Purification and Properties of Aspartate Aminotransferase (E.C. 2.6.1.1) from Skeletal Muscle of *Cirrhina mrigala*

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Aspartate aminotransferase (E. C. 2.6.1.1.) from the skeletal muscle of fresh water fish *Cirrhina mrigala* has been purified 40 fold by ammonium sulphate fractionation, adsorption on alumina C_8 gel and chromatography using DEAE-cellulose column and the properties of the purified enzyme studied. The pH optimum of the enzyme is 7.8. The Km value of aspartic acid and 2-oxoglutaric acid are found to be 2.8×10^{-3} M and 1.0×10^{-4} M respectively. The activity of enzyme is inhibited by p-chloromercurybenzoate, hydroxylamine hydrochloride and sodium cyanide. The inhibition by p-chloromercurybenzoate is reversed by reduced glutathione, B-mercaptoethanol and cysteine. Dicarboxylic acids such as maleic acid, malic acid and succinic acid inhibit the enzyme activity. The enzyme is not activated by any of the metal ions tested and heavy metal ions such as mercury and silver strongly inhibit the enzyme activity.

The tissues of all species of animals contain enzymes which catalyze the process of biological transamination. Transamination reactions represent a prime mechanism for the synthesis and deamination of amino acids in the tissues. The aminotransferases found in the tissues are mainly aspartate aminotransferase, AAT (1-aspartate: 2-oxoglutarate aminotransferase E. C. 2.6.1.1) and alanine aminotransferase, ALAT (1-alanine: 2-oxoglutarate aminotransferase E. C. 2.6.1.2) (Meister, 1962). The purification and properties of aminotransferases from various animal and plant sources and from micro-organisms have been reported by several workers as reported by Chhatbar (1977). Chhatbar (1977) observed that AAT activity was present in sarcoplasma as well in mitochondria of skeletal muscle of fish. Both the isoenzymes had different electrophoretic mobilities and could be separated by electrophoresis.

In this paper purification and properties of sarcoplasmic aspartate aminotransferase from white skeletal muscle of freshwater fish, mrigal is reported.
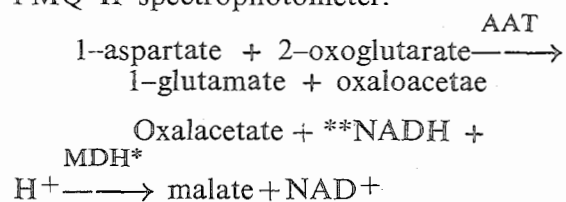
Materials and Methods

The chemicals used were of analytical grade. White skeletal muscle of mrigal immediately killed was the source of the enzyme. All purification experiments were performed at 0 to 5°C. Unless otherwise specified, the buffer used throughout was 0.1 M phosphate buffer, pH 7.6 and the centrifugation was carried out at 10,000 g for 10 min in a refrigerated IEC centrifuge. Percent ammonium sulphate used for fractionation was based on Green & Hughes (1955), though the solutions were maintained at 0 to 5°C. At every stage of purification, the preparation was assayed for protein content and subjected to polyacrylamide disc-gel electrophoresis. Alanine aminotransferase activity was determined (Bergmeyer & Bernt, 1965) at each stage of purification.

Assay of AAT activity

AAT activity was determined by the coupled enzyme reaction according to Bergmeyer & Bernt (1965) using Carl-Zeiss PMQ II spectrophotometer.

$$\text{1-aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AAT}}$$
$$\text{1-glutamate} + \text{oxaloacetae}$$

$$\text{Oxalacetate} + {}^{**}\text{NADH} +$$
$$\text{H}^+ \xrightarrow{\text{MDH*}} \text{malate} + \text{NAD}^+$$

The oxidation of NADH is measured by decrease in absorbancy at 340 mm. One unit

MDH* malate dehydrogenase

NAD^+ nicotinamide adenine dinucleotide

NADH** reduced NAD

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of enzyme activity is defined as that activity which catalyses the oxidation of one micromole of substrate per min at 30°C.

Protein estimation

The protein content in the enzyme preparations were determined with Folin-Ciocalteau reagent according to Lowry *et al.* (1951) using crystalline bovine serum albumin (Sigma) as the standard. Protein content in various fractions obtained by DEAE-cellulose chromatography was expressed as absorbance at 280 nm.

Electrophoresis

Disc electrophoresis in polyacrylamide gel (7.5%) was performed as described by Smith (1960). Electrophoresis on cellulose acetate membrane (CAM) was performed (Smith, 1960) using Beckman model R-101 microzone electrophoresis cell (Beckman Instruments Inc., California, Manual RM-IM-3, 1965).

Purification of aspartate aminotransferase

About 50 g of skeletal muscle was ground with minimum amount of phosphate buffer using a glass mortar and pestle. The partially homogenized tissue was further homogenized using Potter-Elvejehm type of homoganizer operating at variable speed. The homogenate (about 300 ml) was stirred for 2-3 h followed by centrifugation at 500 g for 10 min to separate the large cellular debris. The supernatant was further centrifuged at 20,000 g for 10 min.

First ammonium sulphate fractionation

Solid ammonium sulphate (special for enzyme work, Sarabhai Chemicals) was gradually added to the extract (300 ml) with continuous stirring to 30% saturation. The pH was maintained constant by addition of ammonia solution. The saturated homogenate was stirred for 2 h and centrifuged. The negligible amount of precipitate obtained was discarded. The supernatant was brought to 70% ammonium sulphate saturation and allowed to stand overnight in the cold. The precipitate was separated by centrifugation and dissolved

in phosphate buffer. It was dialysed against phosphate buffer containing 10^{-3} g/l pyridoxal phosphate (PLP) till it was free from ammonium sulphate.

Alumina C_8 treatment

Alumina C_8 gel was prepared as described by Dawson *et al.* (1969). The dialysate obtained was clarified by centrifugation. It was treated with alumina gel (0.2 mg gel/mg protein) and allowed to stand for 30 min. The mixture was centrifuged and the sedimented gel was discarded. The supernatant was further treated four times with fresh gel.

Second ammonium sulphate fractionation

The partially purified preparation was saturated to 70% with ammonium sulphate. The precipitate was separated by centrifugation.

DEAE cellulose chromatography

DEAE — cellulose (Whatman) was subjected to a standard cycle of washes with 0.1 N sodium hydroxide, 0.5 N hydrochloric acid and water as described by Himmelhoch (1971). DEAE–cellulose suspended in 0.01M phosphate buffer, pH 7.5 containing 0.1 M sodium chloride and 10^{-3} g/l PLP was packed in a column (1 x 30 cm) and washed with 0.01 M phosphate buffer containing 0.1 M sodium chloride.

The precipitate obtained in the previous step was dissolved in 50 ml of 0.01 M buffer containing 0.1 M sodium chloride. It was dialysed against the latter supplemented with PLP, till free from ammonium sulphate. 15 ml of the dialysate was applied to the column and eluted with 0.01 M buffer containing 0.1 M sodium chloride at the rate of 5 ml per 30 min. 5 ml eluate was collected in each tube. The concentration of sodium chloride in elution buffer was increased to 0.2 M and 0.3 M respectively. The contents of six tubes (15 to 20) were pooled together and dialysed against distilled water containing PLP to remove salts. The dialysate was saturated to 75% with ammonium sulphate and was stored in cold.

The characteristics of the purified enzyme such as requirement for co-factors, ultra-violet absorption spectrum and electrophoretic mobilities were studied. The enzyme kinetics and the influence of pH, inhibitors, dicarboxylic acids and metal ions on enzyme activity were studied. The standard 3 ml reaction mixture contained 0.1 M phosphate buffer pH 7.6, 2.5×10^{-1} M 1-aspartate, 0.2 M 2-oxoglutarate, 1.2×10^{-2} M NADH, 25 mg MDH and 87 mg of purified enzyme protein. 1 ml of buffer in reaction mixture was replaced with equal volume of inhibitor, dicarboxylic acid or metal ion solution as the case may be.

The influence of aspartic acid concentration on AAT activity was studied by varying its final concentration from 0.0005 M to 0.04 M. The concentration of co-substrate, 2-oxoglutaric acid was kept constant at 0.2 M. The influence of 2-oxoglutaric acid on enzyme activity was determined by varying its concentration between 0.001 M and 0.1 M. The Michaelis constants (apparent Km values) for aspartic acid and 2-oxoglutaric acid were obtained from Lineweaver-Burk plots.

The influence of pH on purified AAT was studied under assay conditions using 0.1 M phosphate buffer and 0.1 M Tris-HCl buffer. An attempt was made to compare the pH behaviour of sarcoplasmic and mitochondrial AAT enzymes. Mitochondria from skeletal muscle of mrigal were isolated by differential centrifugation (Chhatbar, 1977). The mitochondria were

subjected to repeated freezing and thawing followed by incubation with 0.2 ml of 10% Triton x 100 at 37°C for 30 min. This was used as a source of mitochondrial AAT to study the influence of pH on its activity.

Results and Discussion

Table 1 reports the summary of the purification procedure. It is evident that AAT was purified about 40 fold by the methods adopted. The activity precipitated between 30–70% saturation with ammonium sulphate. First fractionation showed about 2.3 fold increase in the activity. At this stage, the fraction also possessed significant level of ALAT activity. Though, the second fractionation did not accomplish any significant concentration in AAT activity, it was useful in concentrating the protein by precipitation. Treatment with alumina gel was found useful for attaining 7 fold purification. Chromatography on DEAE—cellulose achieved maximum purification of AAT. The elution profile of AAT is shown in Fig.1. It indicated a very sharp peak of unadsorbed protein, devoid of AAT activity. It was found that AAT was eluted with 0.2M sodium chloride concentration.

The purified enzyme was relatively stable at lower temperatures of storage. It migrated as a single band of protein in polyacrylamide gel electrophoresis (Fig.2). It was devoid of ALAT activity, though the latter was detected during the first stage of purification. This indicates the

Table 1. *Purification of aspartate aminotransferase E.C. 2.6.1.1 from skeletal muscle of Cirrhina mrigala*

| Step	Volume ml	Enzyme activity U/ml	Total enzyme activity	Protein mg/ml	Specific activity U/mg	Fold purification	Yield %
Homogenate	300	0.48	144.0	10.67	0.045	—	100
First ammonium sulphate fractionation	300	0.55	165.0	5.50	0.100	2.3	115
Alumina C_8 treatment	300	0.40	120.0	1.26	0.317	7.0	83
Second ammonium sulphate fractionation	50	1.80	90.0	5.29	0.340	7.6	63
DEAE-cellulose chromatography (pooled from 3 sets)	90	0.49	44.1	0.27	1.810	40.0	30
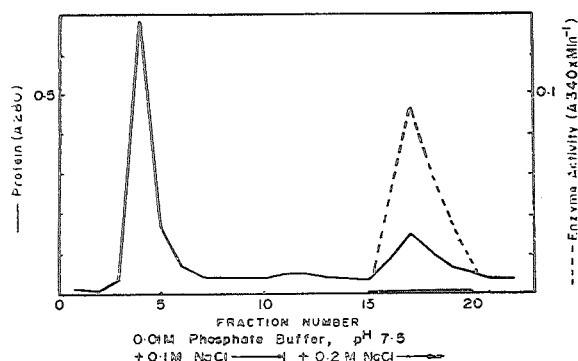
Fig. 1. DEAE cellulose chromatography of fish muscle aspartate aminotransferase

general observation that AAT and ALAT are two distinct enzymes. The purified AAT exhibited absorption peak at 276 nm in ultraviolet region (Fig. 3).

It was observed that the dialysis of the enzyme against phosphate buffer or distilled water resulted in loss of activity. The enzyme could be reactivated by PLP. Bell (1968) observed that AAT from liver of salmon was activated by PLP but not by pyridoxal, pyridoxamine or pyridoxine. In the present studies, it was found advantageous to incorporate PLP in dialysing buffer during purification stages.

Fig. 4 shows electrophoretic mobilities of AAT on CAM at different pH values. It is noted that the enzyme has an iso-electric point at about pH 5. Earlier, Marino et al. (1965) made similar observations with purified ox heart AAT.

Under the standard assay conditions, it was noted that the rate of the enzyme reaction was linear upto 50 min and the product formation was directly proportional to the enzyme concentration upto 29.1 μg protein.

The K_m value of purified AAT for aspartic acid was found to be $2.8 \times 10^{-3} M$ (Fig.5). It was $1.0 \times 10^{-4} M$ for 2-oxo-glutaric acid (Fig.6). Fig. 7 shows that the plot of activity against pH as a bell-shaped curve with pH optimum at 7.8. It also shows pH behaviour of crude mitochondrial AAT and is distinctly different from that of purified sarcoplasmic AAT. It has broad

Fig. 2. Polyacrylamide gel electrophoresis of purified aspartate aminotransferase of fish skeletal muscle

pH optimum between 6.0 and 8.0. These observations confirm the presence of at least two distinctly separate sarcoplasmic and mitochondrial AAT proteins in fish skeletal muscle.

McAllan & Chefurka (1961) found that roach AAT had a marked pH optimum at 8.3. The pH optimum of human intestine AAT was found to be 7.6 using phosphate buffer (Ramaswamy & Radhakrishnan, 1964). Bell (1968) observed that partially purified AAT from salmon liver had no distinct optimum pH but the velocity of enzyme reaction was positively related to increasing pH over the range 5 to 10.

Table 2 presents the influence of inhibitors on AAT activity. It is seen that PCMB inhibits the activity even at low concentration $(10^{-5}M)$. The inhibition by PCMB $(10^{-4}M)$ is reversed by reduced glutathione, B–mercaptoethanol and cysteine (all $10^{-2}M$), thus suggesting the presence of –SH, sulphhydryl groups in the protein molecule and their participation in enzyme activity. Katunuma et al. (1968) have observed that aminotransferases are
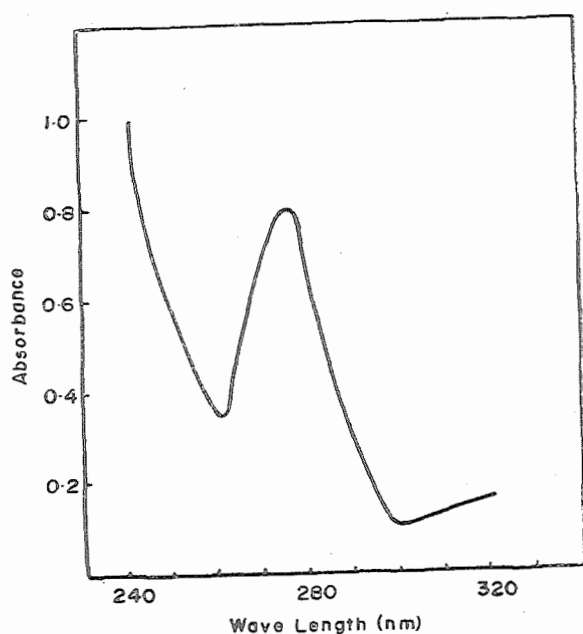
Fig. 3. Ultraviolet absorption spectrum of fish muscle aspartate aminotransferase (enzyme concentration 0.48 mg/ml)
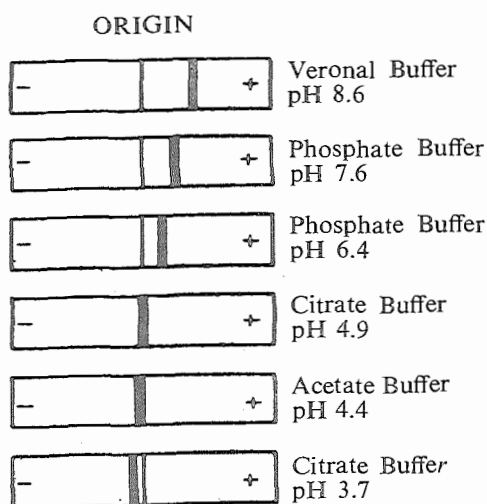
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Fig. 4. Schematic representation of the electrophoretic mobility of fish muscle AAT at different pH values

sensitive to the reagents blocking sulphhydryl and carbonyl groups as well as to the antimetabolites of vitamin B–6.

Fish muscle AAT is inhibited by hydroxylamine hydrochloride at 10^{-3}M concentration whereas it is not substantially inhibited by sodium cyanide. It is also not affected by sodium arsenite.

Table 2. *Influence of inhibitors on fish muscle aspartate aminotransferase*

Inhibitor	Final concentration	*Activity % of control
p-chloromercury-benzoate (PCMB)	10^{-5}	91.00
	10^{-4}	47.00
	10^{-3}	5.80
PCMB (10^{-4})		
+ reduced glutathione	10^{-2}	91.00
+ cystein	10^{-2}	91.00
+ B-mercapto-ethanol	10^{-2}	100.00
Sodium cyanide	10^{-3}	77.00
Hydroxylamine hydrochloride	10^{-3}	5.80
Sodium arsenite	10^{-3}	100.00

* The activity in the control is taken as 100 in each experiment (standard assay conditions)

Table 3. *Influence of dicarboxylic acids on fish muscle aspartate aminotransferase*

Compound	Inhibition %
Maleic acid	62
Malic acid	18
Succinic acid	10
Malonic acid	Nil
Oxalic acid	Nil

* The dicarboxylic acids were present in a final concentration of 4×10^{-2} M in standard assay mixture. The acids were neutralized with 1 N sodium hydroxide prior to addition

The influence of discarboxylic acids on AAT is presented in Table 3. It is observed that the enzyme is strongly inhibited by maleic acid. Malic acid and succinic acid are inhibitory to a certain extent whereas malonic and oxalic acids do not inhibit the enzyme activity. AAT is inhibited by several discarboxylic acids probably due to the formation of an inactive
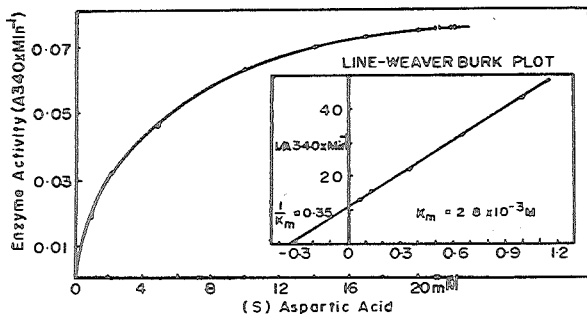
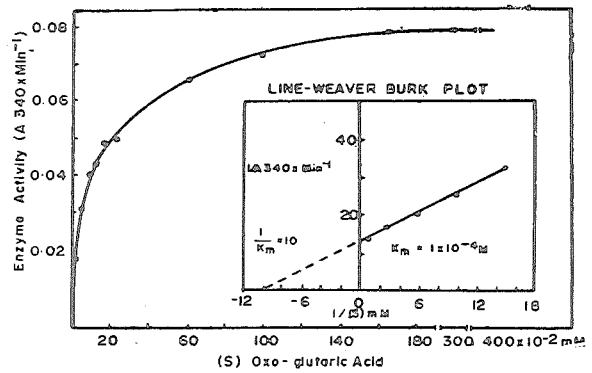Fig. 5. Fish muscle aspartate aminotransferase: Km value for aspartic acid

complex with the enzyme (Jenkins *et al.*, 1959). Michuda & Martinez (1970) found a correlation of the inhibition of transaminations with the affinity of the dicarboxylic acids to the pyridoxal form of the enzyme.

Table 4. * *Influence of metal ions on fish muscle aspartate aminotransferase*

Metal ion	Activity % of control
Mg^{+2}	100
Fe^{+2}	100
Pb^{+2}	20
Ag^{+2}	Nil
Fe^{+3}	97
Mn^{+2}	100
Hg^{+2}	2
Zn^{+2}	100
Cu^{+2}	97

* The final concentration of metal ions in the reaction mixture was 5×10^{-4} M under standard assay conditions. The sulphates of magnesium, ferrous, manganese, zinc and copper; chlorides of mercury and ferric; silver nitrate and lead acetate were used.

The results presented in Table 4 indicate that AAT was not activated by any of the metal ions tested, whereas heavy metal ions such as mercury and silver strongly inhibited the activity.

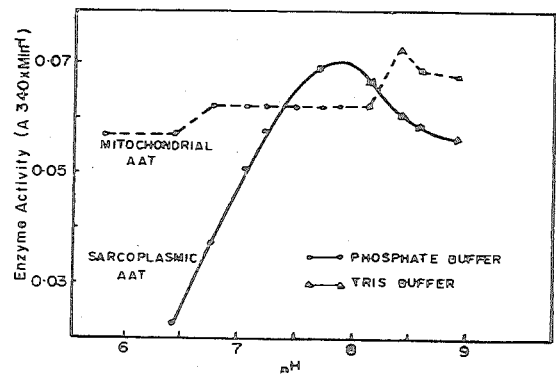Fig. 6. Fish muscle aspartate aminotransferase: Km value for oxo-glutaric acid

Fig. 7. Activity of fish muscle aspartate aminotransferase as a function of pH

The present investigations have shown that the properties and characteristics of AAT of the fish muscle are generally similar to those of AAT from other animal sources.

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