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LENTIL SEED ASPARTATE AMINOTRANSFERASE ISOENZYMES I. ISOLATION and PARTIAL PURIFICATION

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SUMMARY

Three electrophoretically distinct aspartate aminotransferase isoenzymes named AAT-l, AAT-2and AAT-3, were separated from lentil (*Lens culinaris* Medik.) seeds by extraction, ammonium sulphate precipitation, hydroxylapatite and DEAE-cellulose column chromatographies. AAT-1 was purified 228, AAT-2, 42 and AAT-3, 3.8 fold. The isoenzymes were examined by means of polyacrylamide gel electrophoresis.

ÖZET

Elektroforetik bakımdan farklı üç aspartat aminotransferaz izoenzimi yeşil mercimek (*Lens culinaris* Medik.) tohumlarından ekstraksiyon , amonyum sülfatla çöktürme, hidroksilapatit ve DEAE-selüloz sütun kromatografisi yöntemleri kullanılarak ayrıldı. AAT-1, AAT-2 ve AAT-3 olarak adlandırılan bu izoenzimler, sırasıyla 228, 42 ve 3.8 kez saflaştırıldı. İzoenzimler poliakrilamid jel elektroforezi ile incelendi.

Key words: Lentil, *Lens culinaris* Medik., aspartate aminotransferase, isoenzymes

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INTRODUCTION

Owing to its key role in general metabolism, aspartate aminotransferase (AAT, E.C. 2.6.1.1.) which was formerly called glutamic oxaloacetic transaminase (GOT), occurs widely in nature. In animals, cytosolic and mitochondrial forms of AAT have been characterized and purified (1-4). AAT has also been studied in various plants (5, 6) and different isoenzymic forms have been reported. The enzyme has been extensively characterized in wheat germ (7,8), cauliflower (9), cotton seeds (10), pea (11), soybean (12-14), oat (15), lupine (16) and alfalfa (17, 18).

This paper reports the occurence of three aspartate aminotransferase isoenzymes in lentil (*Lens culinaris* Medik.) seeds, their isolation and partial purification.

RESULTS

Hydroxylapatite column chromatography gave a single peak of AAT activity eluted with 50 mM phosphate buffer (Fig. 1). This single fraction was applied to DEAE-cellulose column and three AAT activity peaks were eluted with 50, 100 and 200 mM NaCl respectively. Aspartate aminotransferase fraction eluted with 50 mM NaCl was called AAT-1, AAT fraction eluted with 100 mM NaCl, AAT-2 and the one eluted with 200 mM NaCl, AAT-3 (Fig.2). The rechromatography of AAT-2 on DEAE-cellulose column resulted in a single peak of AAT activity eluted with 50 mM NaCl and named AAT-2a.

Polyacrylamide gel electrophoresis (PAGE) of the different fractions obtained during purification steps are shown in Fig.3. The single fraction eluted from hydroxylapatite column indicated three AAT activity bands and several protein bands (lane 1). Among the fractions eluted from DEAE-cellulose, AAT-1 showed one activity and two protein bands (lane 2), whereas AAT-2 showed three activity and several protein bands (lane 3). On the other hand AAT-3 showed one major activity band corresponding to the major protein band and minor contamination with AAT-1 (lane 4). AAT-2a obtained from rechromatography of AAT-2 on DEAE- cellulose exhibited one main protein band and two activity bands (lane 5).

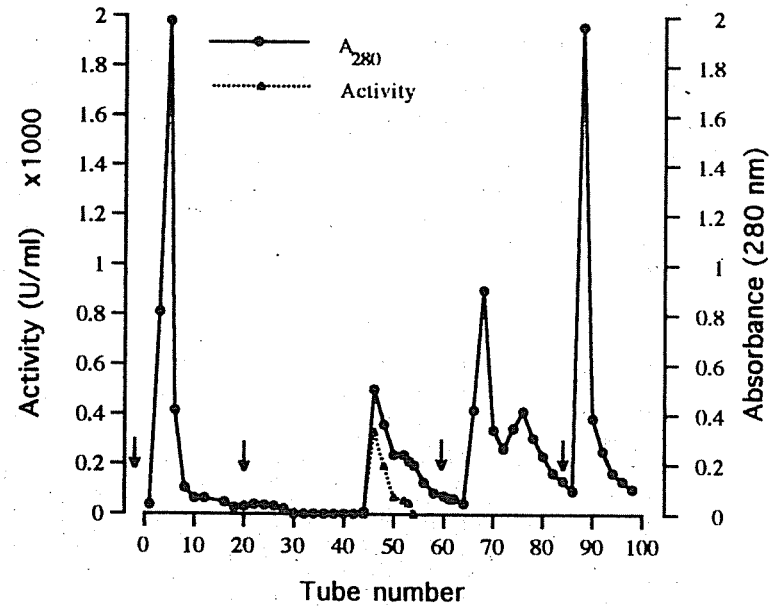

Fig. 1. Hydroxylapatite column chromatography elution profile of lentil seed crude extract 25%-45 % ammonium sulphate cut. Arrows indicate stepwise application of 1, 50, 100 and 200 mM phosphate buffer.

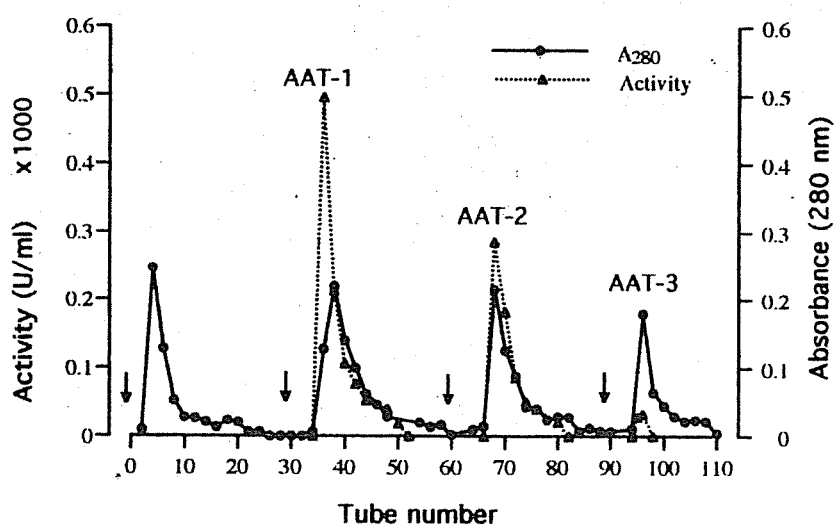

Fig. 2. DEAE- cellulose column chromatography elution profile of lentil seed aspartate amino-transferase active fraction recovered from hydroxylapatite column. Arrows indicate step wise application of 5 mM phosphate buffer of increasing NaCl concentrations (0, 50, 100 and 200 mM). No activity observed at 500 mM NaCl concentration, so the profile is not indicated on the diagram.

The results of partial purification of aspartate aminotransferase isoenzymes from lentil seeds are summarized on Table 1. At the end of this study, AAT-1, AAT-2 and AAT-3 were purified 228, 42 and 3.8 fold respectively. Further chromatography of AAT-2 on DEAE-cellulose resulted in a nearly homogeneous protein fraction but with very low yield and decrease in specific activity.

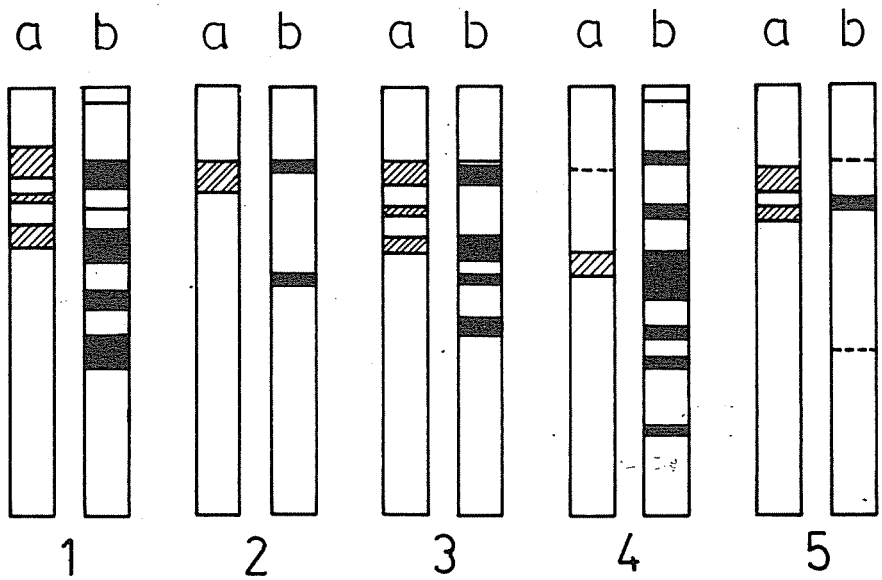

Fig. 3. Schematical presentation of the native PAGE of the different fractions obtained during the course of aspartate aminotransferase purification. Active fraction after hydroxylapatite (lane 1). AAT-1, AAT-2 and AAT-3 after DEAE-cellulose (lanes 2, 3 and 4 respectively), AAT-2a, active fraction after rechromatography of AAT-2 on DEAE-cellulose (lane 5). Lanes a stained for activities and lanes b stained for proteins.

DISCUSSION

Aspartate aminotransferases were purified from some legume root nodules like soybean (12), lupine (16) and alfalfa (17) or seeds (10, 13), but to our knowledge, the occurence and purification of this enzyme in lentil (*Lens culinaris* Medik.) seeds was not achieved to date. In this paper we demonstrate that aspartate aminotransferase exists in three forms in the lentil seeds. These three forms (isoenzymes) were separated by ion exchange chromatography on DEAE-cellulose and named AAT-1, AAT-2 and AAT-3 in order of elution with increa-

Table 1. Purification of aspartate aminotransferase isoenzymes from lentil (*Lens culinaris* Medik.) seeds. All data refer to 100g of lentil meal.

Purification step	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification factor	Recovery (%)
I. Crude extract	1 428 395	5550	257.3	1	100
II. $(NH_4)_2SO_4$ precipitation					
25%-45% cut	363 227	1250	290.6	1.13	25.4
III. Column chromatography					
Hydroxylapatite					
AAT active fraction	239 238	102	2345	9.1	16.7
DEAE-cellulose					
AAT-1 fraction	34 883	3.3	10570	228*	2.44
AAT-2 fraction	47 067	8.9	5288	42*	3.29
AAT-3 fraction	1 999	6.1	327.7	3.8*	0.14
DEAE-cellulose of AAT-2					
AAT-2a fraction	4 041	1.5	2694	21*	0.28

* Purification factors were calculated associated with protein quantities, assuming AAT-1 concentration 18 %, AAT-2 concentration 49 % and AAT-3 33 % crude extract.

sing salt concentration (Fig.2). AAT-2 was the major form of aspartate aminotransferase activity, representing approximately 56 % of the total activity, with AAT-1 and AAT-3, 42 % and 2 % respectively. AAT-1 showed only one activity band and two protein bands on PAGE (Fig. 3, lane 2) one of which corresponded with the activity band and this isoenzyme was not further purified. The fact that AAT-2 exhibited three activity bands on PAGE (Fig. 3, lane 3), leads to consider contamination with AAT-1 and AAT-3. On the other hand, minor contamination of AAT-3 with AAT-1 is also observed (Fig.3, lane 4) but additional purification of this fraction was not undertaken because of its low yield and low activity. Further purification of AAT-2 on DEAE-cellulose, giving a nearly homogenous protein, still showed contamination with AAT-1 as judged by the activity stain on PAGE (Fig. 3, lane 5b). The activity decrease in AAT-2a after rechromatography on DEAE-cellulose may be due to inactivation during chromatography process. Although aspartate aminotransferase occurs generally in two forms in plants (11, 13, 15, 17), in accordance with our results, three forms of the enzyme was also observed in cotton seeds (10) and carrot cells (19), whereas 4 areas of AAT activity were seen in soybean root nodules (12).

MATERIALS and METHODS

Chemicals. β-Nicotinamide adenine dinucleotide disodium salt reduced (NADH, Fluka 43420), malate dehydrogenase (1500 U/mg, Serva 28338), pyridoxal-5'-phosphate (Fluka 82870), L-aspartic acid (Merck 129), 2-oxoglutaric acid (Merck 5194) were used in enzyme assays. In column chromatographies, hydroxylapatite prepared in our laboratory and diethylaminoethyl cellulose (DEAE-cellulose, Servacel 45059) were used. Immersible CX-10 ultrafilters were purchased from Millipore and Visking dialysis tubing (27/32, 24 Å) from Serva.

All other chemicals were of analytical reagent grade.

Protein determination. Protein contents of the samples obtained during the purification processes were determined by measuring the absorbance at 260/280 nm (20) after appropriate dilution of the samples with 5 mM phosphate buffer, pH 7.

Enzyme assay. The routine assay to measure AAT activity involved a nicotinamide adenine dinucleotide (NAD)-dependent malate dehydrogenase-linked reaction according to Karmen (21) and Rej et al. (22). This reaction was

in the direction of aspartate utilization and oxaloacetate produced was converted to malate by malate dehydrogenase. The oxidation of $NADH_2$ was monitored spectrophotometrically at 339 nm using a Shimadzu UV-1208 spectrophotometer. The assay was performed as follows: to a spectrophotometer cuvette 2.5 ml of a mixture containing 54 mM L-aspartate, 12 Units malate dehydrogenase, 0.06 mM $NADH_2$ Na salt, 0.033 mM pyridoxal phosphate and 113 mM trishydroxymethyl aminomethane (Tris) was transferred, 0.25 ml of the enzyme sample was then added. Content of the cuvette was mixed with a thin glass rod and the reaction was initiated by the introduction of 0.25 ml of the solution containing 45 mM 2-oxoglutarate and 125 mM Tris with pH 7.8. The absorbance decrease (ΔA) was recorded every 30 seconds during 5 minutes at 339 nm, final reaction temperature was recorded and "temperature correction factor" (23) was used for the activity calculations. One unit of aspartate aminotransferase activity (Karmen Unit) was expressed as absorbance decrease of 0.001 per ml of the sample per minute at 339 nm of 1 cm light path (21).

Polyacrylamide gel electrophoresis (PAGE). Non-denaturing PAGE was performed on a Pleuger electrophoresis apparatus with the standart Tris-glycine system. 10 fold diluted solution of Tris-glycine buffer (3 % Tris and 14.4 % glycine) of pH 8.6 was used during electrophoresis. The electrophoresis was performed in duplicates in 7.5 % polyacrylamide gels of 6x0.6 cm rods. Protein concentration was 200µg protein/gel. Electrophoresis was carried out initially at 120 V and 5 mA/gel for 30 min. then at 220 V at the same current for 10 min. One of the gels was stained with Amidoblack 10 B and the other with Fast Violet B (24) for the detection of protein bands and aspartate aminotransferase activity zones respectively.

Plant material. Lentil seeds were purchased in the local market and ground to fine powder in a Janke and Kunkel K.G. Type A 10 mill and named as lentil seed meal.

Enzyme purification. Aspartate aminotransferase isoenzymes were purified from lentil seed meal in 5 steps:

1. Isolation. 100 g of lentil seed meal was homogenized for 5 min. in 400 ml of 50 mM phosphate buffer, pH 7, in crushed ice using a Bosch mechanical mixer. The homogenate was centrifuged at 20 000 rpm (45 700x g) for 30 min. in a refrigerated centrifuge (Cryofuge 20-3 Hereaus-Christ) and the supernatant (crude extract =homogenate supernatant) was selected.

Purification steps following the centrifugation were accomplished at 4°C.

2. Ammonium sulphate fractionation. To the crude extract ammonium sulphate was added to 25 % saturation and left overnight in the refrigerator. The precipitate so formed was separated by means of centrifugation at 20 000 rpm for 30 min. and discarded. The supernatant (initial 25% saturation) was brought to 45% saturation by adding $(NH_4)_2SO_4$ crystals and left again overnight at 4°C. The precipitate formed (25%-45% cut) was obtained by centrifugation at 20 000 rpm for 30 min. suspended in 30 ml of 1 mM phosphate buffer of pH 7 and dialysed against the same buffer. Any precipitate formed in the dialysis bag was discarded by centrifugation and the clear supernatant was used for further purification.

3. Hydroxylapatite column chromatography. Hydroxylapatite was prepared in our laboratory according to Tiselius et al. (25). The clear dialysate was applied to hydroxylapatite column of 20x2.5 cm. The elution was performed by washing the column stepwise with 1, 50, 100 and 200 mM phosphate buffers (pH 7). The fraction showing AAT activity was pooled, concentrated either by ultrafiltration through Millipore CX-10 membrane or by placing the solution, filled in a dialysis bag, in sugar and subsequently dialysed against 1 mM phosphate buffer.

4. DEAE-cellulose column chromatography. The pooled fraction with AAT activity, obtained from elution through hydroxylapatite column chromatography, following concentration and dialysis, was applied to DEAE-cellulose column (15x2 cm). Stepwise gradient was performed initially with 5mM phosphate buffer, then 5 mM phosphate buffer containing 50, 100, 200 and 500 mM NaCl. The active portions from each peak of aspartate aminotranferase activity were pooled separately, concentrated, dialysed against 1 mM phosphate buffer and stored at-20°C. The purity of the pooled enzyme fractions was examined by PAGE.

AAT-2 was rechromatographed on DEAE-cellulose, to obtain further purification . Stepwise gradient elution was achieved by using 5 mM phosphate buffer having 50, 100, 150 and 200 mM NaCl concentration. The fraction with AAT activity was pooled and concentrated.

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