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Aspartate aminotransferase isozymes in plants: Comparison of two staining methods in polyacrylamide gels

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Abstract

Two staining methods for aspartate aminotransferase were compared after electrophoretic resolution of its isozymes in polyacrylamide gels. The first one uses L-aspartic acid and Fast Blue BB salt (classical method), the second uses L-cysteine sulfinic acid and a redox system with phenazine methosulfate and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. The seeds of pea, horse bean and soybean were used as a model plant source of the enzyme. The staining method with L-cysteine sulfinic acid is very reliable and more sensitive than the Fast Blue BB method and allows detection at very low isozyme activities in the gel.

Introduction

Aspartate aminotransferase (AAT, E.C. 2.6.1.1., glutamate-oxaloacetate transaminase) is one of the most important enzymes of amino acid metabolism in living organisms, which catalyses the transfer of an amino group between L-aspartic acid and α-oxoglutarate in the presence of the coenzyme pyridoxal-5-phosphate (PLP). This enzyme exists in different isoforms, which are distributed over different cell compartments. Thus, aspartate aminotransferase and its isozymic composition is widely studied from different points of view, including practical use of isozymes in plant genotype identification. For this purpose, isozymes are electrophoretically separated and stained using a suitable staining system, either directly in the gel or using different transfer and/or overlay methods.

In the case of AAT, the most frequent staining method is based on the diazonium dye Fast Blue BB, which forms an insoluble chromophore with oxaloacetate originating from aspartate after transamination (Vallejos 1983, Wetter and Dyck 1983). However, in some cases the available activity of aspartate aminotransferase in the plant tissue is very low and detection of AAT isozymes is not reliable by this staining method without additional operations with the sample (deproteination, concentration) which, on the other hand, are time consuming and can lead to further

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loss of enzyme activity. Such problems are encountered, for example, with the seeds of grain legumes, which have high protein contents together with low aspartate aminotransferase activity, but a similar situation can arise in many other plant tissues.

In 1981, Yagi *et al.* introduced a new sensitive method for staining AAT isozymes for use in clinical biochemistry. This method is based on the finding that L-cysteine sulfinic acid (CSA) can serve as a substrate for aspartate aminotransferase. It is transaminated to β-sulfinyl pyruvate, which decomposes spontaneously to pyruvate and HSO_3^-. HSO_3^- can reduce nitroblue tetrazolium chloride (NBT) in the presence of phenazine methosulfate (PMS) to insoluble formazan. The reaction mixture was further improved (Sakakibara *et al.* 1983) to contain 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in place of NBT and 5-methyl phenazine methosulfate (m-PMS) in place of PMS, which resulted in better photochemical stability (m-PMS) and linearity in intensity staining (MTT). The concentration of L-cysteine sulfinic acid in thr reaction mixture was published as 0.05 M for direct gel staining (Yagi *et al.* 1981) or 0.2 M (Sakakibara *et al.* 1983) when used in 1 cm^3 of the reaction mixture for staining of celluloso-acetate membranes. Although the volume of staining solution can be altered for different indirect staining techniques and the amount of relatively expensive substrate reduced in this way, some workers prefer direct staining of the electrophoretic gels, which requires several tens of millilitres of reaction mixture. The aim of this work was to specify the necessary concentration of L-cysteine sulfinic acid in the reaction mixture for reliable staining of the electrophoretic gels, to compare the results obtained with this sensitive technique with the standard one and to test this method for use in various fields of plant sciences.

Materials and methods

Seeds of pea (*Pisum sativum* L., cv. Bohatýr and cv. Puget), horse bean (*Vicia faba* L., cv. Chlumecký) and soybean (*Glycine max* (L.) Merr., cv. Sluna) were used as a source of aspartate aminotransferase. 100 seeds of each cultivar were finely ground using a *Cyclotec* (*Tecator*) laboratory mill, and seed meal was then extracted with electrophoretic sample loading buffer (62.5 mM TRIS-HCl, pH = 6.8 containing 10 % v/v glycerol) at 100 mg cm^{-3} for pea cultivars and 50 mg cm^{-3} for horse bean and soybean. Homogenates were kept at 4 °C for 1 h and then centrifuged at 4 °C at 30 000 *g* for 10 min. Clear supernatants were used the same day for AAT activity and protein concentration measurements, and for electrophoresis.

Total aspartate aminotransferase activity was determined at 37 °C by th GOT opt. monotest^R (*Boehringer*) which uses the coupled enzymatic reaction of aspartate aminotransferase-malate dehydrogenase (measured at 340 nm). All activity tests were performed at least three times. Protein concentration was measured according to Bradford (1976) in doublets for each data point.

Electrophoresis was carried out on *Midget* (*Pharmacia-LKB*) mini gels (55 × 83 × 0.75 mm for resolving gel) in the native discontinuous buffer system of Laemmli

(1970). The resolving gel consisted of 7.5 % acrylamide to which 10 % (m/v) mannitol was added to improve resolution and sharpness of the isozyme bands (Stejskal, unpublished). The stacking gel was 5 % acrylamide, 1 cm high. A constant current of 10 mA per gel was applied at 8 °C and electrophoresis was prolonged for extra 30 min after the tracking dye bromophenol blue had reached the bottom of the gel to increase distances between individual isozyme bands (total electrophoretic time was 1 h 45 min).

Staining procedures: A technique of Vallejos (1983) was used as a standard staining procedure, except that the pH of the mixture was set at 7.5 (HCl) and used directly for staining without equlibration of the gels.

For L-cysteine sulfinic acid staining, the following components of the mixture were kept constant (given for 100 cm^3 solution): 0.1 M TRIS, 0.005 M α-oxoglutaric acid, 5 mg pyridoxal-5-phosphate, 20 mg MTT, 5 mg PMS. Concentrations of L-cysteine sulfinic acid were altered in the range 0.05 - 0.0025 M.

Procedure: All compounds except PMS were dissolved in 0.1 M TRIS and titrated to pH = 7.5 (HCl). In optimization experiments, three to five sample gel strips were cut and overlayed with 20 ml of staining mixture, to which a corresponding amount of PMS dissolved in 0.1 cm^3 H_2O was added. The staining vessels were kept in the dark at room temperature (25 °C) for different periods of time. In the final experiments, the concentration of L-cysteine sulfinic acid was 0.01 M and 50 cm^3 of staining solution was used for the staining of one mini gel for 90 min. All staining experiments were repeated three times.

L-cysteine sulfinic acid, lot 61H5005, containing 1 mol H_2O per 1 mol acid was obtained from *Sigma*, L-aspartic acid was from *Loba chemie*, Fast Blue BB, α-oxoglutaric acid, pyridoxal-5-phosphate and MTT were from *Serva*, phenazine methosulfate from *Lachema*. All the remaining chemicals were of electrophoretic grade from *Serva*.

Results and discussion

For determination of optimal L-cysteine sulfinic acid concentration in the staining mixture, samples of AAT extracted from pea cv. Bohatýr were chosen, which showed the most disadvantageous ratio of AAT activity to protein content (Table 1.) The critical amount of this sample for loading to 15-well-comb for electrophoresis was found to be 4 - 5 μl (approximately 40 μg of protein) without considerable streaking of the isozyme bands.

Fig. 1 shows the influence of L-cysteine sulfinic acid concentration and staining time on intensity of isozyme bands. The possible increasing of developing time with decreasing CSA concentration is evident. In the case of 0.05 M CSA the staining time is limited to 30 min, after which the staining mixture precipitated together with increasing background staining. Two slow migrating zones of AAT activity are comparably fainter in 0.05 M CSA than in 0.025, 0.01 and 0.005 M CSA, which

could be explained as inhibition by the substrate or by the product, respectively, of enzymatic reaction of these two active isozymes, in contrast to the fast migrating fine triplet, which is equally stained in all concentrations of CSA mentioned above. In the case of 0.0025 M CSA all isozyme bands are fainter than in higher concentrations of CSA, which suggests insufficient amount of the substrate for enzyme reaction and following electron transfer to form insoluble formazan. The reaction mixture

Table 1. Total aspartate aminotransferase activity and protein content in different legume seed extracts (extracted as described in Materials and methods; n = 3).

Cultivar	AAT content [nkat cm^{-3}]	Protein content [nkat cm^{-3}]	AAT [nkat mg^{-1}(protein)]
Bohatýr (pea)	5.82 ± 0.24	8.5	0.68
Puget (pea)	18.07 ± 0.97	10.2	1.77
Chlumecký (horse bean)	10.28 ± 0.37	5.6	1.82
Sluna (soybean)	27.39 ± 1.55	4.7	5.83

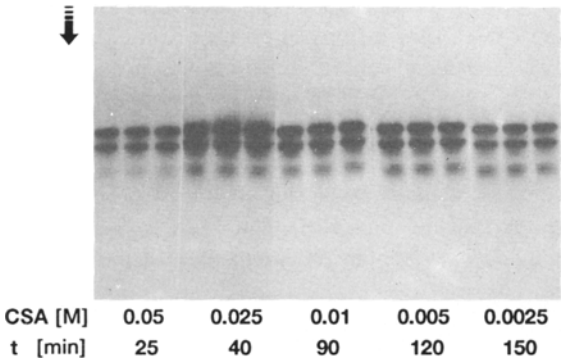

Fig. 1. Influence of L-cysteine sulfinic acid concentration and staining time on developing AAT isozymes. Triplets of the pea seed extract cv. Bohatýr were loaded in 4 µl (23.3 pkat) and stained as described in the text.

containing 0.05 M CSA precipitated and inactivated at 20 - 30 min in contrast to lower CSA concentrations (40 - 150 min). This could be caused either by CSA itself or by overproduction of the reducing agent (HSO_3^-) which can lead to non-specific reduction of MTT in the solution. The blank experiments were not carried out as this was not a subject of the study. Using 0.025, 0.01 and 0.005 M L-cysteine sulfinic acid in the reaction mixture, together with prolonging the developing time, we can obtain practically the same results in intensity and sensitivity of isozyme staining. 0.01 M CSA seems to be a good compromise between the cost of the substrate used and staining time, but 0.005 M CSA also gave acceptable results. 0.025 M CSA is a relatively rapid and sensitive variant without danger of fast background

overstaining. We also tested detection limits for the two procedures (Fig. 2) using serial dilutions of soybean seed sample. We noted a gradual decrease in intensity of isozyme staining over the whole dilution range (0 - 32 ×) with CSA staining, while

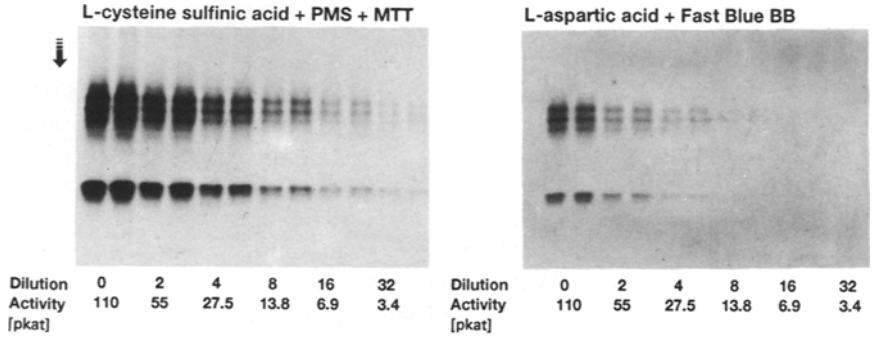

Fig. 2. Comparison of the detection limits for the two staining methods. Samples of seed extract of soybean cv. Sluna were loaded in 4 µl doublets at indicated activities of AAT. Concentration of CSA was 0.01 M, staining time 90 min.

notable bands with classical staining were recorded only till the dilution 8 ×. As comparable result for dilution 32 × (CSA) we can consider 4 - 8 × (Fast Blue BB). This shows that staining using L-cysteine sulfinic acid is approximately 4 - 8 fold more sensitive than the standard one in this arrangement of the experiments.

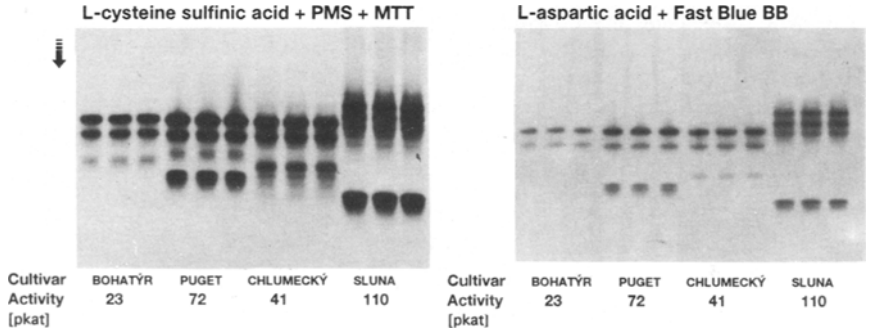

Fig. 3. Comparison of various legume seed samples stained either with L-cysteine sulfinic acid or with L-aspartic acid and Fast Blue BB salt. 4 µl sample triplets were loaded at indicated activities of AAT. Staining conditions for CSA: concentration 0.01 M, time 90 min.

A comparison of standard and L-cysteine sulfinic acid staining of legume seed samples, containing different amounts of AAT activity and protein, is shown in Fig. 3. In all four samples we can see additional fine isoforms of AAT and more expressive staining using CSA, which could not be obtained by the classical method.

Even if the increase in staining sensitivity of AAT isozymes using CSA is not particularly of magnitude large (it did not even reach one order), it is sufficient to detect isoforms with low activity in the samples. Stained bands are violet to dark violet on a clear background in contrast to blue bands on a red background with Fast Blue BB staining, which also makes evaluation of the gels easier. The staining procedure will probably also work on other common electrophoretic media (starch, agarose) as well as on different membranes after blotting of the original electrophoretic gel. Staining times with low CSA concentrations can be shortened by increased staining temperature (37 °C) and the use of m-PMS will probably lead to further possible prolongation of developing time without inactivation of the reaction mixture.

We hope that this paper will contribute to wide use of the described staining method of aspartate aminotransferase isozymes and will help to solve various problems connected with this important enzyme.

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