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ISOLATION AND PURIFICATION OF TWO ASPARTATE AMINOTRANSFERASE ISOENZYMES FROM SOYBEAN SEEDS *

SOYA FASULYESİ TOHUMLARINDAN İKİ ASPARTAT AMİNOTRANSFERAZ İZOENZİMİNİN ELDE EDİLMESİ VE SAFLAŞTIRILMASI *

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Bu çalışmada, soya fasulyesi tohumlarından aspartat aminotransferazın elektroforetik bakımdan farklı iki şekli, AAT_1 ve AAT_2 olarak adlandırılan iki izoenzimi, elde edildi ve sırasıyla 91 ve 16 kez saflaştırıldı. AAT_1, poliakrilamid jel elektroforezinde geniş bir aktivite bandına tekabül eden yerde üç protein bandı gösterdiği halde AAT_2, dar bir aktivite bandına tekabül eden yerde birbirine çok yakın iki protein bandı gösterdi. AAT_1'in üç protein bandı birbirinden birer birer ayrıldığı zaman gerek ayrılan fraksiyonlarda gerek geri kalanlarda aktivite görülmemesi, bu üç protein fraksiyonunun oluşturduğu konformasyonun bozulmuş olmasıyla açıklandı. AAT_2'nin protein bandları birbirine çok yakın olduğu için bu fraksiyonları kesin olarak ayırmak mümkün olmadı.

At least two isoenzyme forms, called cytoplasmic (supernatant, anionic) and mitochondrial (cationic) aspartate aminotransferases (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1.) indispensable in transamination reactions of the animal organism, are known to exist in several animal tissues (1-5). Besides, purification of aminotransferases from plant sources have recently become important (6-10). Although it has been reported that aminotransferase activity occurs in leaves, stems and roots of soybean (11) and at least four forms of the enzyme from the root nodules of the plant have been purified (12), there is no record in literature about the isolation and purification of aspartate aminotransferase from the seeds of soybean. In the present study two aspartate aminotransferase isoenzymes from soybean seeds are isolated and purified.

MATERIAL and METHOD

Chemicals. Hydroxylapatite, prepared in our laboratory according to Levin (13), and diethylaminoethyl cellulose (DEAE-cellulose, Fluka 31807) were used for column chromatography. All the other chemicals were of analytical reagent

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grade. Protein solutions were concentrated by means of Millipore-CX ultrafiltration membrane.

Protein determination. Protein contents of the samples obtained during the purification processes were determined by $E_{280/260}$ method (14) after the samples were diluted with 50 mM phosphate buffer of pH 7 in appropriate proportions.

Enzyme activity tests. Activity experiments of aspartate aminotransferase were carried out according to Karmen (15) and Rej et al. (16). To the spectrophotometer cuvettes of 3 ml capacity was initially added 2.5 ml of the solution of pH 7.8 containing 54 mM L-aspartate, 12 U malate dehydrogenase (MDH), 0.06 mM nicotinamide adenine dinucleotide ($NADH_2$) Na salt, 0.033 mM pyridoxal phosphate and 113 mM tris hydroxymethyl aminomethane (Tris), then 0.25 ml of the enzyme solution. Content of the cuvette was mixed with a thin glass rod and was left for 5 minutes for the constancy of absorbance, then the reaction was initiated by the inclusion of 0.25 ml of the solution containing 45 mM 2-oxoglutarate and 125 mM Tris with pH 7.8. The absorbance decrease was recorded at every 30 seconds during 5 minutes at 339 nm and finally the temperature of the mixture was measured. Whenever the temperature deviated from 25°C, «temperature correction factor» was used (17) for activity calculations. 104 mM Tris solution was utilized as blank. The enzyme activity was expressed as absorbance decrease of 0.001 per ml of the sample per minute at 339 nm of 1 cm light slit.

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis was employed following each purification process, 10 fold diluted solution of Tris-glycine buffer (3% Tris and 14.4 % glycine) of pH 8.6 was used during electrophoresis. Each time, two applications were made from the same sample to 7.5 % polyacrylamide gels of 6×0.6 cm dimensions so that protein concentration was 200 μg protein/gel Electrophoresis was performed initially at 120 V and 5mA/gel for 30 minutes, then at 220 V at the same current for 10 min.. Completion to electrophoresis, one of the gels was stained with Coomassie brilliant blue and the other with Fast violet B according to Schwartz et al. (18) for the detection of protein bands and aspartate aminotransferase activity zones respectively.

Isolation of aspartate aminotransferase from soybean seeds. Soybean *(Glycine max.)* seeds were acquired from Adana (TURKEY). The seeds were ground to fine powder and aspartate aminotransferases (AAT_1 and AAT_2) were purified from the meal in 6 steps:

1. **Extraction.** After the homogenization of 100 g soybean seed meal in 400 ml of 50 mM phosphate buffer, pH 7, for 5 min. in cold, using a mechanical mixer, the homogenate was centrifuged at 20.000 rpm for 30 min. in a refrigerated centrifuge and supernatant (crude extract) was selected.

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

2. **Ammonium sulphate precipitation.** The supernatant was saturated to 0.25 with $(NH_4)_2SO_4$ crystals 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 0.25 saturation) was saturated this time to 0.45 by adding $(NH_4)_2SO_4$ crystals. The precipitate (0.45 saturation), produced overnight in the refrigerator, was obtained by centrifugation

at 20.000 rpm for 30 min., dissolved in 1 mM phosphate buffer of pH 7 and dialysed against the same buffer.

3. **Hydroxylapatite column chromatography.** The dialysate was applied to the hydroxylapatite column, the elution was performed by washing the column with 5 mM, 50 mM and 200 mM phosphate buffers (pH 7) respectively, each buffer volume was three times the column volume. The fractions of the highest enzyme activity were pooled and concentrated.

4. **DEAE-cellulose column chromatography.** The fraction obtained from elution through hydroxylapatite column was applied to DEAE-cellulose column. Stepwise gradient was performed with 5 mM phosphate buffer containing 100 mM and 200 mM NaCl, which resulted in two enzyme active peaks. The purity of the enzyme fractions were examined by PAGE.

5. **DEAE-cellulose column chromatography of AAT_2 fraction.** AAT_2 fraction, obtained from DEAE-cellulose column (step 4) was eluted secondarily through DEAE-cellulose column under different conditions because of detection of some AAT_1 fraction existing during the purity control by means of PAGE. Stepwise gradient elution was achieved by using 5 mM phosphate buffer of 50 mM, 100 mM, 150 mM and 200 mM NaCl concentration. The enzyme active elute was pooled and concentrated.

6. **Purification of AAT_1 and AAT_2 by preparative PAGE.** Purity controls of AAT_1 fraction from DEAE-cellulose column chromatography (step 4) and AAT_2 fraction from secondary DEAE-column chromatography (step 5) by means of analytical PAGE displayed the presence of many protein bands, lacking enzymatic activity. Thereafter, preparative PAGE was applied to AAT_1 and AAT_2 for purification. The experimental details were the same as those described above. Completion to electrophoresis the enzyme active bands were cut and the pure isoenzymes were eluted with 50 mM phosphate buffer, pH 7.

RESULTS

Elution with 50 mM phosphate buffer through hydroxylapatite column resulted in a single enzyme active peak, polyacrylamide gel electrophoresis of that fraction indicated two aspartate aminotransferase active bands and several protein bands. The single fraction from hydroxylapatite column was applied to DEAE-cellulose column and one aspartate aminotransferase active peak was observed at each of the stepwise elution with two different NaCl molarities. Aspartate aminotransferase fraction eluted with 100 mM NaCl was called aspartate aminotransferase 1 (AAT_1) and aspartate aminotransferase eluted with 200 mM NaCl aspartate aminotransferase 2 (AAT_2), (Fig. 1).

It was observed that each of AAT_1 and AAT_2 showed single activity band in polyacrylamide gel electrophoresis, but little AAT_1 activity existed in AAT_2. Therefore AAT_2 was eluted through DEAE-cellulose column for the second time under different conditions, a single and homogeneous peak was obtained with 150 mM NaCl elution. As a result of polyacrylamide gel electrophoresis of this fraction, it was seen that AAT_2 was purified from

AAT_1, but it still contained several inactive protein bands besides one activity band.

After preparative gel electrophoresis of AAT_1 and AAT_2, then their application to polyacrylamide gel electrophoresis for purity control, AAT_1 displayed a single wide activity band corresponding to three protein bands, one strong and two weak, one of which was below and very close to the strong one (Fig. 2). Following slicing and elution of three enzyme active bands of AAT_1 from the gel, no activity was detected in any of the separated bands by repeated polyacrylamide gel electrophoresis. AAT_2, on the other hand, exhibited a single narrower activity band corresponding to two very close protein bands (Fig. 2), which could not be separated from one another.

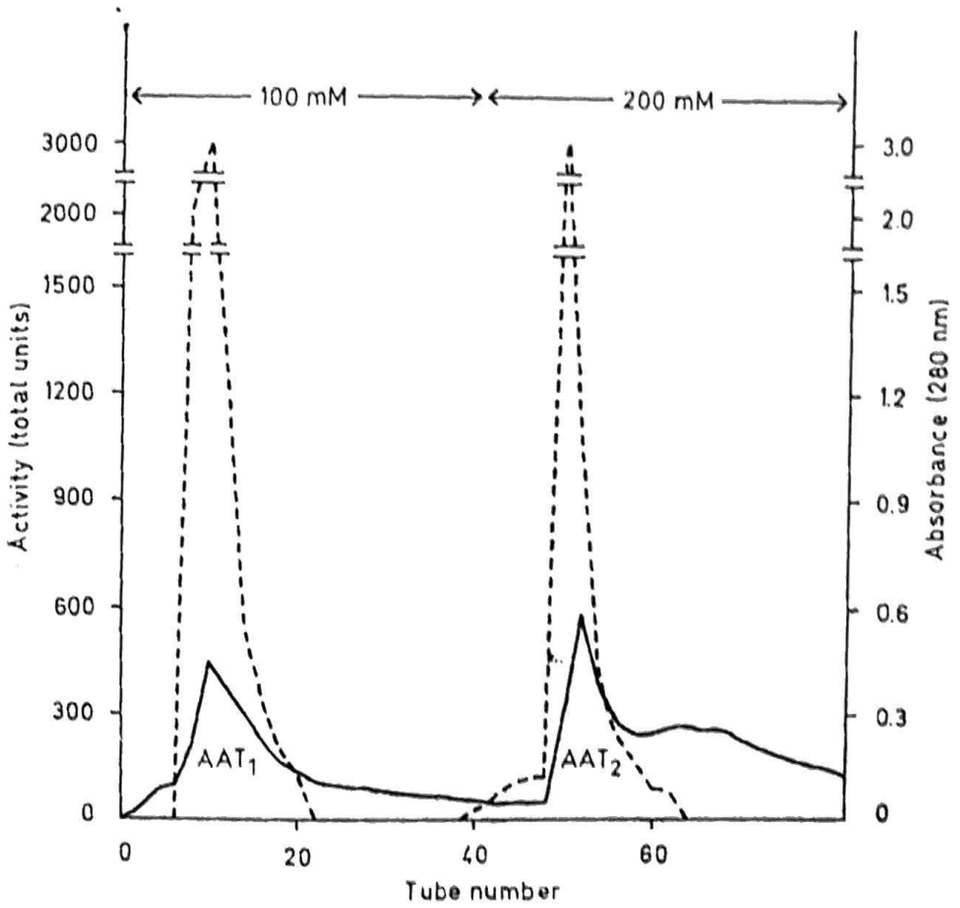


Fig. 1. Chromatography on DEAE-cellulose column of soybean seed aspartate aminotransferase active fraction recovered from hydroxylapatite column. Protein (————), aspartate aminotransferase activity (· · · · · · · · ·).

The results of purification of aspartate aminotransferase isoenzymes from soybean seeds are presented on Table 1. At the end of this study, it was calculated that AAT_1 was purified 91 and AAT_2 16 fold.

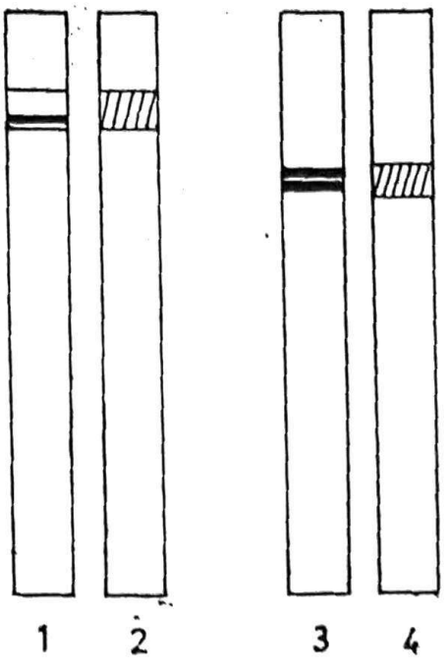


Fig. 2. Polyacrylamide gel electrophoresis of AAT_1 (1 and 2) and AAT_2 (3 and 4) fractions. Gel 1 and 3 stained for protein, gel 2 and 4 stained for activity.

Consequently, two aspartate aminotransferase isoenzymes, AAT_1 and AAT_2, were isolated purely for the first time from soybean seeds.

Table 1. Purification of aspartate aminotransferase isoenzymes from soybean seeds. (All data refer to 100 g of soybean meal)

Step and Fraction	Total activity (U)	Total protein (g)	Specific activity (U/mg)	Purification factor	Recovery (%)
I. Crude extract	1 731 500	13.44	128.8	1	100
II. $(NH_4)_2SO_4$ fraction					
Supernatant (0.25)	1 703 500	11.35	150.1	1.16	98
Precipitate (0.45)	742 400	3.18	233.4	1.81	43
III. Chromatography					
Hydroxylapatite	242 700	266×10^{-3}	912.4	7.08	14
DEAE-cellulose (first)					
AAT_1 fraction	44 350	18.4×10^{-3}	2410.3	53*	2.56
AAT_2 fraction	18 407	33.7×10^{-3}	546.2	6.55*	1.06
DEAE-cellulose (second)					
AAT_2 fraction	4 648	3.54×10^{-3}	1313	15.75*	0.27
IV. Preparative PAGE					
AAT_1 fraction	4 549	1.1×10^{-3}	4135.45	91*	0.26
AAT_2 fraction	659	0.51×10^{-3}	1292.15	15.5*	0.04

*) Purification factors were calculated associated with protein quantities, assuming AAT_1 concentration 35.3 % and AAT_2 64.7 % in crude extract.

Discussion

In the present study detection of two aspartate aminotransferase active bands in consequence of polyacrylamide gel electrophoresis application to the fraction obtained from hydroxylapatite column leads to consider the presence of two isoenzyme forms of the enzyme in soybean seeds. Likewise, isoenzymes of aspartate aminotransferase have been isolated from various animal and plant tissues. Among them are the two aspartate aminotransferases purified from chicken heart or liver with different amino acid contents (5), two and four forms of aspartate aminotransferase respectively from lupine and soybean root nodules (10, 12).

Since it has been observed that three and two protein bands exist after the respective application of polyacrylamide gel electrophoresis to the active bands of AAT_1 and AAT_2 obtained from preparative polyacrylamide gel electrophoresis, it was considered that the isoenzymes AAT_1 and AAT_2 comprise three and two protein fractions respectively. The lack of activity in AAT_1, when one of the protein bands has been separated is attributed to the fact that the activity of the isoenzyme is closely related to the conformation formed by three protein fractions.

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