J. Biochem. Mol. Biol. Vol. 30, No. 6, pp. 438~442 November 30, 1997

Identification of γ-Glutamylamine Cyclotransferase, as the Preform Enzyme at the Dormant Stage, from Soybean (*Glycine max*) Seeds

Hyeog Kang, Sung Joon Park and Young Dong Cho*

Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Korea

(Received October 21, 1997)

Abstract : γ-Glutamylamine cyclotransferase was purified to homogeneity from soybean (*Glycine max*) seeds. To our knowledge, it is the first purification of the enzyme from plant origins. The molecular weight of the enzyme estimated by Sephacryl S-300 gel filtration and SDS-PAGE was 27,000, indicating that the enzyme is a monomer. The optimal pH for activity was 8.6. The Km value for γ-glutamyldansylcadaverine was 11 μM. The enzymatic activity was substantially inhibited by the addition of p-chloromercuribenzoate and partially inhibited by the Cu^{2+} ion. However, neither other modification reagents nor other divalent metal ions affected the enzymatic activity. The comparison between the enzymatic activities of seed extracts treated with cycloheximide and control extracts, and the detection of the same single protein band by western blot analysis at the dormant stage without inhibition with distilled water indicate that γ-glutamylamine cyclotransferase is already present at the dormant stage and gradually activated during germination in soybean seeds.

Key words : dormant stage, γ-glutamylamine cyclotransferase, preform enzyme, soybean

The ε-(γ-glutamyl)lysine crosslinks and protein-bound γ-glutamylamines, formed by the catalytic action of members of a widely distributed group of enzymes called transglutaminases, occur in various tissues and organisms (Beninati *et al.*, 1985; Fesus *et al.*, 1985; Beninat *et al.*, 1988). Despite an accumulation of information on the prodution and distribution of these γ-glutamyl derivatives, there has been little attention directed toward the metabolic fate of these products of transglutaminase action. Because of the resistance to proteolytic digestion, the presence of ε-(γ-glutamyl)lysine and γ-glutamylamines has been reported in the proteolytic digests of acid insoluble materials from various sources (Piacentini *et al.*, 1988; Fesus and Tarcsa, 1989; Carlello *et al.*, 1990).

γ-Glutamylamine cyclotransferase, an enzyme that catalyzes the conversion of γ-glutamylamines to free amines and 5-oxo-proline, was first partially purified and characterized in rabbit kidneys and in various other tissues by Fink *et al.* (1980). The enzymatic activity was also identified in CHO cells (Fesus and Tarcsa, 1989). However, further studies on the enzyme have not been performed.

Although the papers provide much circumstantial evidence indicating that polyamines covalently link to glutamyl residues of endogenous proteins in plants (Signorini *et al.*, 1991; Falcone *et al.*, 1993; Del Duca *et al.*, 1995), no information is available on the existence of γ-glutamylamine cyclotransferase in plants.

We have reported recently the purification of transglutaminase from soybean leaves (Kang and Cho, 1996). As a part of the investigation to establish the metabolic fate of the γ-glutamyl derivatives, which are produced by the action of transglutaminase, in plants, the present paper reports the results of purification and characterization of γ-glutamylamine cyclotransferase from soybean seeds. In this paper we also suggest the possibility that this enzyme is already present at the dormant stage in the seeds by immunoblot analysis and cycloheximide treatment.

Materials and Methods

Plant materials

Soybean seeds (*Glycine max*) were soaked in distilled water and harvested at various time intervals after imbibition.

Assay of γ-glutamylamine cyclotransferase activity

γ-Glutamylamine cyclotransferase activity was measur-

*To whom correspondence should be addressed.
Tel : 82-2-361-2697, Fax : 82-2-313-1608
E-mail : cyd516@bubble.yonsei.ac.kr

ed with some modifications according to the identification method of γ-glutamyldansylcadaverine as described previously (Fink and Folk, 1983; Cariello *et al.*, 1990; Lorand *et al.*, 1992). The seeds were homogenized in a chilled mortar and pestle with 2 to 3 volumes of 10 mM potassium phosphate (pH 7.2) buffer. The homogenate was centrifuged at 15,000 rpm at 4℃ for 15 min and the supernatant was assayed for the enzyme activity. The assay mixture containing 50 μl of plant extract, 5 μl of 1 mM γ-glutamyldansylcadaverine (5 nmole) was taken to a volume of 100 μl with 10 mM potassium phosphate (pH 7.2) buffer containing 10 mM DTT and incubated for 30 min at 37℃. After stopping the reaction with the addition of 500 μl cold acetone, the mixture was stored at -70℃ for 1 hr and then centrifuged at 15,000 rpm for 30 min. The resulting supernatant was concentrated by heating at 65℃ and separated on Silica gel 60 plates with chloroform: methanol: acetic acid (10:2:1, v/v) solvent system. The concentrated samples were also analyzed with HPLC (Waters 600) equipped with a reversed-phase column (Delta pak C_8, 3.9×150 mm). The column was equilibrated at a solvent composition of 40% methanol and 30% each of H_2O and 40 mM potassium acetate (pH 5.5). Two minutes following sample injection, a linear increase of methanol commenced, with a decrease of the other solvents. With a flow rate of 1 ml/min, the methanol concentration was allowed to reach 100% at 22 min. Fluorescence of the effluent was monitored (λ_{exe}=334 nm, λ_{em}=530 nm) with a Waters Model 420 AC detector at the lowest sensitivity. γ-Glutamyldansylcadaverine was kindly provided by Dr. L. Lorand (Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University).

Enzyme purification

All purification steps were carried out at 4℃ except the acetone fractionation step. Soybean seeds were soaked in distilled water and harvested 12 h after imbibition. The seeds (300 g, wet weight) were homogenized in a chilled electric blender with 2 to 3 volumes of 10 mM potassium phosphate (pH 7.2) buffer. The homogenate was filtered through four layers of gauze and clarified by centrifugation (15,000 rpm, 30 min). The supernatant was adjusted to 45% saturation with solid $(NH_4)_2SO_4$ and stirred at 4℃ for 2 h. The solution was then centrifuged and the pellet was discarded. The supernatant was brought to 75% saturation with solid $(NH_4)_2SO_4$ and treated as above, except that the pellet was retained. The obtained pellet was dissolved and dialized overnight in the same buffer. The dialized solution was then applied to a DEAE-Sepharose column. After washing the column, elution was conducted with a linear salt gradient from 0 to 0.3 M NaCl in the same buffer. The active fractions were collected and subjected to 50~75% acetone fractionation at -20℃. After the suspension was centrifuged at 15,000 rpm for 15 min, the pellets were flushed with nitrogen gas and dissolved in the same buffer. The enzyme solution was then dialyzed overnight in the same buffer and loaded on a hydroxyapatite column equilibrated with the same buffer. The active fractions were eluted in the washing step. The active washing fractions were collected and concentrated by ultrafiltration (Amicon's stirred cell, YM-10). The concentrated sample was applied to a S-300 gel filtration column equilibrated with the same buffer. The fractions containing enzyme activity were collected and applied to a Cu^{2+}- chelating column equilibrated with the same buffer containing 0.15 M NaCl. After washing the column with the eqilibration buffer, followed by an acidic buffer (50 mM sodium acetate (pH 4.5), 0.8 M NaCl), elution was carried out with the starting buffer containing 1 mM EDTA. The eluted fractions were concentrated by ultrafiltration and dialized against the starting buffer.

Preparation of antisera and Western blotting

The antibody to purified enzyme was prepared in rat (Sprague-Dawley) as described previously (Harlow and Lane, 1988). Partially purified enzyme and seed extracts were transferred to Immobilon PVDF membrane (Millipore). Binding of the primary antibody was visualized using horseradish peroxidase-conjugated sheep anti-rat IgG and 4-chloro-1-naphtol/hydrogen peroxide as the chromogenic agents.

Results and Discussion

As there has been no information available on the existence of γ-glutamylamine cyclotransferase from plant origins, we began by examining the presence of this enzyme in soybean seeds using γ-glutamyldansylcadaverine as a substrate. Assaying extracts for the presence of this enzyme revealed a pronounced activity in germinating seeds. Figs. 1-A and the 1-B show the chromatographic profiles of the standards and the reaction products produced by incubation of the reaction mixture, respectively. It is evident that active enzyme action takes place in these seed extracts. Incubation of these extracts with the γ-glutamyldansylcadaverine led to the production of the monodansylcadaverine, which is the reaction product. Identification of the monodansylcadaverine was performed by C8 reverse-phase chromatography of the reaction product corresponding to the predicted retention time for the standard compounds. After the preparation of the peaks corresponding to the

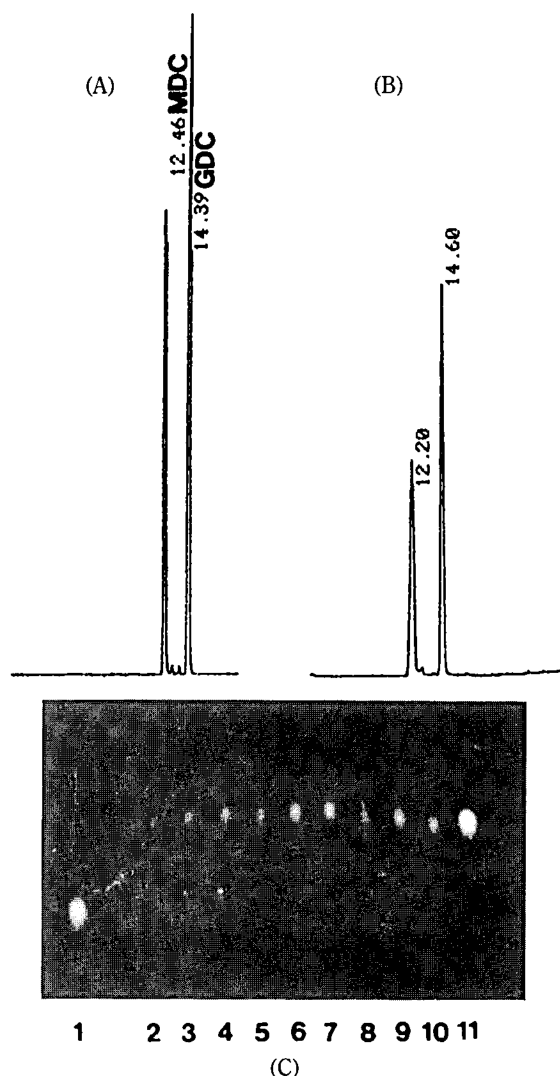


Fig. 1. Identification of γ-glutamylamine cyclotransferase activity in soybean seeds. Seeds were collected at 2 h (Panel A and B) after imbibition in distilled water and homogenized in 10 mM potassium phosphate buffer (pH 7.2). The homogenate was centrifuged at 15,000 rpm at 4°C for 15 min and the supernatant was used as an enzyme source. Panel A represents the elution profiles of a standard mixture containing 3 nmole γ-glutamyldansylcadaverine (GDC) and 3 nmole monodansylcadaverine (MDC). Panel B is the pattern for a reaction mixture which is incubated under standard assay conditions as described in *Materials and Methods*. Panel C shows monodansylcadaverine spots on TLC as a function of the length of the imbibition periods of soybean seeds. The loaded samples were prepared from the peaks corresponding to product by HPLC analysis. Soybean seeds were harvested as follows; 2 h (Lane 2), 4 h (Lane 3), 8 h (Lane 4), 12 h (Lane 5), 12 h (Lane 6), 36 h (Lane 7), 60 h (Lane 8), 84 h (Lane 9) and 132 h (Lane 10). Lane 1 is standard γ-glutamyldansylcadaverine (3 nmole) and Lane 11 is standard monodansylcadaverine (3 nmole).

product in HPLC analysis, the enzyme activity was also assayed by TLC analysis as a function of the length of the imbibition periods (Fig. 1-C). The Rf value of the eluted peaks was identical to that of the standard mo-

Table 1. Purification of γ-glutamylamine cyclotransferase from soybean (*Glycine max*) seeds

Step	Total protein (mg)	Total activity (nmole/h)	Specific activity (nmole/h/mg)	Yield (%)	Purification (-fold)
Crude extract	26,000	4,160	0.16	100	1.0
Ammonium sulfate (45-75%)	7,140	3,045	0.43	73	2.7
DEAE-Sepharose	3,900	2,550	0.66	61	4.1
Acetone (50-75%)	1,312	2,528	1.93	60	12.1
Hydroxyapatite	450	1,202	2.70	29	16.9
S-300 gel column	30	500	16.70	12	104.4
Cu^{2+}-chelating agarose	1.5	160	106.70	3.8	666.9

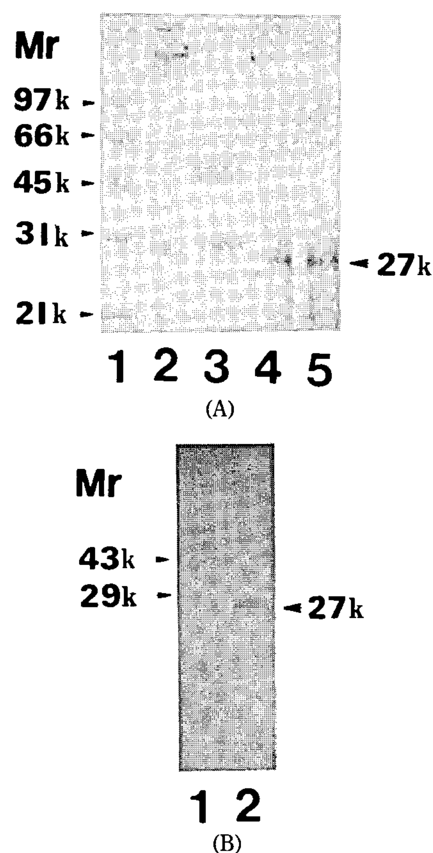


Fig. 2. Detection of purified enzyme by SDS-PAGE and by Western blot analysis. Panel A, the purified enzyme was analyzed by 12.5% SDS-PAGE and stained by silver staining. The molecular mass standards (Lane 1) on the leftside and the molecular mass of the purified enzyme on the rightside are indicated. Lane 3 is prestained marker (upper band: 43 kDa, lower band: 29 kDa). Lane 2, 4 and 5 represent the purified enzymes (5,10 and 20 μg, respectively). Panel B, the partially purified enzyme was subjected to 15% SDS-PAGE and the proteins were blotted onto PVDF membrane. The blot was developed and visualized as described in *Materials and Methods*. The molecular mass of the prestained marker (Lane 1) on the leftside and the molecular mass of the visualized band on the rightside are indicated.

nodansylcadaverine.

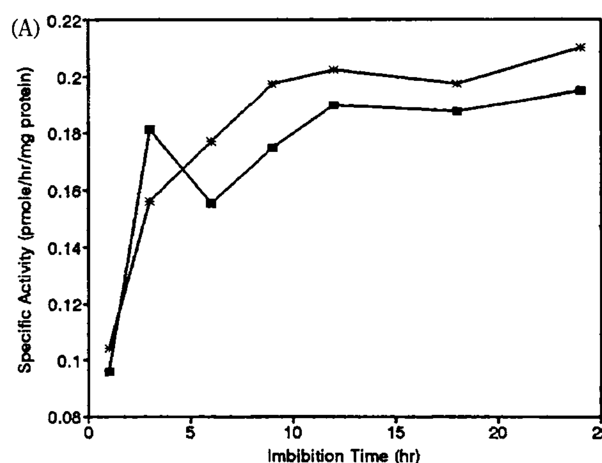
The purification results of γ-glutamylamine cyclotransferase are summarized in Table 1. The Cu^{2+}-chelating column in the purification procedure appeared to be highly specific and resulted in a high purification fold. The apparent molecular weight of native enzyme was determined to be 27,000 by Sephacryl S-300 gel filtration. SDS-PAGE showed a single band and the molecular weight of the enzyme subunit was 27,000 (Fig. 2-A). The partially purified enzyme was subjected to Western blot with rat antibody against this enzyme (Fig. 2-B). Western blot analysis confirmed that this antibody is specific for γ-glutamylamine cyclotransferase. In the rabbit kidney, the molecular weights of the enzyme were determined by the activity present in chromatographic fractions. The exclusion chromatographic properties of kidney enzyme were consistent with an apparent molecular weight of less than 25000 (Fink et al., 1980; Fink and Folk, 1983). Therefore, the size of the purified enzyme from soybean seeds is slightly larger than that from the a rabbit kidney's. With γ-glutamyldansylcadaverine as a substrate, the optimum pH and Km value were 8.6 and 11 μM, respectively. The effects of a number of modification reagents and divalent metal ions on γ-glutamylamine cyclotransferase activity are summarized in Table 2. Phenylmethylsulfonylfluoride that inhibits serine protease did not inhibits the enzyme. Pronounced loss in enzyme activity occurred when the enzyme was treated with p-chloromercuribenzoate. However, other reagents known to modify protein -SH groups, e.g., N-ethylmaleimide and 5,5'-dithiobis (2-nitrobenzoate), were without substantial effect on activity. These results were very similar to those obtain with the enzyme from the kidney. The addition of a Cu^{2+} ion somewhat inhibited the enzyme activity. On the contrary, other divalent metal ions had no effect on the enzyme activity. This enzymatic activity appeared to be present in the early stage after imbibition from the dormant state (Fig. 1-C). To determine whether concurrent protein synthesis is required for this enzyme activity in the dormant soybean seeds, seeds were soaked in dis-

Table 2. Effect of modification reagents and divalent metal ions on the γ-glutamylamine cyclotransferase activity

Treatment	Concentration (mM)	Relative Activity (%)
Control		100
N-ethylmaleimide	0.1	98
	1.0	95
5, 5'-dithiobis-(2-nitrobenzoate)	0.1	100
	1.0	99
p-Chloromercuribenzoate	0.1	45
	1.0	0
Phenylmethylsulfonylfluoride	0.1	100
	1.0	104
Phenylhydrazine	0.1	100
	1.0	100
Dithiothreitol	0.1	107
	1.0	98
Ca^{2+}	0.1	92
	1.0	80
Mg^{2+}	0.1	107
	1.0	117
Cu^{2+}	0.1	77
	1.0	55

Results represent the mean of three independent experiments.

(A)



(B)

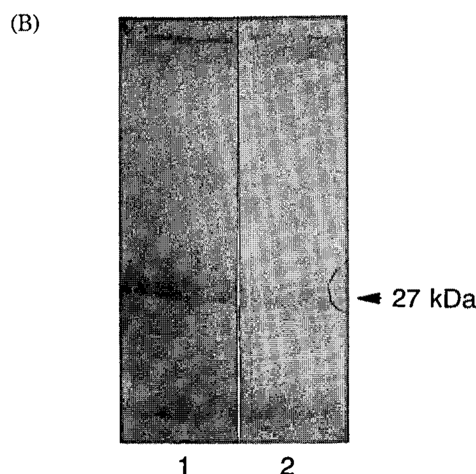


Fig. 3. Effect of cycloheximide on the γ-glutamylamine cyclotransferase specific activity (A) and of imbibition with distilled water on the enzyme synthesis in soybean seeds using Western blot analysis (B). Panel A shows the changes of enzyme specific activity as a function of the length of the imbibition periods of soybean seeds in distilled water (*) and distilled water containing cycloheximide (10 μg/ml) (■). Panel B, the proteins (150 μg per well) were extracted from soybean seeds at the dormant stage (1) and soaked in distilled water for 3 h (2). The dormant seeds were homogenized with 3 volumes of cold acetone. The homogenates were dried and resolubilized with enzyme homogenization buffer. Western blot analysis was performed as described in *Materials and Methods.*

tilled water containing cycloheximide (10 μg/ml) and harvested at various time intervals after inhibition. Control seeds were soaked in distilled water without cycloheximide and harvested at the same time intervals. When the enzymatic activity of seed extracts treated with cycloheximide was compared with that of the control seed extracts, there was no difference (Fig. 3-A). Furthermore, the same single protein band with the molecular weight of 27000 can be also detected in both dormant and germinating seed extracts by Western blotting (Fig. 3-B). Based on the above results, we suggest that γ-glutamylamine cyclotransferase is already present at the dormant stage and activated by unknown factors during germination in soybean seeds.

Acknowledgment

This work was supported by the Korean Ministry of Education (BSRI-95-4420)

References

Beninati, S., Piacentini, M., Argento-Ceru, M. P., Russo-Caia, S. and Autuori, F. (1985) *Biochim. Biophys. Acta* **841**, 120.

Beninati, S., Piacentini, M., Cocuzzi, E. T., Autuori, F. and Folk, J. E. (1988) *Biochim. Biophys. Acta* **952**, 325.

Cariello, L., Velasco, P. T., Wilson, J., Parameswaran, I. N., Karush, F. and Lorand, L. (1990) *Biochemistry* **29**, 5103.

Del Duca, S., Beninati, S. and Serafini-Fracassini, D. (1995) *Biochem. J.* **305**, 233.

Falcone, P., Serafini-Fracassini, D. and Del Duca, S. (1993) *J. Plant Physiol.* **142**, 265.

Fesus, L. and Tarcsa, E. (1989) *Biochem. J.* **263**, 843.

Fesus, L., Szucs, E. F., Barrett, K. E., Metcalfe, D. D. and Folk, J. E. (1985) *J. Biol. Chem.* **260**, 13771.

Fink, M. L. and Folk, J. E. (1983) *Methods Enzymol.* **94**, 451.

Fink, M. L., Chung, S. I. and Folk, J. E. (1980) *Proc. Natl. Acad. Sci. USA.* **77**, 4564.

Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 53-137, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kang, H. and Cho, Y. D. (1996) *Biochem. Biophys. Res. Commun.* **223**, 288.

Lorand, L. Murthy, S. N. P., Parameswaran, K. N., Velasco, P. T. and Wilson, J. (1992) *Biochem. Biophys. Res. Commun.* **186**, 334.

Piacentini, M. and Beninati, S. (1988) *Biochem. J.* **249**, 813.

Signorini, M., Beninati, S. and Bergamini, C. (1991) *J. Plant Physiol.* **137**, 547.