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LENTIL SEEDS ASPARTATE AMINOTRANSFERASE ISOENZYMES II. CHARACTERIZATION

MERCİMEK TOHUMLARI ASPARTAT AMİNOTRANSFERAZ İZOENZİMLERİ II. ÖZELLİKLERİNİN İNCELENMESİ

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Yeşil mercimek (*Lens culinaris* Medik.) tohumlarından elde edilen aspartat aminotransferaz izoenzimlerinin kinetik özellikleri ve bu izoenzimlere bazı inhibitörlerin ve iki değerli bazı katyonların etkisi incelendi. AAT-1, AAT-2 ve AAT-3 olarak adlandırılan izoenzimlerin L-aspartata karşı K_m'lerinin sırasıyla 1.79, 1.58 ve 3.39 mM, 2-oksoglutarata karşı ise her üç izoenzimin K_m'lerinin 0.12 mM olduğu saptandı. AAT-1 ve AAT-2'nin 45°C'ye kadar ısıya dayanıklı oldukları, aktivitelerinin 50°C'den itibaren azalmaya başladığı, optimum pH'nın ise AAT-1 için 7.2, AAT-2 için 6.4-6.8 arasında olduğu bulundu. İzoenzimlerin aktivitesi üzerine etkisi araştırılan tüm maddelerin AAT-1 ve AAT-2'yi çeşitli oranlarda inhibe ettikleri, iki değerli katyonların ise bu izoenzimlerin aktivitesinde herhangi bir değişiklik meydana getirmedikleri gözlemlendi.

Introduction

Studies on aspartate aminotransferases (E.C. 2.6.1.1.) from various plant sources have become important in the last years. As many as five different isoenzymic forms have been reported and the different isoenzymes have been characterized in several plants (1-11).

The present paper is concerned with the kinetic parameters as well as the effect of some inhibitor substances and divalent cations on the activity of aspartate aminotransferase isoenzymes isolated previously in our laboratory from lentil (*Lens culinaris* Medik.) seeds (12).

Materials and Methods

Apparatus

Shimadzu UV-1208 spectrophotometer was used for the measurement of enzyme activity. pH determinations were carried out with Metrohm E-488 pH-meter and heating experiments in a Braun Thermomix BU water bath.

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 enzymatic activity assays. Potassium cyanide (KCN, Merck 4965), iodoacetamide (Fluka 57670), N-ethylmaleimide (Merck 1308), maleic acid (Fluka 63180), D-cycloserine (Fluka 30020) and hydroxylamine HCL (Fluka 55460) were used as inhibitors. All other chemicals were pure reagent grade.

Enzyme preparations

Three separate fractions of aspartate aminotransferase activity were previously separated from lentil seed extract by DEAE-cellulose column chromatography with stepwise NaCl gradient (12). These 3 fractions were separately pooled and named AAT-1, AAT-2 and AAT-3. Because of the very low quantity and poor yield of AAT-3, estimated to account for only 2% of the total activity (12), only Michaelis constants were determined for this isoenzyme. Enzyme preparations were appropriately diluted before each determination.

Standart enzyme assay

Aspartate aminotransferase activity was determined at 25°C by the method of Karmen (13) and Rej et al. (14) as described previously (12). 3 ml reaction mixture contained 45 mM L-aspartate, 0.75 Units malate dehydrogenase, 0.05 mM NADH, 0.0275 mM pyridoxal-5'-phosphate, 3.75 mM 2-oxoglutarate and 104 mM tris(hydroxymethyl) aminomethane (Tris) with a final pH of 7.8. The reactions were started by addition of 2-oxoglutarate. The decrease of absorbance was monitored spectrophotometrically at 339 nm. One enzyme unit is defined as the absorbance decrease of 0.001 per ml of sample per minute.

K_m values

The K_m values for the two substrates L-aspartate and 2-oxoglutarate were determined at 6 different concentrations of both substrates for AAT-1, AAT-2 and AAT-3 respectively. The reaction mixtures were the same as used in the standard enzyme 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. Reactions velocities were plotted on double reciprocal plots and K_m values were determined by linear regression according to Lineweaver and Burk (15).

Effect of temperature on enzyme activity

0.4 ml aliquots of enzyme solutions of AAT-1 and AAT-2 were heated for 30 minutes at temperatures varying from 20°C to 70°C with 5°C intervals. Activities were determined after bringing to room temperature.

Optimum pH studies

The pH optimum of lentil seed AAT-1 and AAT-2 were compared over the pH range 5.0-9.0 by using two overlapping buffer systems: potassium phosphate (0.1 M) and Tris-HCl (0.1 M) for pH 5.0-7.4 and 7.2-9.0 respectively. Final pH was adjusted with either concentrated HCl or NaOH.

Effect of some inhibitors on enzyme activity

The inhibitors used were KCN, iodoacetamide, N-ethylmaleimide, maleic acid, D-cycloserine and hydroxylamine. Inhibitor solutions were prepared in 5 mM phosphate buffer and final pH was adjusted to 7 with concentrated HCl or NaOH. 0.4 ml enzyme-inhibitor mixture was prepared by adding 0.1 ml inhibitor solution to 0.3 ml enzyme solution, in which the final concentration of the inhibitor was 1 mM for hydroxylamine and 10 mM for all other inhibitors tested. Control experiment contained 0.3 ml enzyme solution and 0.1 ml 5 mM phosphate buffer. The enzyme-inhibitor mixtures thus prepared were incubated at 30°C for 10 minutes, then enzme activity was assayed with 0.25 ml of the mixture. For hydroxylamine, the reaction was started with 0.25 ml of the enzyme-inhibitor mixture instead of 2-oxoglutarate to prevent binding of hydroxylamine to the coenzyme pyridoxal-5'-phosphate existing in the solution before the beginning of the reaction.

Influence of some divalent cations on enzyme activity

$MgCl_2$, $CaCl_2$, $MnCl_2$ and $ZnCl_2$ solutions were prepared in distilled water. 0.1 ml of these solutions was added to 0.3 ml enzyme solution so that final concentration of the cation in 0.4 ml was 1 mM. This enzyme-cation mixture was incubated at 30°C for 10 minutes. Enzyme activity experiment was performed with 0.25 ml of this solution. Control experiment contained 0.3 ml enzyme solution and 0.1 ml distilled water.

Results

The substrate kinetics of AAT-1, AAT-2 and AAT-3 for two different substrates are presented on Table 1. All three isoenzymes showed similar K_m values with respect to 2-oxoglutarate, whereas AAT-3 showed two fold lower affinity for aspartate than the two other isoenzymes.

AAT-1 and AAT-2 were resistant to temperatures up to 45°C with a slight activation at 30°C. Inactivation began beyond 50°C and activity was totally lost at 70°C.

The activities of AAT-1 and AAT-2 were affected by pH. AAT-1 displayed maximal activity at pH 7.2 in both phosphate and Tris-HCl buffers and the activity in phosphate buffer was higher than that in Tris - HCl buffer. In

Table 1. Apparent Michaelis constants of aspartate aminotransferase isoenzymes from lentil seeds. Each value represents the mean of three separate experiments.

Substrate	K_m(mM)		
	AAT-1	AAT-2	AAT-3
L-Aspartate	1.79	1.58	3.39
2-Oxoglutarate	0.12	0.12	0.12

contrast, AAT-2 exhibited lower activity in phosphate buffer than in Tris-HCl buffer with a maximum between pH 6.4-6.8.

A slight inhibition (6 to 27%) of both AAT-1 and AAT-2 were observed with all inhibitors tested. Only hydroxylamine, a well known inhibitor of pyridoxal dependent enzymes, gave a marked inhibition of 70% and 62% for AAT-1and AAT-2 respectively (Table 2).

Table 2. Effect of various inhibitors on the activity of aspartate aminotransferase isoenzymes from lentil seeds. All values are the means ± standart deviation (S.D.) of five different determinations.

Inhibitor	Conc. (mM)	% Activity	
		AAT-1	AAT-2
None (control)	-	100	100
KCN	10	72.9±14.6	87.5±6.9
Iodoacetamide	10	89.3±6.5	100.3±7.9*
N-Ethylmaleimide	10	83.5±8.2**	94.1±8.3*
Maleic acid	10	76.9±15.3	95.5±11.2*
D-Cycloserine	10	84.1±13.4	72.4±6.3
Hydroxylamine	1	30.0±7.9	37.9±4.9

* Stastistically insignificant values. All other values were statistically significant.

** Mean of 4 experiments

Studies indicated that magnesium, calcium, manganese and zinc did not significantly effect the activity of AAT-1 and AAT-2 (Table 3).

Table 3. Effect of some divalent cations on the activity of aspartate aminotransferase isoenzymes from lentil seeds. All values are the means ± S.D. of five different determinations.

Added cation	Conc. (mM)	% Activity*	
		AAT-1	AAT-2
None (control)	1	100	100
Mg^{2+}	1	101.3±11.1	97.2±8.6
Ca^{2+}	1	95.5±12.5	100.2±9.8
Mn^{2+}	1	97.5±6.7	106.3±6.8
Zn^{2+}	1	99.8±8.5	93.0±7.0

* All values were statistically insignificant.

Discussion

The present study indicates that aspartate aminotransferase isoenzymes from lentil seeds have many properties similar to the corresponding enzymes from animals and other plants.

The apparent Michaelis constants of AAT-1, AAT-2 and AAT-3 determined from the Lineweaver-Burk plots (Table 1) are in agreement with data from other aspartate aminotransferases from animal (16-20) and plant (4-6, 10, 21) tissues. It can be seen that all three isoenzymes showed greater affinity for 2-oxoglutarate than for L-aspartate. No significant discrepancies in the Michaelis constants could be determined among the three enzymes (Table 1), which suggest that the likely differences in conformation between them apparently do not lie in the region directly involved in the catalytic process.

Our findings that inactivation of AAT-1 and AAT-2 is proceeding above 50°C, is similar to those of Cruickshank et al. (1) for wheat germ aspartate aminotransferase, whereas soybean aspartate aminotransferase isoenzymes

purified previously in our laboratory were inactivated above 45°C (10).

Optimum pH of aspartate aminotransferases purifed from various plant sources were reported to vary over a broad range from pH 6.8 to 9.5 (1, 3, 4-7, 9, 22). In the present study, optimum pH of 7.2 for AAT-1 is comparable to those of aspartate aminotransferases from soybean root nodules (5) and oat leaves (6). The higher activity of AAT-2 in Tris-HCl buffer in comparison to phosphate buffer, as for the aspartate aminotransferase from oat leaves (6), human sera (23) and soybean seeds (10), can be explained by the inhibitory effect of phosphate ions previously reported (24).

The inhibition of both AAT-1 and AAT-2 by KCN (Table 2), in accordance with several aminotransferases from other plant sources (1, 4, 5, 10), may be due to the combining of cyanide to the prosthetic group, pyridoxal phosphate, of aminotransferases, the mechanism which has been previously reported (25). The slight inhibition recorded after treatment with iodoacetamide and N-ethylmaleimide (Table 2) suggests that lentil seeds aspartate aminotransferase isoenzymes are more sensitive to -SH group experiments than those of the soybean seeds (10). On the other hand, the fact that AAT-1 was more sensitive to inhibition by -SH group reactives than AAT-2 (Table 2) as the isoenzymes from soybean seeds (10), conducts to propose that AAT-1 molecule may have more -SH groups essential for enzymic activity than AAT-2 molecule.

In contrast to soybean seeds isoenzymes (26), AAT-1 is more sensitive to inhibition by maleic acid than AAT-2 (Table 2), but the extent of inhibition is less than the one for pig heart aminotransferases (27, 28). This may be explained by the differentiation in catalytic sites of the plant isoenzymes due to different protein conformation in comparison with the mammalian ones.

Hydroxylamine and D-cycloserine, an antibiotic which is readily degraded to hydroxylamine upon acid hydrolysis (29), are well known transaminase inhibitors which inhibit these enzymes by binding to their cofactor pyridoxal-5'-phosphate (30-32). In the present study, the fact that AAT-1 and AAT-2 were inhibited nearly to the same extent by D-cycloserine (at 10 mM inhibitor concentration) is in accordance with our previous findings for soybean seeds isoenzymes (26). On the other hand, hydroxylamine proved to be a much more potent inhibitor than cycloserine causing 70% and 62% inhibition of AAT-1 and AAT-2 respectively at 1 mM inhibitor concentration (Table 2).

Although activation of aspartate aminotransferase isoenzymes from other sources have been reported (4), in this study, in agreement with other studies (5, 10), no stimulation of either AAT-1 or AAT-2 was detected with Mg^{2+}, Ca^{2+}, Mn^{2+} and Zn^{2+} ions (Table 3).

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