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QUARTERLY

Purification of arginase from *Aspergillus nidulans**

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Arginase (EC 3.5.3.1) of *Aspergillus nidulans*, the enzyme which enables the fungus to use arginine as the sole nitrogen source was purified to homogeneity. Molecular mass of the purified arginase subunit is 40 kDa and is similar to that reported for the *Neurospora crassa* (38.3 kDa) and *Saccharomyces cerevisiae* (39 kDa) enzymes. The native molecular mass of arginase is 125 kDa. The subunit/native molecular mass ratio suggests a trimeric form of the protein.

The arginase protein was cleaved and partially sequenced. Two out of the six polypeptides sequenced show a high degree of homology to conserved domains in arginases from other species.

Arginase (arginine amidinohydrolase, EC 3.5.3.1) catalyses hydrolysis of arginine to ornithine and urea. There are two arginase isoenzymes in ureotelic organisms. The liver enzyme participates in the urea cycle and the kidney enzyme probably enables the organism to use arginine as a source of proline or glutamate [1].

In storage tissues of numerous plants, arginine constitutes a large part of the nitrogen reserve. Arginase enables its use as a carbon and nitrogen source during germination (seeds) or sprouting (tubers or bulbs) [2].

In lower organisms, such as yeasts and filamentous fungi, arginase enables the use of arginine as a nitrogen source. In *A. nidulans* exogenous arginine causes a several fold induction of arginase. Homoarginine, citrulline, canavanine and lysine can also act as arginase inducers [3]. Kinetic studies showed that the induction of arginase in *A. nidulans* was dependent on synthesis of mRNA and its subsequent translation. The functional half-life of arginase mRNA was estimated to be 2.7 min [4].

Here we report the purification and partial characterisation of the *A. nidulans* arginase. (A preliminary report of this work has appeared [5].)

MATERIALS AND METHODS

The arginase from *A. nidulans* was purified as follows:

–*1. Crude extract.* Mycelium of the proA6, pabaA9, biA1 strain was grown at 30°C in minimal medium supplemented with proline, PA-

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[1]Abbreviations: PABA, *para*-aminobenzoic acid; PMSF, phenylmethylsulfonyl fluoride.

BA[1], biotin [6] and 10 mM arginine as the sole nitrogen source. Under these conditions we observed 15-fold induction of arginase above the basal level. About 100 g of mycelium was ground in a mortar with glass powder and buffer A containing 50 mM Tris/HCl, pH 7.5, 20 mM arginine, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF and 5 mM $MnCl_2$ (15 ml per 100 g of mycelia). The crude extract was obtained by centrifugation at 8000 × *g* for 20 min at 4°C.

–2. *Heat treatment.* The crude extract was divided into 3 ml aliquots and heated for 10 min at 50°C. The solution was immediately cooled on ice for 20 min and denatured proteins were removed by centrifugation at 8000 × *g* for 15 min at 4°C.

–3. *Ammonium sulfate precipitation.* The solution from step 2 was diluted with 2 volumes of buffer A and solid $(NH_4)_2SO_4$ was added to 0.5 (w/v) saturation. The solution was stirred for 30 min at 0°C. The precipitated proteins were removed by centrifugation at 8000 × *g* for 15 min at 4°C. To the supernatant solid $(NH_4)_2SO_4$ was added to 0.6 (w/v) saturation and stirred again for 30 min at 0°C. After centrifugation at 8000 × *g* for 15 min at 4°C, the pellet was resuspended in 1 ml of buffer B containing 40 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF and 20% (v/v) glycerol. The resuspended precipitate was stored at –20°C. Steps 1–3 were repeated until a total of about 400 mg of protein was obtained.

–4. *Bio-Gel P-60 gel filtration.* Combined material from step 3 (32 ml) was desalted and purified from proteins smaller than 60 kDa on a Bio-Gel P-60 column (100–200 mesh). The material was applied at 4°C to a 2.6 cm × 50 cm column equilibrated with buffer B. Proteins were eluted with the same buffer.

–5. *DEAE-cellulose salt gradient chromatography.* The enzymatically active fractions from step 4 were applied at 4°C to the 1.6 cm × 30 cm DE-52 (Whatman) column equilibrated with buffer B. The column was washed with 30 ml of buffer B containing 0.05 M KCl. Arginase was eluted with 400 ml of a 0.05–0.3 M KCl linear gradient in buffer B.

–6. *Extraction from polyacrylamide gels.* The enzymatically active fractions from step 5 were loaded on polyacrylamide gels (4.5% w/v stacking gel and 6% w/v resolving gel) containing 20% glycerol (v/v) under non-denaturing conditions [7] and subjected to electrophoresis. Gel pieces containing a single protein band were extracted by overnight incubation with buffer B. Acrylamide was removed by centrifugation and the sample containing arginase was identified by the enzymatic reaction. After elution, the samples were concentrated on membrane cones Centriflo CF25 (Amicon).

During the whole purification procedure protein was assayed according to Bradford [8] and arginase by the procedure of Bartnik [9].

The purified arginase was digested and partially sequenced as follows:

– *A. Protein digestion.* Protein (1 nmole) was digested overnight at 37°C by 1 µg of endoproteinase Asp N (Boehringer Mannheim) in 10 mM Tris/HCl buffer, pH 7.8.

– *B. Peptide purification.* A140B Solvent delivery system (ABI Foster City) was used for separation of peptides which were then detected using a photo diode array detector (990 Waters). They were separated on a C8 RP300 reverse phase (ABI) column (2.1 mm × 22 cm). Column was elutad by a linear gradient of acetonitrile (1%–80%) for 70 min, at a flowrate of 200 µl/min.

– *C. Peptide sequencing.* Peptides were sequenced in a 470 A gas-liquid protein sequencer (ABI). Phenylthiohydantoin derivatives of amino acids were analysed on a 120A PTH analyser (ABI).

RESULTS AND DISCUSSION

Salt gradient chromatography of arginase on DEAE-cellulose is presented in Fig. 1.

Purification of arginase is summarized in Table 1 and illustrated in Fig. 2. Arginase of *A. nidulans* was purified to homogeneity. Yield of purification is very low (0.1%). The aim of experiment was to obtain the protein as pure as possible. Because of that we used only one of the most active fractions after the DEAE-cellulose chromatography for the further gel extraction.

The sequence of six sequenced arginase polypeptides is presented in Fig. 3.

Molecular mass of the purified arginase subunit determined by SDS-polyacrylamide gel electrophoresis (Fig. 4) is 40 kDa. It is similar to

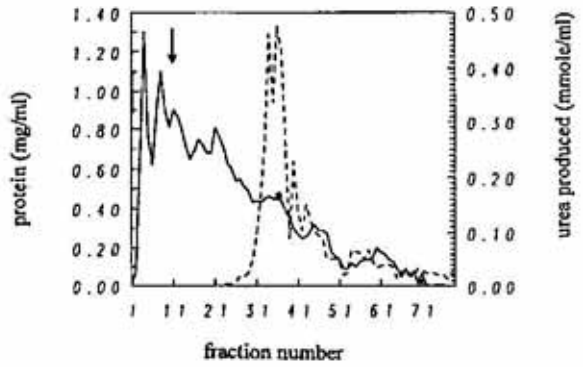

Fig. 1. DE-52 salt gradient chromatography.
---, Arginase activity assayed as reported by Bartnik [9]; —, protein content assayed as reported by Bradford [8] The arrow marks the beginning of the 0.05–0.3 M KCl linear gradient.

that reported for *N. crassa* arginase, 38.3 kDa [10], and for the *S. cerevisiae* enzyme, 39 kDa [11]. It is also close to the values reported for

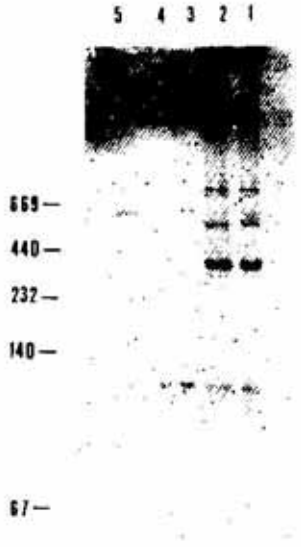

Fig. 2. Purification of arginase and determination of its native molecular mass by electrophoresis on polyacrylamide gel under non-denaturating conditions.
1, Ammonium sulfate precipitate (50 µg protein); 2, enzymatically active fractions after Bio-Gel P-60 column (50 µg protein); 3, the most active fraction after DE-52 salt gradient chromatography (10 µg protein); 4, arginase extracted from the polyacrylamide gel; 5, high molecular weight calibration kit (Pharmacia). The molecular weight of standards is indicated ($\times 10^{-3}$).

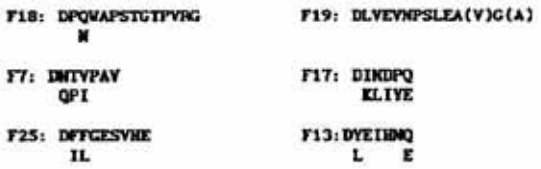

Fig. 3. The sequence of the six isolated A. nidulans arginase polypeptides.

arginase subunits from other organisms such as *Bacillus licheniformis*, 33 kDa [12], *Iris hollandica*, 36.5 kDa [13], rat liver, 30 kDa [14], rabbit liver, 36.5 kDa [15] and human liver, 35 kDa [16]. Only for soybean arginase a much larger subunit of 60 kDa was reported [17].

The native molecular mass of purified arginase determined by polyacrylamide gel electrophoresis under non-denaturating conditions as described above is 125 kDa (Fig. 2). The subunit/native molecular mass ratio indicates a trimeric form of the enzyme.

Two out of the six sequenced *A. nidulans* arginase polypeptides (F18 and F19) show a high degree of homology to the conserved domains in human and *S. cerevisiae* arginases (Fig. 5).

It is possible to classify other hitherto purified arginases into two groups. The first consists of

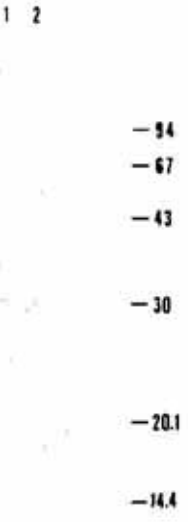

Fig. 4. The arginase subunit molecular mass determination by SDS-polyacrylamide gel electrophoresis.
1, Purified arginase; 2, low molecular weight calibration kit (Pharmacia). The molecular weight of standards is indicated ($\times 10^{-3}$).

Table 1
Purification of the A. nidulans arginase

Step	Volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	514	6107	18243	3.0	1	100
Heat treatment	446	1607	13939	8.8	2.9	76.4
$(NH_4)_2SO_4$ 0.5–0.6 saturation	32	429	3861	9	3	21.2
Bio-Gel P-60	38	281	3962	14.1	4.7	21.7
DE-52[b]	2.8	1.2	129	100	33.3	0.7
Gel extraction	1.85	0.1	15	150	50	0.1

a, 1 unit (U) — one activity unit is the arginase activity which produces 1 µmole of urea per minute under the standard conditions; b, only one of the most active fractions (no. 34) was used for the gel elution.

proteins of smaller molecular mass (about 120 kDa) which have a trimeric or tetrameric structure [11, 14–16]. The second group consists of larger proteins as a rule hexamers [10, 12, 13] of molecular mass about 200–250 kDa.

The other two so far characterised fungal arginases belong to two different groups. The *S.*

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human -          MSAKSRTIGIIGAPFSKGQPRGGVEEGPTVLRKAGL------------LEKLKEQE
yeast - METGPHYNYYKNRELSIVLAPFSGGQGKLGVEKGPKYMLKHGLQTSIEDLGWSTELEPSMDEA

human - CDV-KDYGDLPFADIPNDSPFQIVKNPRS--VGKASEQLAGKVAQVKKNGRISLVLGGDHSLA
yeast - QFVGKLKMEKDSTTGGSSVMIDGVKAKRADLVGEATKLVYNSVSKVVQANRFPLTLGGDHSIA

human - IGSISGHARVHPDLGVIWVDAHTDINTPLTTTSGNLHGQPVSFLLKELKGKIPDVPGFSWVTP
yeast - IGTVSAVLDKYPDAGLLWIDAHADINTIESTPSGNLHGCPVSFLMGLNKDVPHCPESLKWVPG

human - CISAKDIVYIGLRDVDPGEHYILKTLGIKYFSMTEVDRLGIGKVME--ETLSYLLGRKKRPIH
yeast - NLSPKKIAYIGLRDVDAGEKKILKDLGIAAFSMYHVDKYGINAVIEMAMKAVHPETNGEGPIM

human - LSFDVDGLDPSFTPATGTPVVGGLTYREGLYITEEIYKTGLLSGLDIMEVNPSLGKTPEEVTR
yeast - CSYDVDGVDPLYIPATGTPVRGGLTLREGLFLVERLAESGNLIALDVVECNPDLAIHDIHVSN

Asp   -          DPQWAPSTGTPVRG                    DLVEVNPSLEAVGA
                          (F18)                          (F19)

human - TVNTAVAITLACFGLAREGNHKPIDYLNPPK
yeast - TISAGCAIARCALGETLL
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Fig. 5. Comparison of amino-acids sequence of the human liver [18] and yeast [19] arginases. Two A. nidulans arginase polypeptides (F18 and F19) are shown.

cerevisiae arginase is a trimer of molecular mass 114 kDa [11] similarly as the enzyme from *A. nidulans*, whereas the *N. crassa* enzyme is a hexamer of molecular mass 266 kDa [10].

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