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Partial purification and some properties of aspartate aminotransferase from the mantle tissue of *Mytilus galloprovincialis* Lam.

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Abstract: Aspartate aminotransferase (E.C.2.6.1.1; AST), is a pyridoxal phosphate dependent enzyme that occurs in virtually all organisms and plays a key role in intermediary nitrogen metabolism. Although AST was purified from a variety of plant and animal sources, it has not been purified previously from mantle tissue of *Mytilus galloprovincialis* Lam. In the present study we have partially purified AST from the mantle tissue of *M. galloprovincialis* and investigated some kinetic properties of the enzyme. The partially purified enzyme showed three protein and a single activity band in polyacrylamide gel electrophoresis. It was found that the enzyme exhibited maximum activity at 15ºC and 35ºC and that the activity was decreased at 40ºC and totally lost at 55ºC. AST activity was maximum at pH 7.4 in Tris-HCl buffer. Km values of AST for aspartate and 2-oxoglutarate were 1.64 mM and $2.2x10^{-2}$ mM, respectively, and Vmax values for the same substrates were 0.12 U/mL and 0.168 U/mL, respectively.

Key words: aspartate aminotransferase*, Mytilus galloprovincialis* Lam., mussel, enzyme purification

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Introduction

Aspartate aminotransferase (E.C.2.6.1.1; AST) which was formerly called as glutamic oxalacetic transaminase (GOT), is a pyridoxal phosphate dependent enzyme that occurs in virtually all organisms and plays a key role in intermediary nitrogen metabolism. It catalyzes the interconversion of L-aspartate and L-glutamate with the corresponding α-keto acids. AST is widely distributed among animals, plants and microorganisms (1-6). In eukaryotes, the enzyme exists as two different isoenzymes in cytoplasm and mitochondria, which are named cytosolic-AST and mitochondrial-AST (2). The seasonal dinamics of enzyme activity is traced throughout the annual cycle of mussels. Changes in the AST activity were observed only in the mitochondrial fraction which had maximum activity in winter and minimal one in summer (7). Pastore et al. (8), suggested that the mitochondrial and cytosolic GOT activity had an increasing trend and cytosolic glutamic pyruvic transaminase (GPT) activity had a decreasing trend in autumn.

Although AST was purified from a variety of plant and animal sources, it has not been purified previously from mantle tissue of *Mytilus galloprovincialis* Lam. In the present study we have partially purified AST from the mantle tissue of *M. galloprovincialis* and investigated some kinetic properties of the enzyme.

Materials and methods

Chemicals

β-Nicotinamide adenine dinucleotide disodium salt reduced ($NADH_2$-Na_2, Fluka 43420), pyridoxal 5'-phosphate (Fluka 82870), 2-oxoglutaric acid (Merck 5194), L-aspartic acid (Merck 129) and malate dehydrogenase (1500 U/mg, Serva 28338) were used in enzymatic activity assays. Hydroxylapatite was prepared in our laboratory according to the modified method of Tiselius et al. (9). Bovine serum albumin (Fluka 05470) was used as a standard for protein determination. All the other chemicals were of analytical reagent grade.

Purification of AST from the mantle tissue of M. galloprovincialis

Mussels, which had been collected from Rumelikavağı-Bosphorus, were obtained from İstanbul Su Ürünleri Hali-Kumkapı. AST was purified from the mantle tissue of the mussels in three steps:

I. Extraction: 15 mussels were separated from their shells and the mantle tissues were separated from the other tissues. The mantle tissues were cut with scissors, washed with distilled water, dried on a filter paper and weighed (45.45 g). The mantle pieces were homogenized with cold saline (200 mL) by means of a Bosch homogenizer and the homogenate was stirred via magnetic stirrer for 30 min at +4ºC. After standing overnight at +4ºC, the homogenate was centrifuged at 20000 rpm for 30 min at 0ºC. The supernatant was filtered and the mussel mantle crude extract (280 mL) was obtained.

II. Ammonium sulphate fractionation: Ammonium sulphate was added to the crude extract to 30% saturation and left overnight at +4ºC. The precipitate so formed was separated and discarded by means of centrifugation at 20000 rpm for 30 min at 0ºC. The supernatant was brought to 65% saturation by adding ammonium sulphate and left again overnight at +4ºC. The precipitate thus formed (30-65% cut) was separated by centrifugation under the same conditions, dissolved in a minimum volume of saline and dialysed during the day[1] against 1 mM of the Na-K phosphate buffer by changing the buffer every 2 hours (Sigma dialysis tubing, 25mm x 16mm, D-9777). Any precipitate formed in the dialysis bag was discarded by centrifugation and the clear supernatant called 30-65% ammonium sulphate cut (17 mL) was used for further purification.

III. Hydroxylapatite column chromatography: 13 mL of the 30-65% ammonium sulphate cut containing 200 mg protein was applied to the hydroxylapatite column and the elution was performed by washing the column stepwise with 5 mM, 10 mM, 20 mM, 50 mM, 100 mM and 200 mM Na-K phosphate buffer (pH 7). 3.5 mL fractions were collected and elution profile was drawn after measuring the absorbance of every tube at 280 nm. Besides, AST activity was determined in each tube and fractions showing

[1] The mussel extract contains cellulase which destroys the dialysis bag made of cellulose if the dialysis process last for a longer period. This is also why DEAE-cellulose chromatography is not suitable for the purification of the mussel.

the highest enzyme activity were pooled. The purified mussel mantle AST was stored at -20ºC.

Protein determination

Protein contents of the samples during extraction and ammonium sulphate precipitation processes were determined according to the method of Lowry et al. (10) whereas the fractions obtained through column chromatography were analyzed for protein by E 280/260 method (11).

Enzyme assay

AST activity was determined by the method of Karmen (12) modified by us (4) and Rej et al. (13). The reaction mixture (2.5 mL) containing 54 mM L-aspartate, 12 units malate dehydrogenase, 0.06 mM $NADH_2$, 0.033 mM pyridoxal 5'-phosphate, and 113 mM Tris (hydroxymethyl)-aminomethane (Tris) with a final pH of 7.8 was placed in a spectrophotometer cuvette. Then 0.25 mL of the enzyme solution was added and the content of the cuvette was mixed with a thin glass rod. The reaction was started by addition of 0.25 mL of a solution containing 45 mM 2-oxoglutarate and 125 mM Tris. The decrease of absorbance at 339 nm during 5 minutes was monitored spectrophotometrically. Final reaction temperature was recorded and "temperature correction factor" (14) was used for the activity calculations. One unit of AST activity (Karmen Unit) was expressed as absorbance decrease of 0.001 per mL of the sample per minute of 1 cm light path.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE was performed acccording to Ornstein (15) and Davis (16). Proteins (100 µg/gel) were electrophoretically resolved on 7.5% polyacylamide gels of 6x0.6 cm dimensions with Tris-glycine buffer (0.025 M Tris, 0,192 M glycine, pH 8.5). Electrophoresis was carried out at 4ºC in a cold chamber, at 5 mA/gel for 50 min. One of the two gels was stained with Amidoblack 10B to determined the protein bands. The other gel was subjected to activity staining with Fast violet B (17).

Kinetic properties of AST

I. Effect of temperature on enzyme activity: 0.4 mL aliquots of the enzyme solution was diluted with 1 mM Na-K phosphate buffer to 150 U/mL activity, heated for 30 minutes at varying temperatures between 5ºC and 60ºC with 5ºC intervals. The enzyme activities were determined as described above.

II. Effect of pH on enzyme activity: The activity of AST was determined between pH 5 and 9. 0.1 M K-K phosphate buffer was used between pH 5 and 7, and 0.1 M Tris-HCl buffer for between pH 7.2 and 9. 7.5 mL of the buffer solution of desired pH were mixed with 2.5 mL of a neutral aspartate solution, containing $NADH_2$, pyridoxal-5'-phosphate, malate dehydrogenase and Tris. 2.5 mL of this mixture was transferred to a spectrophotometer cuvette, 0.25 mL 2-oxoglutarate solution was added and the reaction was initiated with 0.25 mL of the enzyme solution properly diluted to show an activity of 150 U/mL. The pH's were rechecked after the assays had been completed.

III. Substrate specificity: Michaelis constants (Km) values of AST for L-aspartate and 2-oxoglutarate were determined by using 6 different concentrations of both substrates. The reaction mixtures were the same as used in the standard assay. The difference was that L-aspartate concentration was varied from 0.45 mM to 45 mM when 2-oxoglutarate concentration was kept constant and 2-oxoglutarate concentration was changed from 0.0375 mM to 3.75 mM with constant L-aspartate concentration. Reaction velocities were plotted on double reciprocal plots and the values of Km and maximum velocity (Vmax) were determined by linear regression according to Lineweaver and Burk. The method of least squares was used to determine the Km values.

Results and discussion

Hydroxylapatite column chromatography of the 30-65% ammonium sulfate cut of the mussel mantle tissue, resulted in a single peak of AST activity eluted with 100 mM phosphate buffer (Figure 1).

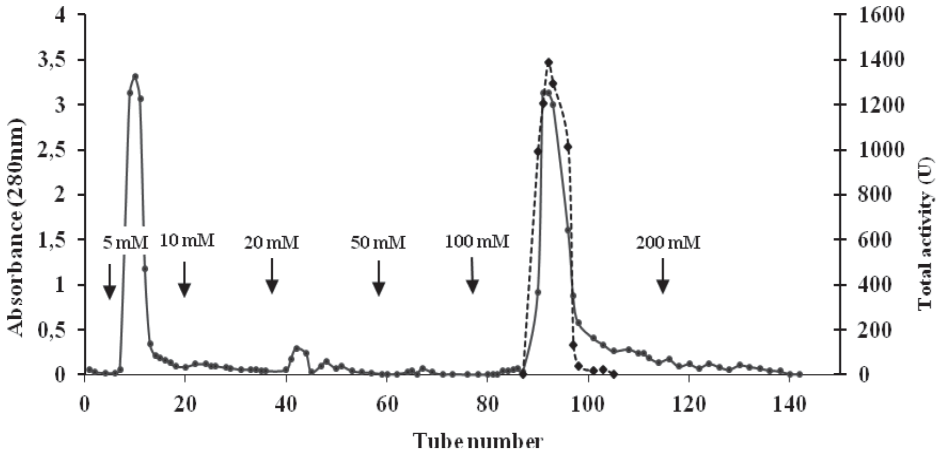

Figure 1. Hydroxylapatite column chromatography of the 30-65% ammonium sulphate cut of *M. galloprovincialis* mantle tissue. Column dimentions: 2x14.5 cm; Applicate: 200 mg protein; Flow rate: 40 mL/h; Protein (-●-) and AST activity (---◆---).

The results of partial purification of AST from the mantle tissue of *M. galloprovincialis* are summarized in Table 1. AST was purified in three steps and 443 fold purification was obtained. After the purification process, the specific activity of AST increased from 103 U/mg to 45656 U/mg. Enzyme activity of the crude extract was found lower than the activity of the 30-65% ammoniun sulphate cut. This lower activity can be explained by the presence of an inhibitor in mussel tissues which was eliminated in the other purification steps (18).

Table 1. Purification of aspartate aminotransferase (AST) from the mantle tissue of *M. galloprovincialis* (All data refer to 45.45 g mantle tissue obtained from 15 mussels).

Purification step	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Purification factor**
I. Crude extract	462	46750	103	1
II. 30-65% ammonium sulphate cut	256	325530	1274	12
III. Hydroxylapatite (100 mM Na-K-phosphate buffer eluate)	18	842800	45656	443

It was observed that mussel mantle AST displayed one broad activity band and three protein bands, two strong and one weak, in native PAGE (Figure 2).

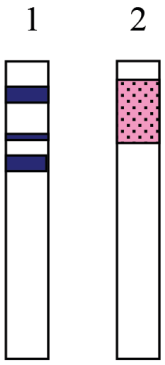

Figure 2. Schematic presentation of the PAGE of aspartate aminotransferase (AST) from the mantle tissue of *M. galloprovincialis* (Gel 1 stained for protein; gel 2 stained for activity).

The partly purified AST exhibited two maximum activity peaks, one at 15ºC and the other at 35ºC, the activity decreased with increase in temperature and was completely inactive at 55ºC (Table 2). It was reported that AST obtained from animal and plant sources were resistant to temperatures up to 75ºC (19,20) and 40-55ºC (21-23), respectively. In a previous study, we found that AST from the hepatopancreas of *M. galloprovincialis* exhibited maximum activities at 25ºC and 40ºC; and that the activity completely dissappeared at 60ºC (18). In the present study, the highest enzyme activity obtained at 15ºC and complete loss of activity at 55ºC, which may be attributed to the mussels life in the cold seas. Similarly, α-amylase purified from the same mussel species in our department was found to exhibit maximum activity at 1ºC (24).

Table 2. Effect of temperature on the activity of aspartate aminotransferase (AST) from the mantle tissue of *M. galloprovincialis*.

t °C	**Control**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**
% Activity	100	86.1	91.8	92.7	89.9	87.5	89.7	91.6	80.8	43.4	20.7	0	0

The partly purified AST showed maximum activity at pH 7.4 in Tris-HCl buffer (Figure 3). We also found that the enzyme activity was greater in Tris-HCl buffer than in phosphate buffer. Optimum pH values of AST vary according to the used sources (2,18,21,22), our results are consistent with other studies on *M. galloprovincialis* mussels (7).

Km and Vmax values of mussel mantle AST against two substrates L-aspartate and 2-oxoglutarate were given in Table 3. The Km value of mussel mantle AST for L-aspartate are higher than that of 2-oxoglutarate. Km values were different for the two substrates, L-aspartate and 2-oxoglutarate and it was previously reported that the enzyme is highly specific for 2-oxoglutarate both in the animal (7,25,26) and plant-derived enzymes (21,23).

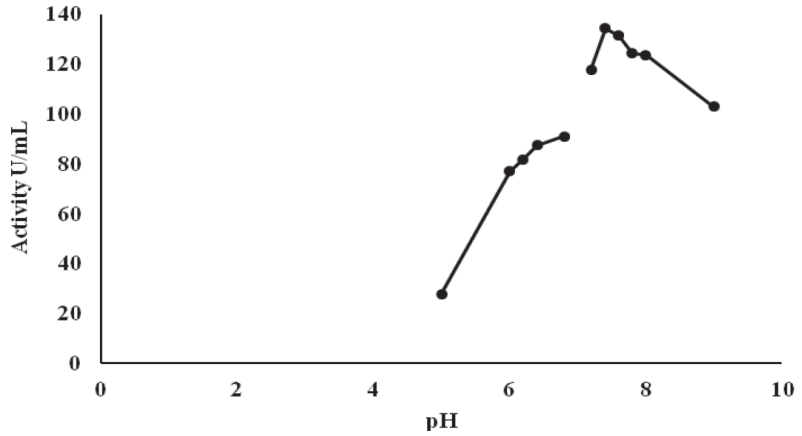

Figure 3. Effect of pH on the activity of aspartate aminotransferase (AST) from the mantle tissue of *M. galloprovincialis*.

Table 3. Km and Vmax values of aspartate aminotransferase (AST) from the mantle tissue of *M. galloprovincialis*.

	Km (mM)	**Vmax (U/mL)**
L-Aspartate	1.64	0.12
2-Oxoglutarate	0.22	0.168

Acknowledgements

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