Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 32-36

PURIFICATION OF MONOMERIC AGMATINE IMINOHYDROLASE FROM SOYBEAN

Kyung Hee Park and Young Dong Cho

Department of Biochemistry, College of Science, Yonsei University

Seoul 120-749, Korea

Received November 16, 1990

SUMMARY : Agmatine iminohydrolase (EC 3.5.3.12) was purified to homogeneity from the cytosol of soybean (*Glycine max*) axes by chromatographic separations on Sephadex G-25, Bio-rex 70, and agmatine-affinity columns. The enzyme was homogeneous by the criteria of analytical gel electrophoresis. Molecular weights estimated by Sephadex G-100 gel and sodium dodecyl sulfate polyacrylamide gel electrophoresis were 70,000, indicating that the soybean axes enzyme is a monomer, in contrast to the dimeric enzymes from corn and rice. The isoelectric point determined by gel electrofocusing was 7.5, higher than that of the corn enzyme (4.7). The optimal pH and temperature for activity were 6.5 and 50℃, respectively. The enzyme has high specificity for agmatine, and the Km for agmatine was 2.5×10^{-3} molar. The enzyme was sensitive to Cu^{2+} and also was inhibited by p-hydroxymercuribenzoate.

Putrescine in higher plants seems to be formed via N-carbamylputrescine from agmatine (1, 2). Agmatine iminohydrolase (EC 3.5.3.12), which catalyzes the hydrolysis of agmatine into N-carbamylputrescine and ammonia, seems to be one of the critical enzymes in putrescine biosynthesis. The enzyme of this pathway has been isolated and studied from rice (3), corn (4) and groundnut (5). In the case of the enzymes from rice (3) and corn (4), extensive studies on the enzyme have shown that it is dimeric enzyme. To the best of our knowledge, purification of monomeric agmatine iminohydrolase has not been reported. However, we have concrete evidence of the presence of a monomeric agmatine iminohydrolase in soybean axes. Therefore, in this article, we describe its purification and partial characterization of the enzyme from soybean axes for further studies. Results are compared to those for the corn and rice enzyme, with the dimer taken as a reference since there has been no report on the monomeric enzyme.

METHODS

Plant Materials. Seedlings of soybean (*Glycine max*) were grown in plastic trays at 25℃ for 3 days in the dark. All plants were watered daily (6).

Enzyme Assay. The enzyme assay was based on the estimation of ammonia released from agmatine by the enzyme. A Warburg flask was used for the enzymatic reaction and for the

0006-291X/91 $1.50

subsequent ammonia distillation, and the ammonia was determined with Nessler's reagent. The standard assay mixture, consisting of 200 μmol potassium phosphate buffer (pH 6.5), 30 μmol agmatine sulfate, 1 mg chloramphenicol, 1 mg streptomycin sulfate and enzyme solution in a total volume of 2.0 ml, was placed in the outer compartment of the Warburg flask. The central compartment contained 2 ml of 0.01N HCl. The flasks were sealed and incubated with 20 rpm shaking for 2 hr at 37℃. At the end of this period, 1 ml of saturated K_2CO_3 was added to the outer compartment. After shaking for a further 30 min at 30 rpm, the ammonia which had distilled to the HCl solution in the inner compartment was determined with Nessler's reagent (7). In control experiments, omission of the substrate was used. One unit is defined as the amount of enzyme liberating 1 μmol ammonia per hour. Agmatine iminohydrolase activity was a linear function of both incubation time and concentration under these conditions.

Enzyme Purification. Purification of the enzyme was carried out at 4℃. Plant axes (400 g) were blended in a chilled electric mixer with 2 volumes (v/w) of the extracting buffer [50 mM phosphate buffer (pH 6.5) containing 6% glycerol, 5 mM 2-mercaptoethanol and 0.1 mM EDTA]. The homogenate was filtered through four layers of gauze and clarified by centrifugation (13,000xg, 20min). The supernatant was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and stirred at 4℃ for 5 hr. The solution was then centrifuged and the pellet was discarded. The supernatant was brought to 50% saturation with solid $(NH_4)_2SO_4$ and treated as above, except that the pellet was retained. The pellet containing the enzyme was dissolved with 5 ml of the extracting buffer. Then 5 ml of the enzyme solution was applied to a Sephadex G-25 column (2.4 × 54 cm) equilibrated with extracting buffer. The flow rate was 20 ml per hour. Active fractions of Sephadex G-25 filtration were applied to a Bio-rex 70 column (2.6 × 12 cm) previously equilibrated with the extracting buffer. The column was washed with the same buffer. Elution of the enzyme was achieved with 800 ml of a linear gradient of 0.1 M to 0.4 M KCl made up in the extracting buffer. Active fractions were pooled and then dialyzed against 1 ℓ of the extracting buffer.

Agmatine-CH-Sepharose 4B was prepared according to the method of Pharmacia Fine Chemicals. The agmatine was coupled to activated CH-Sepharose 4B by gently shaking 10g of the swollen gel in 50 mM agmatine, dissolved in coupling buffer $NaHCO_3$ (0.1 M, pH 8.0) containing 0.5 M NaCl, for 24 hr at room temperature. The reacted gel was washed alternatively with high and low pH buffer solutions four or five times. Formate buffer (0.1 M, pH 4.0) and Tris buffer (0.1 M, pH 8.0), each containing 0.5 M NaCl, were used.

The dialyzed enzyme of Bio-rex 70 eluates was applied to an agmatine-CH-Sepharose 4B affinity column (2 × 8 cm) equilibrated with the extracting buffer. After 80 ml of the extracting buffer was passed through the column, the bound enzyme was eluted with 50 ml of the same buffer containing 5 mM agmatine sulfate. The flow rate was approximately 15 ml per hour. Active fractions were pooled and dialyzed against the extracting buffer.

Protein Determination. Protein was determined by the Lowry procedure (8), with BSA as the standard.

Determination of Molecular Weight. The molecular weight of the enzyme was estimated by gel filtration through a Sephadex G-100 column (1 × 90 cm) according to the method of Andrew (9). The column was calibrated with γ-globulin (150 Kd), BSA (66 Kd), ovalbumin (45 Kd) and myoglobulin (19 Kd) as markers of known molecular weight. Disc gel and SDS electrophoresis were carried out according to Laemmli (10).

RESULTS AND DISCUSSION

At the onset of germination, enzyme activity was very low; thereafter, activity increased rapidly. Subsequently, enzymatic activity reached its maximum three days after germination and declined slightly as germination progressed. The results of typical purification procedure for three-day-old soybean axes agmatine iminohydrolase are presented in Table 1. Because methods employed by

Table 1. Purification of agmatine iminohydrolase from soybean axes

Stage of purification	Total Volume	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	ml	mg	unit	units/ mg	%	-fold
Crude	865	2768	5320	1.92	100	1
Sephadex G-25	80	240	1376	5.73	25.9	2.98
Bio-rex 70	247	13.5	862	63.7	16.2	33.3
Affinity	30	0.54	93	172.2	1.75	89.7

Chaudhuri and Ghosh to purify the enzyme from rice seedlings (3) and Yanagisawa and Suzuki, from corn seedlings (4) were not suitable for the purification of the enzyme from soybean axes, we have modified these procedures. Accordingly, we have not used DEAE-cellulose and Sephadex G-100 columns and instead used Bio-rex 70, which was not used in their attempts. We could not find any evidence of the presence of agmatine iminohydrolase isozymes in the purification steps. This result could not be compared with others because there has been no report on monomeric agmatine iminohydrolase. The purity of the enzyme was judged by our observation that the enzyme migrated as a sharp, single band in polyacrylamide disc gel electrophoresis (Fig. 1). We suggest that our purification protocol is effective for the monomeric enzyme, when compared with other methods, and that it may be useful in studies where purified monomeric agmatine iminohydrolase is required for further enzyme studies, production of antibodies, etc. The molecular weight of the enzyme determined by Sephadex G-100 gel filtration was estimated to be 70,000; and SDS gel electrophoresis also showed that the molecular weight of the enzyme was 70,000.

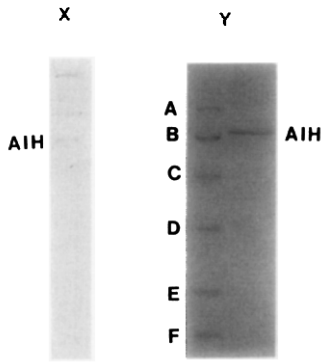

Fig. 1. Polyacrylamide gel electrophoresis of purified soybean agmatine iminohydrolase.
X, The untreated enzyme was electrophoresed in a 12.5% gel ; Y, enzyme treated with 2% SDS, 5% 2-mercaptoethanol and 10% glycerol at 100℃ for 10 min. A, phosphorylase b (96KD) ; B, bovine serum albumin (66KD) ; C, ovalbumin (45KD) ; D, carbonic anhydrase (31KD) ; E, soybean trypsin inhibitor (21KD) ; F, lactalbumin (14KD) ; AIH, agmatine iminohydrolase (X, 5μg; Y, 30μg).

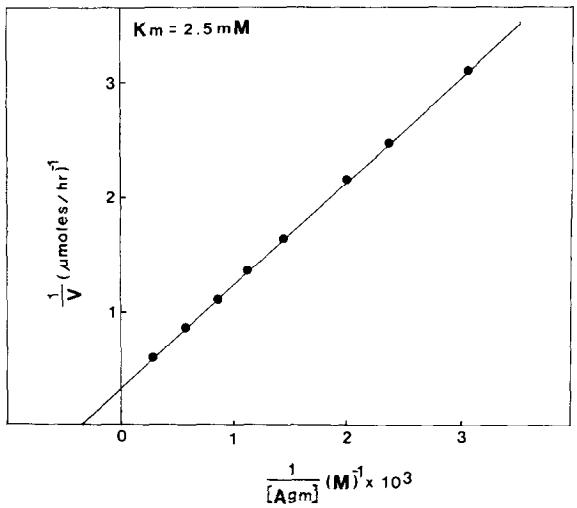

Fig. 2. Lineweaver-Burk plot of initial velocities measured at concentrations of agmatine for agmatine iminohydrolase.

suggesting that the enzyme in soybean axes is a monomer. Agmatine iminohydrolase was first reported in maize seedlings in 1969 (11), but was not been homogeneously purified until 1981 (4). The enzyme was also isolated from rice seedlings (3). Molecular weights of the native enzymes from rice and corn were shown to be 183,000 and 85,000, respectively, and both were dimers of identical subunits. The enzyme from the rice seedlings was observed to follow typical Michaelis-Menten kinetics with a Km value of 1.5×10^{-2} M (3). As expected, agmatine iminohydrolase from soybean axes was also observed to obey typical Michaelis-Menten kinetics with a Km value of 2.5×10^{-3} M (Fig. 2), which is larger than that from the corn seedlings (1.9×10^{-4} M).

The isoelectric point of the enzyme from the corn seedlings determined by the gel-electrofocusing method was 4.7 (4), while the enzyme from the soybean axes had an isoelectric point of 7.5. However, there was no significant difference in optimum pH between the three enzymes: the rice enzyme, 6.0 and the corn and soybean enzymes, 6.5. The optimum temperature for the activity of the enzyme from rice was 28℃, which attracted our attention since the enzyme from corn and soybean had temperature optimums of 60℃ and 50℃, respectively.

The purified enzyme from the corn lost 30% of its activity in three days at 4℃ (4). Coincidently, the enzyme from soybean was observed to lose almost the same degree of activity under identical conditions. The soybean enzyme has high specificity for agmatine, while several analogues such as arginine, creatine, and citrulline were not attacked by this enzyme, which agrees with the findings on corn. The enzyme from soybean was inhibited by several cations, Cu^{2+}, Co^{2+}, Zn^{2+},

which were in response to the increase of their concentrations. Such trends were also reported in the corn and rice seedlings.

The enzyme activity from the rice was inhibited by spermidine and spermine(3) whereas the enzymes from the corn and the soybean were innocuous. N-ethylmaleimide(NEM), iodoacetamide, and p-hydroxymercuribenzoate(p-HMB) were incubated at 37℃ for 10min. The inhibition was the most extensive in p-HMB. Such trends were also observed in the corn(4).

Such partial characterizations show that the enzyme from the soybean axes, a monomer, has a few similarities to the corn and rice enzymes, dimers, described above. A possible reason for the differences observed between the monomer and dimers may be the difference in amino acid compositions and structures, as well as the difference in the number of subunits. Further studies are needed.

ACKNOWLEDGMENT

We are pleased to acknowledge the support of the KOSEF(880510).

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