (4.55); J, amyloglucosidase (3.5).

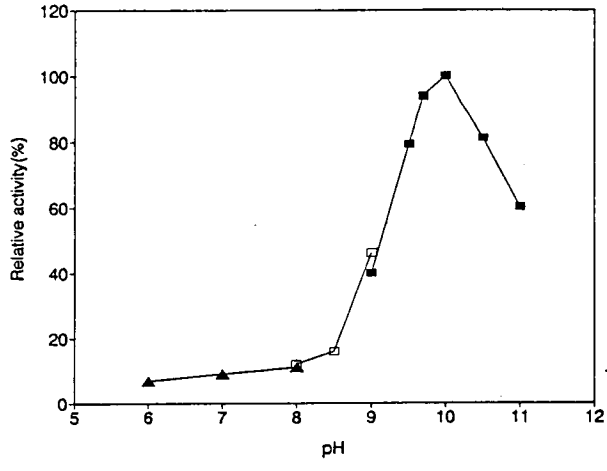

Fig. 5. Effect of pH on *S. pombe* arginase. Following buffers were: (▲—▲), 50 mM potassium phosphate; (□—□), 50 mM Tris-HCl; (■—■), 50 mM glycine-NaOH.

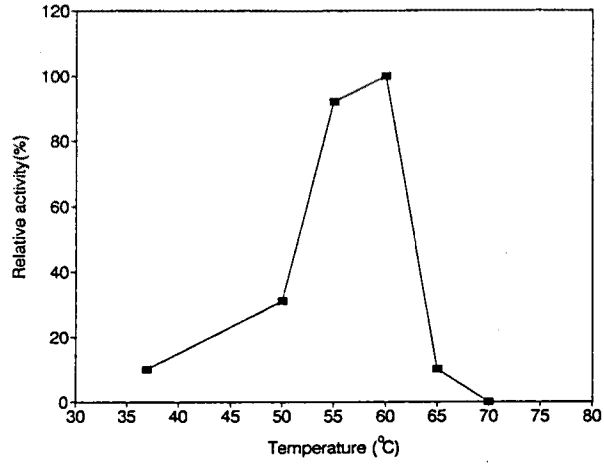

Fig. 6. Effect of temperature on *S. pombe* arginase.

zyme was determined to be 170,000 by FPLC superose 6 gel filtration (Fig. 3). These results indicate that the enzyme is a tetramer with identical subunits. The enzyme from mammalian liver has a native molecular weight of 110,000~115,000. Estimates of the subunit size vary from 31,000 to 35,000 (Jackson *et al.*, 1986). On the other hand, bacterial arginase has a native molecular weight of 260,000~300,000 and subunit size of 31,000~39,000 (Abdelal, 1979).

Isoelectric focusing

The isoelctric point of *S. pombe* arginase was determined to be 5.4 by Phast Gel IEF (Fig. 4). This value is lower than that of the eucaryotic arginase (pI 5.7~6.0) (Rossi *et al.*, 1983).

Physicochemical properties

The activity of purified enzyme was optimal at pH 10.0 (Fig. 5). This optimum pH for catalysis is large compared with those of mammalian arginase and other bacterial arginases (pH 8.5~9.5) (Jackson *et al.*, 1979). The purified enzyme was stable at −20℃ for several months without loss of activity. The enzyme activity

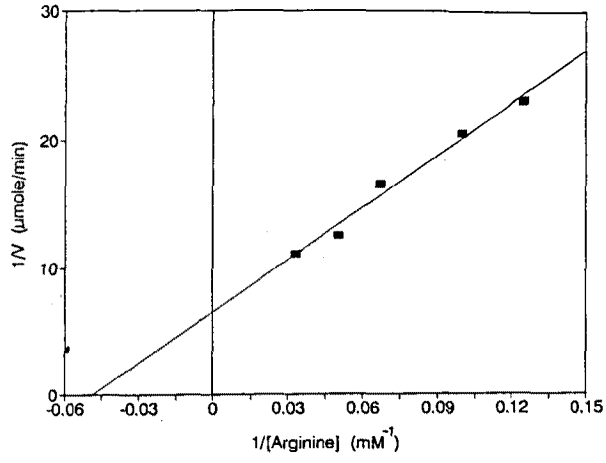

Fig. 7. Kinetic property of arginase from *S. pombe*. Double reciprocal plot was obtained by assaying the activity in the presence of L-arginine at the concentrations indicated in the figure.

Table 2. Effect of arginine metabolites on the arginase activity from *S. pombe*

Addition	Relative activity (%)
None	100
L-Ornithine	87
L-Glutamate	24
L-Proline	100
L-Citrulline	113
L-Agmatine	111

Arginase activity was determined in the presence of the compounds listed in the at a final concentration of 5 mM.

was optimal at 60℃ (Fig. 6). In many microorganisms, the optimum temperature for the arginase reaction is 30~37℃ (Abdelal *et al.*, 1979) but that of the thermophile bacteria, Bacillus caldovelox, is 60℃ (Patchett *et al.*, 1991).

Kinetic property

Arginase from *S. pombe* exhibited hyperbolic saturation kinetics with an apparent K_m value for L-arginine of 18 mM (Fig. 7) which is similar to those of the *S. cerevisiae* (12.5 mM) and *Rhodobacter capsulatus* (16 mM) arginases (Pennickx *et al.*, 1974; Moreno-Vivian *et al.*, 1992). On the other hand, *N. crassa* and soybean arginases have large K_m values (83~131 mM) (Borkovich *et al.*, 1987; Kang and Cho, 1990). Uricotelic arginase has a large native molecular weight (260~300 kDa) and K_m value (83~131 mM) whereas the uereotelic arginase has a small native molecular weight (110~120 kDa) and K_m value (5~20 mM). Therefore, these results, taken together with a native molecular weight and apparent K_m value, suggest that arginase from *S. pmbe* is of the ureotelic type.

Effect of arginine metabolites

Purified arginase was slightly inhibited by L-ornithine whereas strong inhibition by L-glutamate was observed (Table 2). In yeast, arginase catalyzes the conversion of arginine to ornithine and urea. Ornithine is further degraded to glutamate and proline. A arginine is derived from citrulline by ornithine transcarbamylase. Many arginases are reported to be inhibited by L-ornithine (Beruter *et al.*, 1978; Moreno-Vivian *et al.*, 1972; Patchett *et al.*, 1991) but the strong inhibition of arginase by L-glutamate is not reported. These results suggest that L-glutamate may play a certain role in the regulation of *S. pombe* arginase activity.

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