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Isolation, Purification, and Crystallization of Aspartate Aminotransferase from Wheat Grain

V. M. Kochkina

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, Moscow 119991, Russia; fax: (7-095) 135-1405; E-mail: belj@eimb.ru

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Abstract—A procedure for isolation and purification of aspartate aminotransferase from wheat grain includes chromatography on DEAE cellulose, acidification–alkalization, precipitation with protamine sulfate, fractionation with ammonium sulfate, and chromatography on hydroxyapatite. The yield of protein was 27% with 95% purity. Crystals of the enzyme ($0.05 \times 0.025 \times 0.015$ mm^3) were obtained from ammonium sulfate solution.

Key words: wheat grain, aspartate aminotransferase, purification, crystallization

Aminotransferases (transaminases) are key enzymes of cell metabolism catalyzing transfer of the amino group from amino to keto acid. The transfer is performed by pyridoxal-5-phosphate, a cofactor of transaminases. Of a large number (more than 70) of aminotransferases, aminotransferase from animal tissue catalyzing transamination between L-aspartate and 2-oxoglutarate is best studied [1]. The structure of the enzyme complexes with the substrates and their analogs [1-3] and the structure of free aspartate aminotransferase [4] have been determined by high resolution X-ray analysis.

In contrast to the animal and bacterial aminotransferases, which are widely studied, data on plant aminotransferases are practically absent. Thus, the latter are described on only eight pages of 640 comprising a book by Christen and Metzler [1]. No reports on the studies of plant aminotransferases were delivered at the international symposia on pyridoxal enzymes during the last 25 years.

Although the data on aminotransferases of higher plants and their properties are scarce and not systematized, they are known to play a role in fixation and transfer of nitrogen in plant organs and tissues and its storage in seeds and to participate in biosynthesis of alkaloids and porphyrins [1, 5]. The enzyme has been localized in all organs and tissues of higher plants and found in all cell organelles. For purification of plant transferases, standard procedures have been established [5]. Most plant transaminase preparations are not pure enough and were isolated in very small quantities. This seems to be caused by a low content of aminotransferase in plants and its ability to form complexes with other enzymes; this property hinders purification of aminotransferase and requires processing of large amounts of raw material. For optimizing conditions for crystallization of the enzyme, especially when its physicochemical properties are not well studied, tens of even hundreds of milligrams of highly purified enzyme preparation are needed.

In the present work we have isolated, purified, and crystallized aspartate aminotransferase from wheat grain.

MATERIALS AND METHODS

Activity of the enzyme was estimated by two spectrophotometric methods. The direct method is based on the measurements of increased absorbance of the solution at 280 nm due to formation of oxaloacetate during transamination between aspartate and 2-oxoglutarate [6]. Activity was measured for 3 min in a cuvette thermostatted at 25°C.

The amount of the enzyme causing increase in absorbance by 0.001 absorbance unit per 1 min in a cuvette with the optical path of 1 cm was defined as the activity unit (U). The indirect method in the conjugated system is based on reduction of oxaloacetate formed during transamination by the action of malate dehydrogenase in NADH [7]. The reaction rate was assayed by decrease in absorbance at 340 nm caused by oxidation of NADH. The measurements were performed after each

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30 sec for 3 min after beginning of the reaction at 25°C. The amount of the enzyme catalyzing oxidation of 1 μmol of NADH per 1 min in a cuvette with the optical path of 1 cm was defined as the activity unit (U). A sample 1 ml in volume contained 10 μmol of L-aspartate, 3 μmol of 2-oxoglutarate, and 30 μg of cofactor dissolved in 0.05 M potassium phosphate buffer, pH 8.5. The indirect method is an order of magnitude more sensitive than the direct one.

The protein concentration in the enzyme preparations was determined spectrophotometrically using LKB 4050 and 111 spectrophotometers (Sweden). The absorbance of 1 mg protein at 280 nm was defined as the optical unit.

Electrophoresis in the polyacrylamide gel under denaturing conditions was performed according to Laemmli [8].

The following reagents were used in this study: DEAE cellulose DE-52 from Whatman (England); L-aspartate, 2-oxoglutarate, hydroxyapatite, and NADH from Sigma (USA); protamine sulfate and malate dehydrogenase from Serva (Germany). Other reagents were of extra pure grade and produced in Russia.

RESULTS AND DISCUSSION

Wheat grains were disintegrated in a hand mill, and five volumes of 0.01 M Tris-HCl or 0.01 M potassium-phosphate buffer, pH 7.5, containing 50 μg of cofactor per 1 ml of solution were added to the flour. The enzyme was extracted for 3 h at room temperature. After extraction the flour was removed by centrifugation at 4500*g* for 20 min. Extraction of the enzyme by buffer solutions instead of aqueous ones [5] has some advantages, since protein is acid-unstable and on water extraction losses will be observed even at the first stage of isolation.

DEAE cellulose equilibrated with the buffer analogous to the buffer for extraction was added to the extract. Ion-exchange chromatography was performed "in volume", taking for calculations 30 mg of protein per 1 ml of resin. For protein adsorption, the extract–cellulose mixture was incubated for 20-30 min with slow stirring. Then resin was isolated by centrifugation at 4500*g* for 20 min. The enzyme was eluted from the resin with 0.2 M potassium-phosphate buffer, pH 8.0, containing 60 μg of cofactor per 1 ml of solution, overnight at 4°C. It should be noted that under the chosen conditions, only 20% of the enzyme is adsorbed on the resin, and increased amount of resin does not cause increased adsorption. The specific activity of the eluted enzyme increases almost fivefold compared with the specific activity of the raw material.

After removal of the cellulose, the supernatant was decanted and acidified with 0.1 M acetic acid to pH 5.2. The precipitate thus formed was removed by centrifugation at 4500*g* for 20 min, and the solution was alkalized to pH 8. The "acidification–alkalization" procedure allowed ~35% decrease in the total amount of protein while the yield of the enzyme remained the same; as a result, the specific activity of the enzyme increased.

To the supernatant alkalized to pH 8.0, protamine sulfate was added to the final concentration 0.25-0.3%. The precipitate thus formed was isolated by centrifugation at 4500*g* for 20 min. The stage of precipitation with protamine sulfate does not change specific enzyme activity, only negligible decrease in protein content is observed in preparations.

Ammonium sulfate was added to the supernatant to 5% saturation (saturation percentage is given for 25°C); for precipitate formation, the mixture was incubated for 20 min. The precipitate was removed by centrifugation at

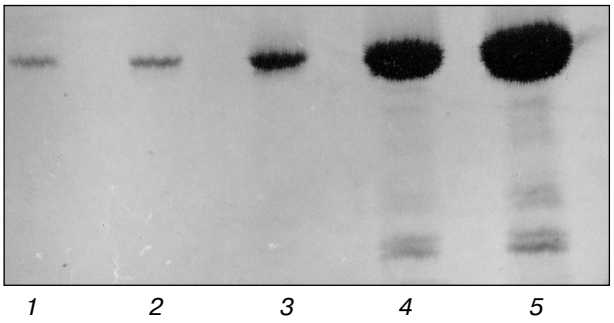

Fig. 1. Electrophoregram of the enzyme in polyacrylamide gel. Mass of applied protein (lanes *1-5*): 5, 10, 50, 250, 500 μg.

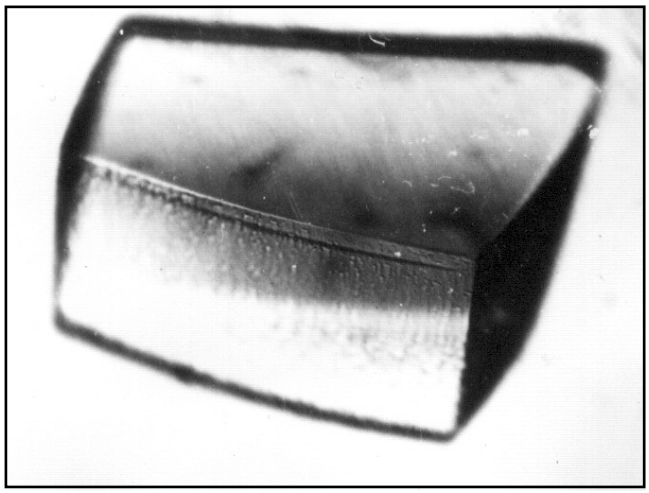

Fig. 2. Crystals of the enzyme grown from ammonium sulfate solution (magnification ×1000).

Purification of aspartate aminotransferase from 50 g of wheat grain

Stage of purification	Protein, mg	Actvity*	
		total, U	specific, U/mg
Initial extract	2350	200	0.085
Chromatography on DEAE cellulose	89	35.4	0.4
Supernatant of the extract	1762	164	0.094
Acidification–alkalization	1575	160	0.1
Precipitation with protamine sulfate	1457	159	0.11
Fractionation with ammonium sulfate (5-55% saturation)	517	130	0.25
(20-55% saturation)	258	106	0.48
Chromatography on hydroxyapatite	14	54	3.6

* Activity assayed according to Karmen [7].

4500*g* for 20 min, and in the supernatant saturation with ammonium sulfate was increased to 55%. The required mass was applied during 30 min, and the mixture was incubated overnight at 4°C for formation of precipitate. The precipitate was harvested by centrifugation at 4500*g* for 30 min and dissolved in the minimal volume of 0.2 M potassium-phosphate buffer, pH 8.8, containing 60 μg of cofactor per 1 ml of buffer.

The enzyme preparation was fractionated with ammonium sulfate for a second time. The fraction precipitated between 20 and 55% ammonium sulfate saturation exhibited the maximal specific activity. The precipitate was harvested by centrifugation at 4500*g* for 30 min and dissolved in 0.5 mM potassium-phosphate buffer, pH 7.5, containing 60 μg of cofactor per 1 ml of buffer.

Fractionation with ammonium sulfate resulted in almost fivefold increase in the specific activity of the enzyme. It should be noted that 35-47% loss of the enzyme activity is observed during fractionation.

The preparations of the enzyme eluted from DEAE cellulose and salted out between 20 and 55% ammonium sulfate saturation were pooled as having comparable specific activities and chromatographed on hydroxyapatite "in volume". Hydroxyapatite equilibrated with 0.01 M potassium-phosphate buffer, pH 7.5 (taking 30 mg of protein per 1 ml of hydroxyapatite) was added into the pooled enzyme solution.

The mixture was incubated with stirring for 30 min, then the hydroxyapatite was removed by centrifugation at 4500*g* for 30 min. The transaminase was eluted from the hydroxyapatite with 0.15 M potassium-phosphate buffer, pH 7.5, containing 60 μg of cofactor per 1 ml of buffer. After chromatography on hydroxyapatite, the specific activity of the enzyme was 3.6 U/mg of protein according to Karmen [7] and 1500 U/mg according to Jenkins [6]. The yield of the enzyme was 27%. According to the data of horizontal electrophoresis in polyacrylamide (Fig. 1), the enzyme had 95% purity.

The data on purification of aspartate aminotransferase from 50 g of wheat grain are presented in the table.

The transaminase preparation was used for optimizing crystallization conditions. Crystals were obtained by equilibrium diffusion from a free surface [9] using PEG 6000 as a precipitator and also by vapor diffusion in a "sitting" drop [10] using ammonium sulfate at 4°C as a precipitator. Precipitation with PEG 6000 resulted in very small crystals without any definite habit.

Crystallization by equilibrium diffusion in a "sitting" drop was performed in weighting bottles 35-40 mm in height and 35-60 mm in diameter. All weighting bottles and solutions used for crystallization were pre-cooled to 4°C to prevent the temperature change in the vessels. Cooled enzyme solution (10-12 mg/ml) was placed into the wells on cooled non-siliconized glasses (0.2-2 ml depending on well size). Glass or plastic Petri dishes with diameter lesser than that of a weighting bottle were placed in the latter as carriers for glasses with wells. Cold 45% ammonium sulfate prepared with buffer, pH 7.5, was placed in a reservoir of a weighting bottle (3-5 ml depending on the volume of a bottle). A lid of a weighing bottle was lubricated with vacuum silicone lubricant for better impermeability, closed tightly, and placed in cold at 4°C. The crystal nuclei appeared after 7-10 days as solutions in the wells and the bottle reservoirs were equalized. Crystals with dimensions $0.05 \times 0.025 \times 0.015$ mm^3 grew after 3-4 weeks (Fig. 2).

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