

Mitochondrial membrane-bound activity of arginase is independent of nitrogen excretion pattern in ureogenic and non-ureogenic vertebrates

Mishra Suman & Mishra Rajnikant*

Biochemistry and Molecular Biology Laboratory, Department of Zoology, Banaras Hindu University,
Varanasi-221 005, Uttar Pradesh, India

Received 19 January 2015; revised 30 July 2015

Arginase, that regulates metabolism of arginine, is widely distributed in organisms. The two major isoforms, cytosolic Arginase-I, and mitochondrial Arginase-II have been characterized well. However, reports also suggest another mitochondrial membrane-bound arginase which is extracted by washing the mitochondria with KCl. Here, we studied this mitochondrial membrane-bound arginase among vertebrates. Our observations support that arginase activity is predominant in cytosol which is designated as Arginase-I. The mitochondrial membrane-bound Arginase (mbArg) which resembles Arginase-II seems independent of nitrogen excretion pattern because of its presence both in ureogenic and non-ureogenic vertebrates.

Keywords: Arginase isoforms, *Calotes versicolor*, Garden-lizard, *Gallus gallus*, *Heteropneustes fossilis*, mbArg, *Mus musculus*, *Rana tigrina*, Reptiles

Arginase [EC 3.5.3.1] catalyzes the hydrolysis of arginine into ornithine and urea¹ and regulates the level of L-arginine^{2,3}. Among the two isoform of arginase, arginase-I (ARG I) influences removal of nitrogenous waste in the form of urea, and arginase-II (ARG II), regulates biosynthesis of ornithine, proline and glutamate⁴. Reports also suggest presence of more than two isoforms^{5,6} of arginase based on immunological properties⁷, net charge⁸ or localization to the membrane of mitochondria of fishes⁹, birds^{10,11} or mammals^{12,13}. The mitochondrial membrane-bound arginase has been suggested to be associated with inner mitochondrial membrane¹⁰ which can be extracted by including 150 mM KCl in homogenizing medium⁹. In mammals, 66% of the mitochondrial arginase is associated to outer mitochondrial membrane¹² or 90% of the mitochondrial arginase remains bound to mitochondrial membrane¹³. Reports also suggest plasma membrane-bound arginase having no immuno-cross reactivity with anti-liver arginase¹⁴. In this study, we investigated the presence and biological significance of mitochondrial membrane-bound arginase (mbArg). This report describes comparative analysis of arginase and its mitochondrial membrane-bound form in representative types of fish, amphibian, birds, reptiles and mammals.

Materials and Methods

Animals and tissue collection

Tissues of representative types of Amphibians (*Rana tigrina*), Aves (*Gallus gallus*), Pisces (*Heteropneustes fossilis*), Reptiles (*Calotes versicolor*), and Mammals (*Mus musculus*), were either collected during practical classes or purchased from commercial resources. Fish and mice were maintained as per the Institutional Animal Ethical guidelines. Animals were sacrificed to dissect out liver, washed with saline and stored at -80°C . All the chemicals were of analytical grade purchased from Sigma, Himedia, SRL and other indigenous sources.

Isolation of mitochondria and mitochondrial membrane bound fraction of Arginase:

Homogenate (20%) of liver was prepared in homogenizing buffer containing 30 mM Tris HCl (pH 7.2), 1 mM EDTA, 250 mM Sucrose, 50 mM mannitol and protease inhibitor cocktail (Sigma-Aldrich) using a Potter-Elvehjem type glass homogenizer with a motor-driven teflon pestle. Cytosolic and mitochondrial fractions were separated according to previously described method¹⁵. The mitochondrial pellet was washed twice with homogenizing buffer to remove the cytosolic contamination from mitochondrial pellet. For isolation of mitochondrial membrane bound fraction the mitochondrial pellet was washed with 100 mM KCl centrifuged at

*Correspondence:

Phone: +91 542 6702505; Fax: +91 542 2368174

E-mail: rmishraa@bhu.ac.in

12000×g for 15 min. The supernatant was considered mitochondrial membrane-bound fraction, and pellet was evaluated as washed mitochondrial fraction which was suspended in homogenizing buffer without mannitol. These fractions were used for enzyme assay and protein profile analysis.

Assay of succinate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase and arginase

Arginase assay was performed according to the method of Brown and Cohen¹⁶ with modification as reported by Saha and Rath¹⁷. The reaction mixture was consisting of 25 mM sodium glycinate buffer (pH 9.5), 2.5 mM MnCl₂, 25 mM L-arginine and suitably diluted enzyme extract in total volume of 2 mL which was incubated for 15 min at 30°C. The reaction was terminated by adding 10% perchloric acid, and protein was removed by centrifugation. Urea was estimated in the supernatant. A unit of arginase activity was defined as that amount of enzyme, which produced one μ mole of urea per hour at 30°C. The extraction procedure of mitochondrial membrane bound form is checked by the mitochondrial and cytosolic marker enzyme assay. Glutamate dehydrogenase (GDH) activity was assayed by the spectrophotometric method of Olson and Anfinsen¹⁸, and succinate dehydrogenase (SDH) by the method of Ells *et al.*¹⁹. However, enzyme specific staining of GDH and SDH were performed by the method of Okwumabua *et al.*²⁰ as a marker enzyme of mitochondrial fraction. Enzyme specific staining of lactate dehydrogenase (LDH) was performed as described by Bharti and Mishra²¹.

Protein profile and RT-PCR analysis

The protein concentration was estimated by the method of Bradford *et al.*²² using bovine serum albumin as the standard. Proteins from different fractions were resolved on 12% denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were visualized by staining with Coomassie Brilliant Blue-R250 (CBB R-250). Total RNA was extracted from liver of all organisms using TRIzol (Invitrogen), and reverse transcribed using Oligo-dT primer according to suggested protocol for first strand cDNA synthesis kit (Fermentas). Expression of arginase-I (*ARG I*) and arginase-II (*ARG II*) was analysed through RT-PCR. β -actin was used as control experimental. The primer-sets used in this study have been summarized in Table 1.

Table 1—Forward and reverse primers used (5'–3') to amplify genes of interest in RT-PCR experiments

Gene name	Primer sequences	Prod. size
<i>ARG I</i> F	5'-GTCCAGAAGAATGGAAGAGTCAG-3'	300 bp
<i>ARG I</i> R	5'-GTTCCCCAGGGTCTACGTCTCG-3'	
<i>ARG II</i> F	5'-GGCTACAGCTGTGTCCACATGG-3'	286 bp
<i>ARG II</i> R	5'-CAGGAGGCTCCACATCTCTCAG-3'	
β ACTIN F	5'-TGACGGGGTCACCCACACTGT-3'	600 bp
β ACTIN R	5'-CTACAAGCATTTGCGGACGATGGAGGG-3'	

Statistical analysis

The data were presented as mean $\pm$ SD. Data were analysed using one-way ANOVA followed by Tukey's test.

Results

Arginase activity was observed in cytosolic, mitochondrial and mitochondrial membrane-bound fractions of fish (Fig. 1A), frog (Fig. 1B), chicken (Fig. 1C), garden-lizard (Fig. 1D), and mouse (Fig. 1E). The arginase activity was higher in ureogenic (mouse, frog and fish) vertebrate (Figs. 1 A, B and E) in comparison to non-ureogenic (chicken and garden-lizard) vertebrates (Figs. 1 C and D). However, the transcript of arginase was detected in all representative types [Figs. 1 A(i) and E(i)]. The *ARG I* and *ARG II* both transcripts were observed in fish [Fig. 1A(i)], frog [Fig. 1B(i)], chicken (Fig. 1C(i)), and mouse [Fig. 1E(i)], but only *ARG II* was detectable in garden-lizard [Fig. 1D(i)]. The arginase activity in KCl associated fraction, supposed to be bound to mitochondrial membrane was higher in liver of fish (20%) followed by mouse (17%), frog (15%), garden-lizard (7%) and chicken (6%) as compared to arginase activity in crude extract (Fig. 2A). The activity of mitochondrial membrane-bound arginase was higher in mouse (65%), followed by fish (30%), frog (30%), garden-lizard (15%) and chicken (15%) as compared to the arginase activity of total mitochondrial fraction (Fig. 2B). On the basis of the spectrophotometric analysis [Figs. 3A(i) and B(i)], the mitochondrial membrane bound arginase seems as the different isoform but the enzyme-specific staining reveals the presence of enzymes from mitochondrial-matrix in membrane-bound fraction [Figs. 3 A(ii) and B(ii)]. However, cytosolic marker enzyme like LDH was negligible in the mitochondrial and membrane-bound fraction (Fig. 3C) as compared to the cytosol. The polypeptides of 98, 86, 54, 36, 27, 24 and 20 kDa were modulated in mitochondrial membrane bound fraction (Fig. 3D).

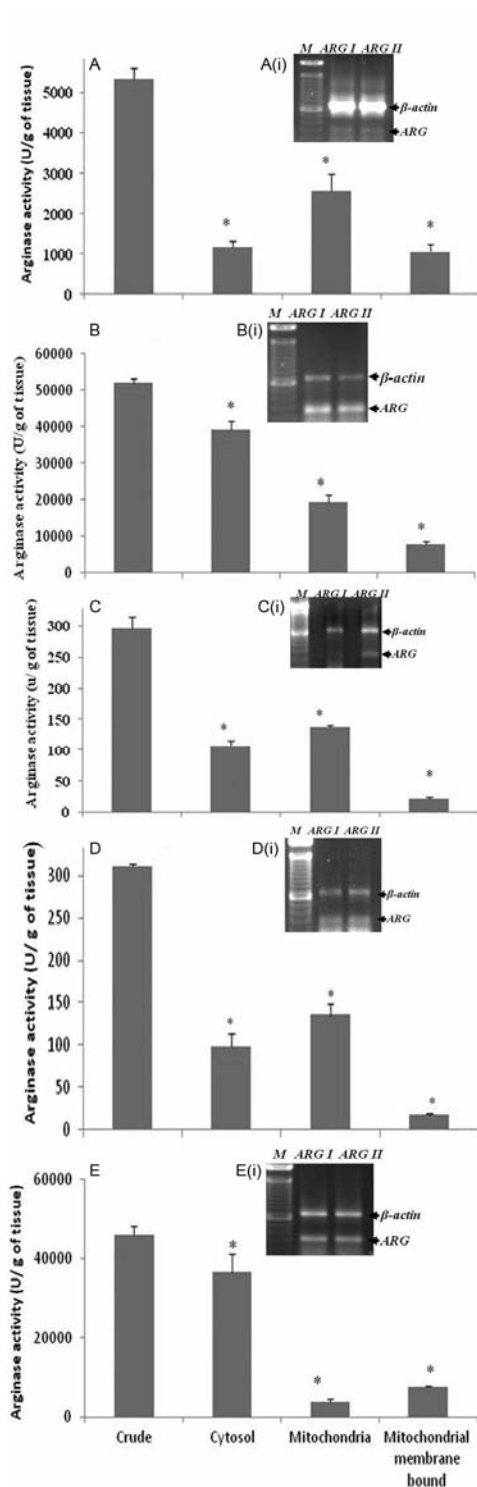


Fig. 1—Analysis of activity of arginase in different sub-cellular fraction of liver of (A) *Heteropneustes fossilis*, and (Ai) its expression; (B) *Rana tigrina*, and (Bi) its expression; (C) *Calotes versicolor*, and (Ci) its expression; (D) *Gallus gallus*, and (Di) its expression; (E) *Mus musculus*, and (Ei) its expression. [Values are plotted as mean $\pm$ S.D. * P < 0.01, ** P < 0.05]

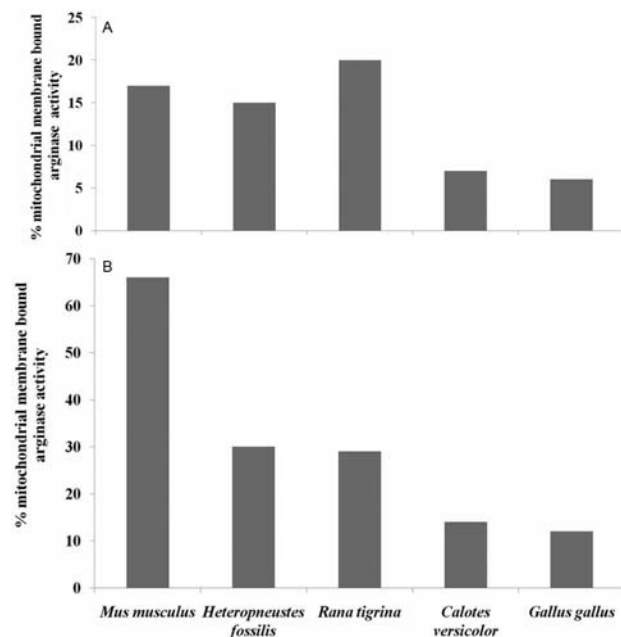


Fig. 2—The relative activity (%) of mitochondrial membrane bound arginase in five organisms as compared to (A) total activity of arginase present in crude; and (B) total activity of arginase present in mitochondria.

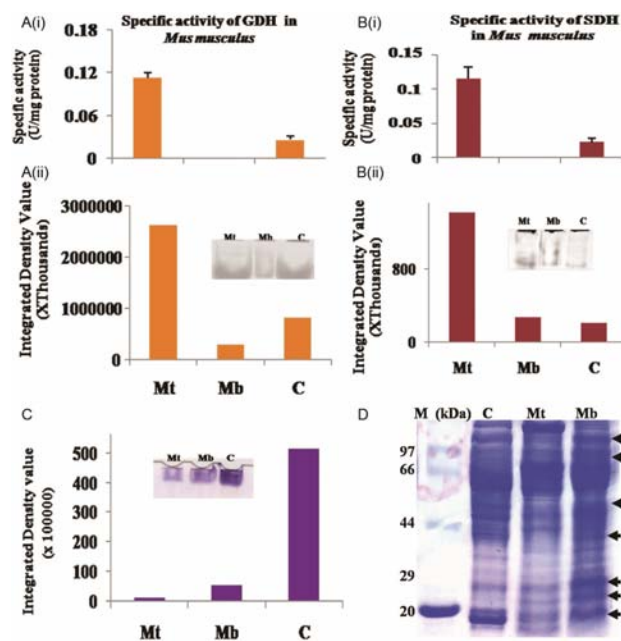


Fig. 3—Analysis of extraction procedure of mitochondrial membrane bound fraction from liver of *Mus musculus* (mammal) by described method. Activity of GDH in different sub-cellular fraction, by (Ai) spectrophotometric method, and (Aii) enzyme specific staining, and Integrated density value of GDH by enzyme specific method; Activity of SDH in different sub-cellular fraction, by (Bi) spectrophotometric method, and (Bii) enzyme specific staining, Integrated density value of SDH of enzyme specific method; (C) Activity of LDH in different sub-cellular fraction, by enzyme specific staining, Integrated density value of LDH of enzyme specific method; and (D) protein profile analysis of different sub-cellular fraction of liver of *Mus musculus*.

Discussion

The presence of arginase-II along with arginase-I in ureogenic (mouse, frog and fish) vertebrate [Figs. 1A(i), B(i) and E(i)] in comparison to the non-ureogenic (chicken and garden-lizard) vertebrates [Figs. 1C(i) and D(i)] suggested specific physiological function dependent on physiological fate of arginine and ornithine. However, reports describe cytosolic arginase (arginase-I) in hepatic tissue²³⁻²⁵ and mitochondrial arginase (arginase-II) in extra hepatic tissue^{26,27}. It was also interesting to observe distribution of both isoforms in hepatic tissue of ureogenic as well as non-ureogenic vertebrates (Fig. 1). In non-ureogenic vertebrates, arginase-II appears to regulate amino acid synthesis via glutamate and alpha ketoglutarate. However, in marine fish mitochondrial arginase-II may be critical for production of urea for osmotic balance. In reptiles and birds, mitochondrial arginase-II supports the view of influencing the production of ornithuric acid and uric acid along with glutamate, proline and polyamines metabolism. Reports on distribution and isoforms of nitrogen metabolizing enzymes support the findings because they have been influenced by the mode of the nitrogen detoxification pattern^{4,23,28} and environmental adaptation of organism^{4,29,30} in vertebrates. Results enlighten that both arginase isoforms (arginase-I and arginase-II) are present in liver of vertebrates. The presence of mitochondrial membrane bound arginase (mbArg) testifies its physiological significance (Fig. 4) in the transport of arginine and ornithine³¹. The observation of active fraction of LDH in the mitochondrial membrane-bound fraction (Fig. 3C) appears negligible as compared to the cytosolic fraction but it may be due to mitochondrial-LDH associated with mitochondrial outer membrane³². The presence of mitochondrial matrix protein in the mitochondrial membrane bound fraction (Fig. 3D) indicates the leakage of mitochondrial matrix proteins under influence of KCl.

The presence of transcripts of arginase-II transcripts [Figs. 1A(i), B(i), C(i), D(i) and E(i)] in all the five representative types (fish, frog, bird, reptiles and mammals), and arginase-I in fish, frog, chicken and mammals is in agreement that the different sub-cellular location of an enzyme critically enhance the efficiency of metabolism³⁰. However, the association of arginase with mitochondrial membrane was proposed to regulate the ureogenesis¹⁴ and the abundancy of arginase not to the nitrogen excretion

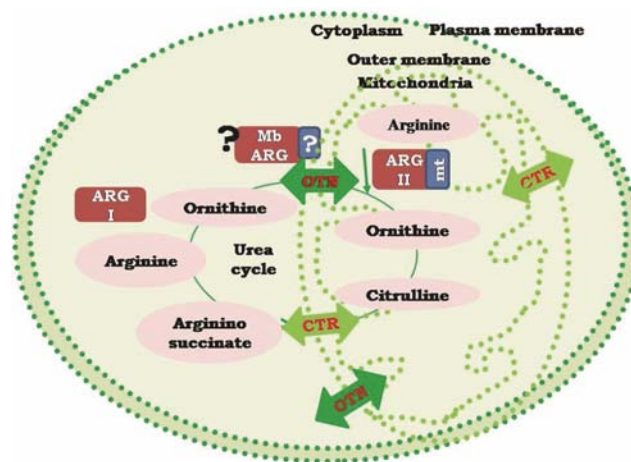


Fig. 4—Schematic representation of the presence of Arginase-I (ARG I), Arginase-II (ARG II) and mitochondrial membrane bound form (MbARG). [CTR= citrulline transporter, ORN= ornithine transporter. Arginase-II having the mitochondrial targeting sequence that enables them to get localized in mitochondria, while Arginase-I not having any mitochondrial targeting sequence and localized in cytosol. Question mark with star raises the lack in literature related to physiological significance of MbARG and question mark in targeting site (in box) showed the probable change in the mitochondrial targeting sequence, as any modification in mitochondrial targeting sequence will change the efficiency of mitochondrial localization]

pattern²⁸. It is presumed that the association of mitochondrial membrane-bound arginase could be useful to enhance the efficiency of transport of arginine and ornithine from cytosol to mitochondrial matrix and vice versa to maintain the homeostasis of these two metabolites at different physiological conditions (Fig. 4).

Acknowledgement

One of the authors, SM acknowledges guidance and suggestions of Prof. BK Ratha during the initial phase of doctoral work. The financial supports from UGC-CAS, DBT-ISLS, UGC-UPE to RM, and UGC-JRF (RFSMS) to SM are gratefully acknowledged.

References

- 1 Kossel A & Dakin HD, Über die Arginase Hoppe Seylers. *Z Physiol Chem*, 41 (1904) 321.
- 2 Nlu G & Muntian EM, The role of bivalent cations in the binding of hexokinase II isoenzyme to mitochondrial membranes. *Biokhimiia*, 51 (1986) 801.
- 3 Brdiczka D, Function of the outer mitochondrial compartment in regulation of energy metabolism. *Biochim Biophys Acta*, 1187 (1994) 264.
- 4 Jenkinson CP, Grody WW & Cederbaum SD, Comparative properties of arginases. *Comp Biochem Physiol B*, 114 (1996) 107.
- 5 Gideon D, Matthews NG, Koopman WJH, Pines O & Vardimon D, Weak mitochondrial targeting sequence

- determines tissue-specific subcellular localization of glutamine synthetase in liver and brain cells. *J Cell Sci*, 123 (2009) 351.
- 6 Uren RT, Dewson G, Christine Bonzon C, Lithgow T, Newmeyer DD & Kluck RM, Mitochondrial Release of Pro-apoptotic Proteins electrostatic interactions can hold cytochrome *c* but not smac/diablo to mitochondrial membranes. *J Biol Chem*, 280 (2005) 2266.
 - 7 Porembska Z & Zamecka E, Immunological properties of rat arginases. *Acta Biochim Polon*, 31 (1984) 223.
 - 8 Herzfeld A & Raper SM, The heterogeneity of arginase in rat tissue. *Biochem J*, 15 (1976) 469.
 - 9 Srivastava S & Ratha BK, Unique hepatic cytosolic arginase evolved Independently in ureogenic fresh water air breathing teleost *Heteropneustes fossilis*. *PLOS ONE*, 8 (6) (2013) e66057. doi: 10.1371/journal.pone.0066057.
 - 10 Grazi E, Magri E & Balboni G, On the control of arginine metabolism in chicken and liver. *Eur J Biochem*, 60 (1975) 431.
 - 11 Porembska Z, Skrzypek-Osiecka I & Rahden-Staron I, Subcellular localization of arginase in rat liver. *Acta Biochim Polon*, 27 (1980) 203.
 - 12 Cheung CW & Rajman L, Arginine, mitochondrial arginase, and the control of carbamoyl phosphate synthesis. *Arch Biochem Biophys*, 209 (1981) 643.
 - 13 Nissim I, Luhovyy B, Horyn O, Daikhin, Y, Nissim I & Yudkoff M, The role of mitochondrially bound arginase in the regulation of urea synthesis: studies with [U-¹⁵N₄] arginine isolated mitochondria and perfused rat liver. *J Biol Chem*, 280 (2005) 7715.
 - 14 Fuentes JM, Campo ML & Soler G, Physico-chemical properties of hepatocyte plasma- membrane bound arginase. *Arch Int Physiol Biochim Biophys*, 99 (1991) 413.
 - 15 Mishra M & Shukla SP, Effect of endosulphan on bioenergetic properties of liver mitochondria from the freshwater catfish *Clarias batrachus*. *Pestic Biochem Physiol*, 50 (1994) 240.
 - 16 Brown Jr GW & Cohen PP, Comparative biochemistry of urea synthesis I Methods for the quantitative assay of urea cycle enzymes in liver. *J Biol Chem*, 234 (1959) 1769.
 - 17 Saha N & Ratha BK, Active ureogenesis in a fresh water air-breathing teleost *Heteropneustes fossilis*. *J Exp Zool*, 241 (1987) 137.
 - 18 Olson JA & Anfinsen CB, The crystallization and characterization of L-glutamic acid dehydrogenase. *J Biol Chem*, 19 (1952) 67.
 - 19 Ells HA, A colorimetric method for the assay of soluble succinic dehydrogenase and pyridinenucleotide-linked dehydrogenases. *Arch of Biochem Biophys*, 85 (1959) 561.
 - 20 Okwumabua O, Persaud JS & Reddy PG, Cloning and Characterization of the Gene Encoding the Glutamate Dehydrogenase of *Streptococcus suis* Serotype 2. *Clin Diagn Lab Immunol*, 8 (2001) 251.
 - 21 Bharti B & Mishra R, Lymphoma affects enzymes and protein profile of non lymphatic tissues in mice. *Int J Int Biol*, 3 (2008) 175.
 - 22 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248.
 - 23 Casey CA & Anderson PM, Sub-mitochondrial localization of arginase and other enzymes associated with urea synthesis and nitrogen metabolism in liver of *Squalus acanthias*. *Comp Biochem Physiol B*, 82 (1985) 307.
 - 24 Gasiorowska I, Porembska Z, Jachimowicz J & Mochnacka I, Isoenzymes of arginase in rat liver tissues. *Acta Biochim Pol*, 17 (1970) 19.
 - 25 Xu Q, Baker BS & Tata JR, Development and hormonal regulation of the *Xenopus* liver-type arginase gene. *Eur J Biochem*, 211 (1993) 891.
 - 26 Gotoh T, Sonoki T, Nagasaki A, Terada K, Takiguchi M & Mori M, Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. *FEBS Lett*, 395 (1996) 119.
 - 27 Joerink M, Savelkoul HF & Wiegertjes GF, Evolutionary conservation of alternative activation of macrophages: structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L). *Mol Immunol*, 43 (2006) 116.
 - 28 Venkatakrishnan G & Reddy SRR, Arginase isoforms in frog and lizard tissues. *Indian J Biochem Biophys*, 47 (2010) 13.
 - 29 Ouzounis CA & Kyrpides NC, On the evolution of arginase and related enzymes. *J Mol Evol*, 39 (1994) 101.
 - 30 Volff JN, Genome evolution and biodiversity in teleost fish. *Heredity* (Edinb), 94 (2005) 280.
 - 31 Paulus H, The evolutionary history of the ornithine cycle as a determinant of its structure and regulation. *Curr Top Cell Reg*, 2 (1983) 177.
 - 32 Elustondo PA, White AE, Hughes ME, Brebner K, Pavlov E & Kane DA, Physical and Functional Association of Lactate Dehydrogenase (LDH) with Skeletal Muscle Mitochondria. *J Biol Chem*, 288 (2013) 25309.