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PARTIAL PURIFICATION and CHARACTERIZATION of ASPARTATE AMINOTRANSFERASE from the HEPATOPANCREAS of the MUSSEL *Mytilus galloprovincialis* Lam.

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SUMMARY

In this study aspartate and alanine aminotransferase enzymes of *Mytilus galloprovincialis* Lam., the mussel species specific to the Turkish coast were examined. Because it was found that aspartate aminotransferase activity in the hepatopancreas was superior to alanine aminotransferase while surveying the activities of these enzymes in the whole body, hepatopancreas and mantle tissues; among these two enzymes, aspartate aminotransferase was partially purified from hepatopancreas and its kinetic properties were studied.

Molluscs were collected daily from the Rumelikavağı coast of the Bosphorus and hepatopancreases were homogenized with 0.9% NaCl solution after dissection from the body. The fraction obtained after 35%-65% ammonium sulphate precipitation was applied to hydroxylapatite column. The elution performed by increasing molarity gradient of the phosphate buffer, pH 6.8, resulted in the recovery of the aspartate aminotransferase activity in 100 mM phosphate buffer pool. The 83 fold purified enzyme was applied to polyacrylamide gel electrophoresis and the existence of two activity bands indicated two isoenzymes of the aspartate aminotransferase.

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Effect of temperature on the activity of the enzyme was examined and it was found that the enzyme exhibited maximum activities at 25 °C and 40 °C; the activity completely dissappeared at 60 °C. Aspartate aminotransferase activity was maximum at pH 7.6, Km values for aspartate and 2-oksoglutarate were 1.7 mM and $6.6x10^{-2}$ mM, Vmax for the same substrates were 0.162 U/ml and 0.149 U/ml, respectively.

ÖZET

Bu çalışmada, Türkiye sahillerinde bulunan *Mytilus galloprovincialis* Lam. midye türünün aspartat ve alanin aminotransferazları incelendi. Tüm doku, hepatopankreas ve manto kısımlarında yapılan araştırmada, aspartat aminotransferazın hepatopankreastaki aktivitesinin alanin aminotransferaza göre fazla olmasından dolayı, midye hepatopankreasından aspartat aminotransferaz kısmen saflaştırıldı ve bazı kinetik özellikleri incelendi.

Deneyin yapılacağı gün İstanbul Boğazı Rumelikavağı kıyılarından toplanan midyelerin hepatopankreas kısımları çıkarılarak, %0.9 NaCl ile, homojenize edildi. Aspartat aminotransferaz aktivitesi gösteren homojenizatın %35-%65 amonyum sülfat kesiti hidroksilapatit kolona uygulandı. pH'sı 6.8 olan ve artan molaritede fosfat tamponu ile yapılan basamaklı elüsyon sonucunda, aspartat aminotransferaz 100 mM fosfat tamponu ile elüe edildi. Bu işlem sonunda, midye hepatopankreasından 83 kez saflaştırılan aspartat aminotransferazın poliakrilamid jel elektroforezinde iki aktivite bandı göstermesi nedeniyle iki izoenzim içerdiği sonucuna varıldı.

Aspartat aminotransferaz üzerine temperatürün etkisi incelendiğinde, enzimin 25 °C ve 40 °C'lerde maksimum aktivite gösterdiği ve aktivitenin 60 °C'de tamamen kaybolduğu görüldü. Enzimin optimum pH'sının pH 7.6'da olduğu; aspartat ve 2-oksoglutarata karşı Km değerlerinin sırasıyla, 1.7 mM ve $6.6x10^{-2}$ mM, aynı substratlara karşı Vmax değerlerinin ise 0.162 U/ml ve 0.149 U/ml olduğu bulundu.

Key words: Mussel, *Mytilus galloprovincialis* Lam., aspartate aminotransferase, alanine aminotransferase, transaminase

INTRODUCTION

Aspartate aminotransferase (EC 2.6.1.1; AAT) which was formerly called glutamic oxaloacetic transaminase (GOT), occurs widely in nature and has been studied most extensively. In eukaryotes, the enzyme exists in two isoenzymic forms, one localized in the cytoplasm and the other in the mitochondria of mammalian cells (1,2). The measurement of these isoenzymes in human serum reportedly is of clinical significance

in assessing tissue damage in diseases including myocardial infarction and hepatitis. Characterization of aspartate aminotransferase, through kinetic and clinical investigations is thoroughly reviewed (3).

Aspartate aminotransferase has been found in all tissues of all molluscs investigated. The levels of activity in molluscan tissues vary from very high in muscle of some cephalopods to very low in the tissues of some fresh water bivalves and gastropods (4,5). An intracellular localization and aminotransferase activity in some tissues of the Black Sea mussels were studied and it was shown that alanine aminotransferase (ALAT; GPT) was localized mainly in the cytoplasmic fraction whereas a considerable quantity of aspartate aminotransferase was localized in mitochondria (6). The same workers examined transaminase activity in tissues of *Mytilus galloprovincialis* Lam. in standart and hypoxic conditions and found an alanine /aspartate ratio of 1/1 (7).

Although aspartate and alanine aminotransferases were purified from a variety of plant and animal sources, they haven't been purified to date from *Mytilus galloprovincialis* Lam. which is a mussel specific to the coasts of Turkey. In this study, the occurence of aspartate and alanine aminotransferase activities in the whole tissue, mantle and hepatopancreas of the mussel *Mytilus galloprovincialis* Lam., collected from Istanbul Bosphorus Rumelikavağı coasts, were determined and the most active enzyme was purified and characterized from the tissue which contained the highest activity of this enzyme.

MATERIAL AND METHODS

Chemicals. β-Nicotinamide adenine dinucleotide disodium salt reduced ($NADH_2$-Na_2, Fluka 43420), pyridoxal-5'-phosphate (Fluka 82870), 2-oxoglutaric acid were used in enzyme assays. L-aspartic acid (Merck 129), malate dehydrogenase (1500 U/mg; Serva 28338) and L-alanine (Fluka 05130), lactate dehydrogenase (860 U/mg; Sigma L-2500) were used for the activity measurements of aspartate aminotransferase and alanine aminotransferase respectively. Hydroxylapatite prepared in our laboratory was used in column chromatographies. Bovine serum albumin (Fluka 05470) was used as a standart for protein determination. All other chemicals were of analytical reagent grade.

Protein determination. Protein contents of the samples during extraction and ammonium sulphate precipitation processes were determined according to the method of Lowry (8) whereas the protein fractions obtained through column chromatography were analyzed for protein by E 280/260 method (9).

Enzyme assay. The routine enzyme assay involved a nicotinamide adenine dinucleotide (NAD)-dependent malate dehydrogenase-linked reaction to measure aspartate

aminotransferase activity and a lactate dehydrogenase-linked reaction to measure alanine aminotransferase activity, according to the method of Karmen (10) modified by us (11) and Rej et al (12). To a spectrophotometer cuvette 2.5 ml of a solution containing 54 mM L-aspartate, 12 Units malate dehydrogenase (for AAT) or 54 mM L-alanine, 160 Units lactate dehyrogenase (for ALAT), 0.06 mM $NADH_2$, 0.033 mM pyridoxal-5'-phosphate, and 113 mM Tris with a final pH of 7.8. For AAT, 0.25 ml of the enzyme solution was first 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. For ALAT, 2-oxoglutarate solution was added first and the reaction was initiated by the addition of 0.25 ml of the enzyme solution. The decrease of absorbance at 339 nm during 5 minutes was monitored spectrophotometrically. One unit of aminotransferase 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 on a Pleuger electrophoresis apparatus acccording to Ornstein (13) and Davis (14). 10 fold diluted solution of Tris-glycine buffer (3% Tris and 14.4% glycine, pH 8.5) was used during electrophoresis. Electrophoresis was performed in 7.5% polyacrylamide gels of 6 x 0.6 cm rods. 50µl samples in 40% saccharose were applicated to the gels by means of a Hamilton injector. Electrophoresis was carried out at 4 °C in a cold chamber, at 5 mA/gel for 50 mins. The gels were stained with Amidoblack 10B for protein and with Fast violet B (15) for the detection of aspartate aminotransferase activity.

Determination of the occurence of aspartate and alanine aminotransferase activities in different tissues of Mytilus galloprovincialis **Lam.** 25 mussels freshly collected at the day of the experiment from Istanbul Bosphorus Rumelikavağı coast, were separated from their shells. The whole mussel tissue was washed, dried and weighed (184.13 g). After the addition of 150 ml cold saline, the whole tissue was homogenized at 14 000 rpm by means of a Bosch homogenizer. An additional 150 ml saline was added to the homogenate and it was stirred via magnetic stirrer for 30 mins in a cold room. After standing overnight at 4 °C, the homogenate was centrifuged at 20 000 rpm at -20 °C for 30 mins (Cryofuge 20-3 Hereaus-Christ). The supernatant was filtered (345 ml) and called **mussel whole tissue crude extract.**

Mantle and hepatopancreas tissues of another group of 25 mussels were separated. The mantle tissues (47.66 g) were homogenized with a total of 100 ml of saline as previously described for the whole tissue extract. The extract thus obtained (80 ml) was called **mussel mantle tissue crude extract.** The hepatopancreases were treated with a total of 100 ml of saline and **mussel hepatopancreas crude extract** was prepared in the same manner.

The protein contents, aspartate and alanine aminotransferase activities of the three extracts were determined.

***Purification of aspartate aminotransferase from the hepatopancreas of Mytilus galloprovincialis* Lam.** The enzyme was purified from the hepatopancreas of the mussel in three steps:

I. Extraction. 43 mussels were separated from their shells and the hepatopancreases were separated from other tissues by carefully cutting with scissors. The hepatopancreases were homogenized with a total of 500 ml cold saline and the **mussel hepatopancreas crude extract** was obtained as described above.

II. Ammonium sulphate fractionation. To the crude extract ammonium sulphate was added to 35% saturation and left overnight in the refrigerator. The precipitate so formed was separated and discarded by means of centrifugation at 20 000 rpm at –20 °C for 30 mins. The supernatant was brought to 65% saturation by adding $(NH_4)_2SO_4$ crystals and left again overnight at 4 °C. The precipitate formed (35%-65% cut) was obtained by centrifugation in the same conditions and dissolved in a minimum volume of 50 mM K-K phosphate buffer (pH 7.8). The solution thus obtained was dialysed in one day[1] against 5 mM of the same buffer by changing the buffer every 2 hours. Any precipitate formed in the dialysis bag was discarded by centrifugation and the clear supernatant called **35%-65% ammonium sulphate cut** was used for further purification.

The protein content and aspartate aminotransferase activity of the crude hepatopancreas extract and the 35%-65% ammonium sulphate cut were determined before hydroxylapatite column chromatography.

III. Hydroxylapatite column chromatography. Hydroxylapatite was prepared in our laboratory according to Tiselius, Hjerten and Levin (16). A column of 3 x 20 cm was filled with hydroxylapatite and equilibrated by washing with 5 volumes of 1 mM K-K phosphate buffer (pH 6.8). 17 ml of the 35%-65% ammonium sulphate cut containing ~ 600 mg protein was applied to the column and the elution was performed by washing the column stepwise with 1 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM, 200 mM and 1 M of the same buffer. 8 ml fractions were collected at a flow rate of 48 ml/h and elution profile was drawn after measuring the absorbance of every tube at 280 nm. Besides, AAT activity was determined in each tube and fractions showing enzyme activity were pooled, concentrated by placing the solution, filled in a dialysis bag, in sugar and subsequently dialysed against 1 mM phosphate buffer (pH 7). The purified **mussel hepatopancreas AAT** was stored at –20 °C.

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

Effect of temperature on enzyme activity. 0.4 ml aliquots of an enzyme solution diluted to 150 U/ml activity, were heated for 30 minutes at temperatures varying between 10 °C – 60 °C with 5 °C intervals. Activities were determined after bringing to room temperature.

Effect of pH on enzyme activity. The activity of AAT was examined between pH 5-9. 0.1 M K-K phosphate buffer was used between pH 5-7 and 0.1 M Tris-HCl buffer for pH 7.2-9. The buffer solution of desired pH (3 ml) were mixed with 1 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.

K_m values. The K_m values for two substrates L-aspartate and 2-oxoglutarate were determined at 6 different concentrations of both substrates. The reaction mixtures were the same as used in the standart 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 K_m values were determined by linear regression according to Lineweaver and Burk.

RESULTS

Both total and specific activity of alanine aminotransferase were found lesser than aspartate aminotransferase activity in all of the three mussel tissues examined (Table 1).

Table 1: Protein contents, aspartate and alanine aminotransferase activities in extracts of whole tissue, mantle tissue and hepatopancreas of *Mytilus galloprovincialis* Lam.

			Aspartate Aminotransferase		Alanine aminotransferase	
	Volume (ml)	Total Protein (mg)	Total U	U/mg	Total U	U/mg
Whole tissue (23 mussels)	360	6048	1477800	244.3	113400	18.8
Mantle (10 mussels)	100	377	157900	446.7	31500	83.6
Hepatopankreas (10 mussels)	100	413	168400	382.3	21000	50.9

Hydroxylapatite column chromatography of mussel hepatopancreas aspartate aminotransferase 35%-65% ammonium sulphate cut, gave a single peak of AAT activity eluted with 100 mM phosphate buffer (Fig. 1).

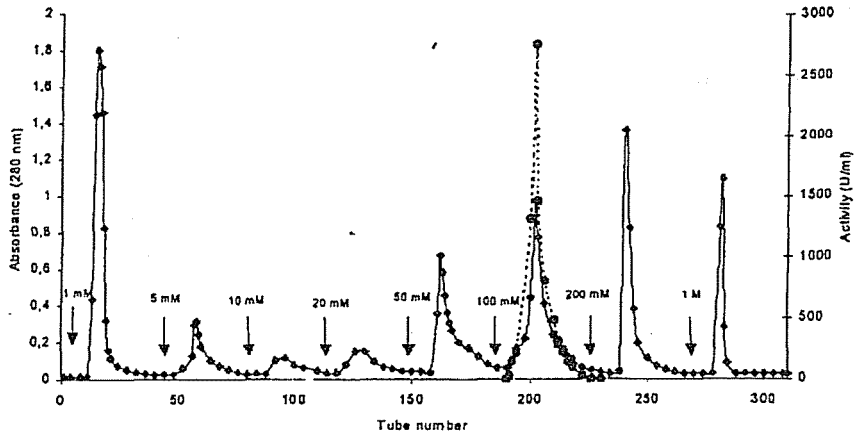

Figure 1: Hydroxylapatite column chromatography elution profile of *Mytilus galloprovincialis* Lam. hepatopancreas 35%-65% ammonium sulphate cut.
Column dimensions: 3 x 20 cm.
Applicate: 612 mg protein
Flow rate: 48 ml/h
Protein (–◆–); enzyme activity (---●---).

The results of partial purification of aspartate aminotransferase from *Mytilus galloprovincialis* Lam. hepatopancreas are summarized in Table 2.

Table 2: Purification of aspartate aminotransferase from the hepatopancreas of the mussel *Mytilus galloprovincialis* Lam.
(All data refer to 100 g hepatopancreas tissue obtained from 43 mussels)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification factor
I. Crude extract	9198	190 000	20.7	100	1
II. 35%-65% Ammonium sulphate cut	1687	488 000	289.3	256.8	14
III. Hydroxylapatite column chromatography 100 mM K- phosphate buffer eluate	262	450 000	1718	236.8	83

Polyacrylamide gel electrophoresis (PAGE) of the samples obtained during the three purification steps are shown in Fig. 2. The crude extract exhibited 5 protein bands but no activity band. This can be explained by the low total AAT activity of the crude extract in comparison with 35%-65% ammonium sulphate cut (Table 2). The single fraction eluted from hydroxylapatite column indicated two activity bands when stained with Fast violet B for AAT activity (15) and 4 protein bands two of which correspond to the activity bands (Fig 2).

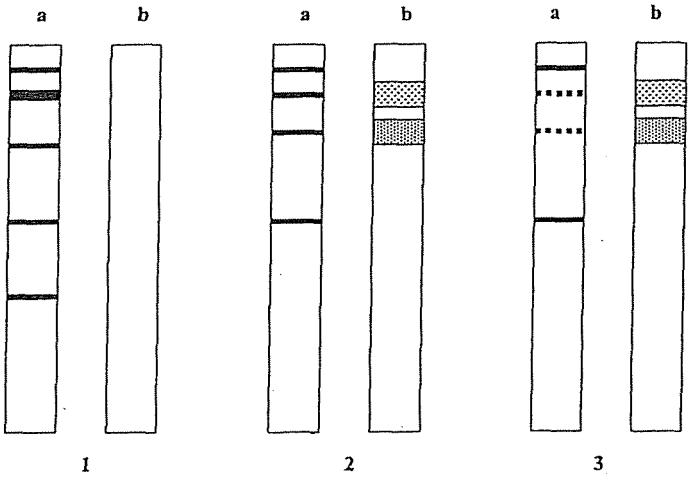

Figure 2: Schematic presentation of the native PAGE of the different fractions obtained during AAT purification.
1. Crude extract. 2. 35%-65% ammonium sulphate cut. 3. Hydroxylapatite 100 mM eluate. (a: stained in blue for protein with Amidoblack 10B, b: stained in pink for AAT activity with Fast violet B).

Aspartate aminotransferase ehibited maximum activity at 25 °C and 40 °C, the activity decreased below room temperature (25 °C) as well as at 30-35 °C and showed a slight activation at 40 °C. Inactivation began beyond 50 °C and activity was totally lost at 60 °C (Table 3).

Table 3: Effect of temperature on the activity of *Mytilus galloprovincialis* Lam. hepatopancreas aspartate aminotransferase.

t °C	Control	10	15	20	25	30	35	40	45	50	55	60
%Activity	100	74.3	85.7	87	100	87.6	74.3	100.9	72	44.4	7.5	0

Mussel hepatopancreas aspartate aminotransferase showed maximum activity at pH 7.6 (Fig. 3).

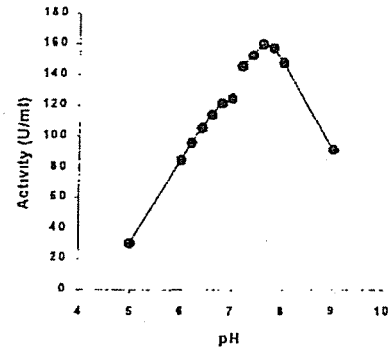

Figure 3: Effect of pH on the activity of *Mytilus galloprovincialis* Lam. hepatopancreas aspartate aminotransferase.

The substrate kinetics of mussel hepatopancreas AAT for two different substrates are presented in Table 4.

Table 4: Apparent Michaelis constants of *Mytilus galloprovincialis* Lam. hepatopancreas aspartate aminotransferase against two substrates L-aspartate and 2-oxoglutarate.

	Km (mM)	Vmax (U/ml)
L- Aspartate	1.7	0.162
2- Oxoglutarate	6.6×10^{-2}	0.149

DISCUSSION

Aspartate and alanine aminotransferase ratios in *M. galloprovincialis* was used to determine the pollution of the sea water (7,17). This pollution was as well understood by the determination of trace elements and antioxydant enzymes (18), heavy metals and stability of lysosomal membranes in the gastrointestinal tract (19) or cholinesterase activity (20). But to our knowledge, neither of these two enzymes were purified and characterized from the mussel.

In our study, the whole tissue as well as mantle and hepatopancreas tissues of the mussel were analysed for alanine and aspartate aminotransferase activities and aspartate aminotransferase activity was higher than alanine aminotransferase in all of the three tissues (Table 1). For this reason this prevalent enzyme was purified and characterized from the hepatopancreas of the mussel. On the other hand, it was reported that the difference in AAT/ALAT ratio in mussels collected from Sivastopol coasts could be an indicator of water pollution (17). The fact that in our study this ratio is higher than 1 in all tissues studied, can prove once again the high pollution of the Bosphorus.

Aspartate aminotransferase was purified in three steps and 83 fold purification was achieved (Table 2). The fact that enzyme activity was very low in the crude extract (no activity bands could be detected on PAGE) leads to consider that the mussel contains an inhibitor which was eliminated in the other steps during the purification process.

The two activity bands seen after PAGE, demonstrates that aspartate aminotransferase, like in other plant (21-24) or animal (1,25) sources, exists in two isoenzymic forms in the mussel.

My*tilus galloprovincialis* Lam. hepatopancreas aspartate aminotransferase has many properties similar to the corresponding enzyme isolated from animal and plants. Literature reports plant enzymes to be resistant to temperatures up to 40-55 °C (26-28) and animal enzymes to 75 °C (5,29). In our study, in accordance with literature, aspartate aminotransferase showed maximum activity at 25 °C and 40 °C and losts it's activity at 60 °C (Table 3).The reason for two maximum activities can be the occurence of the cytosolic enzyme as two isoenzymes like cited in the literature (25). The fact that mussel AAT showed maximum activity at 25 °C, agrees also with the temperature correction factor taken as 1 at this temperature, in the widely used enzyme assay described by Frankel and Reitman (30).

Goromosova and Tamozhnyaya reported the optimum pH's of *Mytilus galloprovincialis* species in the cytoplasmic fraction to vary over a wide range, from pH 7.5 to pH 9 (31). In another study made on dolphin muscle, optimum pH's of 7.3-8.3 and 6.3-7.3 were reported for supernatant (anionic) and mitochondrial (cationic) aspartate aminotransferases respectively (32). As our enzyme was cytosolic, optimum pH of 7.6 (Fig 3) agrees with the values found for other cytosolic aspartate aminotransferases. Since we found only one peak of optimum pH, we can assume that both subunits of the cytosolic enzyme have the same optimum pH. The apparent Michaelis constants of the mussel AAT determined from the Lineweaver-Burk plots (Table 4) are in agreement with data from other aspartate aminotransferases from animal (5,33-35) and plant (21,23,26,27,31) sources. Since our enzyme was also cytosolic, the K_m values agree also with K_m values of cytosolic enzyme given by literature.

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