BIOLOGIA PLANTARUM 48 (4): 531-535, 2004

Purification and characterization of *S*-adenosyl-L-methionine nicotinic acid-*N*-methyltransferase from leaves of *Glycine max*

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Abstract

Trigonelline (TRG), which act as a cell cycle regulator and a compatible solute in response to salinity and water-stress, is the N-methyl conjugate of nicotinic acid the formation of which is catalyzed by S-adenosyl-L-methionine nicotinic acid-N-methyltransferase. The enzyme was purified 2650-fold from soybean (*Glycine max* L.) leaves with a recovery of 4 %. The purification procedure included ammonium sulfate (45 - 60 %) precipitation, linear gradient *DEAE-Sepharose* chromatography, adenosine-agarose affinity chromatography, hydroxyapatite chromatography and gel filtration (*Sephacryl-S-200*). The purified enzyme preparation showed a major band with a molecular mass of 41.5 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis that is related to the enzyme activity. The native enzyme had a molecular mass of about 85 kDa as estimated by gel filtration. The K_m values for S-adenosyl-L-methionine and nicotinic acid were 31 and 12.5 μM, respectively. The purified enzyme showed optimum activity at pH 6.5 and temperature of 40 - 45 °C. High concentration of dithiothreitol (10 mM) and glycerol (20 %) stabilize the enzyme during purification and storage. Hg^{2+} strongly inhibits enzyme activity.

Additional key words: alkaloid, enzyme purification, *Fabaceae*, soybean, trigonelline.

Introduction

The nicotinamide biochemical network is fundamental to cellular physiology and encompasses essential molecules such as NAD^+, $NADP^+$, nicotinamide, nicotinic acid and the alkaloid trigonelline (TRG) (Cho *et al*. 1999, 2001, Wood 1999). TRG is the *N*-methyl conjugate of nicotinic acid synthesized from nicotinic acid by *S*-adenosyl-L-methionine nicotinic acid-*N*-methyl-transferase (NNMT) (EC 2.1.1.7) (Joshi and Handler 1960). NNMT is enzymatically related to a number of methyltransferases in plants that utilize *S*-adenosyl-methionine as the methyl group donor (Hanson and Roje 2001). Although long considered a storage form of nicotinic acid, the ability of exogenous TRG to affect the plant cell cycle (Evans and Tramontano 1984, Mazzuca *et al*. 1997, 2000, Minorsky 2002) and mediate leaf movement (Ueda and Yamamura 1999) has been well documented. TRG is also an important component of flavor in roasted coffee (*Coffea arabica* L. and *Coffea canephora* L.).

The ability to synthesize and accumulate TRG is widely distributed in plants and has well been documented within members of the *Rubiaceae* (Joshi and Handler 1960) and *Fabaceae* (including *Glycine max*) (Tramontano *et al*. 1985, Ueda *et al*. 1995, Cho *et al*. 2003). In soybean, TRG and the amino acid proline have been shown to accumulate in response to NaCl- and water-stress and are postulated to function as compatible solutes and/or osmoprotectants (Evans and Tramontano 1981, Naidu *et al*. 1992, Wood 1999, Cho *et al*. 2001, Malenčić *et al*. 2002, Rai 2002). Recently, we have identified 2 quantitative trait loci (QTL) associated with

Received 30 December 2002, *accepted* 20 June 2003.
Abbreviations: NiA - nicotinic acid; NNMT - *S*-adenosyl-L-methionine nicotinic acid-*N*-methyltransferase; PAGE - polyacrylamide gel electrophoresis; PPFD - photosynthetic photon flux density; SAM - *S*-adenosyl-L-methionine; TRG - trigonelline.
Acknowledgments: This work was supported in part by grants to AJW from the Illinois Soybean Program Operating Board (98-ISPOB-24-212-3) and Illinois Council on Food and Agricultural Research (project number 00E-079-3).
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foliar TRG accumulation in field grown soybean (Cho *et al*. 2002). In order to more fully understand TRG biosynthesis in plants, we describe a 2 650-fold purification and biochemical characterization of NNMT from soybean leaves.

Materials and methods

Plants and chemicals: Soybean (*Glycine max* L. cv. Forrest) seeds were obtained from Dr. D.A. Lightfoot (Southern Illinois University, Carbondale, IL, USA). Soybean plants were grown on open benches in the Horticulture Research Center greenhouse (SIU): min. temperature of 22 °C, max. temperature of 29 °C; photosynthetic photon flux density (PPFD) of approximately 900 μmol m^{-2} s^{-1}, watered 1 - 2 times per day, and pulse fed to soil saturation once every four weeks with a N:P:K (20:10:20) fertilizer mix.

5'AMP-agarose (C-8 attachment), bovine alkaline phosphatase, S-adenosyl-L-methionine (SAM), nicotinic acid (NiA), nicotinamide, trigonelline (TRG), [7-^{14}C]-nicotinic acid (1.74×10^{12} Bq mol^{-1}), [7-^{14}C]-nicotinamide (1.74×10^{12} Bq mol^{-1}), *Dowex 1 × 8* (200 mesh, formate form), and gel filtration calibration standards (*MW-GF-200 kit*) were obtained from *Sigma Chemical Co*. (St Louis, MO, USA). *HiPrep 16/10 DEAE*-agarose, *Sephacryl S-200*, and *Sephadex G-25* (PD-10) were purchased from *Amersham Pharmacia Biotech* (Piscataway, NJ, USA). Hydroxyapatite (*Macro-Prep Ceramic Hydroxyapatite Type I*, 80 μm) was obtained from *Bio-Rad* (Richmond, CA, USA).

Enzyme assays and product identification: Enzyme activities were measured essentially as described by Upmeier *et al*. (1988) with some modification. The reaction mixture consisted of 0.1 cm^3 enzyme preparation, 50 nmol NiA containing 7.4×10^4 Bq [7-^{14}C]-NiA and 75 nmol SAM, 50 mM Tris-HCl (pH 7.0) in a total volume of 0.15 cm^3. The mixture was incubated for 1 h at 35 °C, stopped by boiling and denatured proteins were removed by centrifugation. The supernatant was applied to a 2 cm^3 *Dowex 1 × 8* (200 mesh) column equilibrated with deionized water. TRG was eluted by washing with 5 cm^3 deionized water. Radioactivity in this fraction was measured by liquid scintillation counting to quantitate the enzyme activity. The enzyme assay for nicotinamide amidohydrolase activity contained 0.1 cm^3 enzyme preparation, 100 nmol nicotinamide containing 7.4×10^4 Bq [7-^{14}C]-nicotinamide in a total volume of 0.15 cm^3 buffer B (20 mM Tris-HCl, 10 mM dithiotreitol (DTT), 20 % glycerol (v/v), pH 8.0). The enzyme reaction and separation of product were the same as described above. After eluting the remaining substrate with 15 cm^3 distilled water, the product could be eluted by 15 cm^3 8 M formic acid. Paper chromatography was performed as described by Joshi and Handler (1960) to identify TRG, NiA and nicotinamide. TRG product was also purified and quantified spectrophotometrically (A_{264}) using a *Cary Model 50 BIO UV-VIS* spectrophotometer (*Varian, Inc*., Palo Alto, California) as described by Cho *et al*. (1999).

Preparation of adenosine-agarose affinity column: 5'-adenosine-agarose was prepared as described by James *et al*. (1995). After transferring to a 1.5 × 10 cm column, the gel was washed with 100 cm^3 of 0.5 M NaCl and 100 cm^3 of water, and equilibrated with buffer B. For storage, ethanol was added to a final concentration of 20 % (v/v).

Enzyme purification: All solutions used for purification were made with deionized water and all the purification procedures were carried out at 4 °C. For extraction and ammonium sulfate fractionation soybean leaves were homogenized in a chilled mortar with buffer A (1 cm^3 g^{-1}(f.m.) (100 mM Tris-HCl, 20 mM DTT, 5 % glycerol (v/v), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and 10 % (m/m) quartz sand. The homogenate was filtered through four layers of cheesecloth and centrifuged at 14 000 *g* for 30 min. Solid ammonium sulfate was added slowly to the supernatant to a final 45 % saturation and stirred for another 20 min. Precipitated proteins were removed by centrifugation for 30 min at 14 000 *g*. Solid ammonium sulfate was added to the supernatant to obtain 60 % saturation and stirred for another 20 min. After centrifugation (14 000 *g*, 30 min), the pellet was dissolved in a minimum amount of buffer B and desalted on *Sephadex G-25* (PD-10, *Amersham Pharmacia Biotech*).

Ion exchange on DEAE-agarose: The desalted solution was applied to an anion ion exchange column (*HiPrep 16/10 DEAE*-agarose) previously equilibrated with buffer B. The column was washed with 100 cm^3 of buffer B and then eluted with 200 cm^3 linear gradient of NaCl (0 - 200 mM) in buffer B (1 cm^3 min^{-1}). Three cm^3 fractions were collected and fractions with high activity were pooled and concentrated by ultrafiltration (*Centriprep YM-3*, *Millipore*, Bedford, MA, USA) and subsequently desalted by PD-10.

Affinity chromatography on adenosine-agarose: The desalted sample was applied (0.5 cm^3 min^{-1}) to a 5 cm^3 adenosine-agarose column equilibrated in buffer B. The column was washed with buffer B until the $A_{280} < 0.05$. NNMT was eluted (0.5 cm^3 min^{-1}) with 30 cm^3 of buffer B containing 3 mM SAM. The eluate was concentrated

by ultrafiltration (*Centriprep YM-3*, *Millipore*) to about 2.5 cm^3.

Hydroxyapatite chromatography: The concentrated sample was changed into buffer C (5 mM potassium phosphate, 10 mM DTT, 20 % (v/v) glycerol, pH 8.0) by passing through PD-10 previously equilibrated with buffer C. The sample was applied to a hydroxyapatite column (*Macro-Prep Ceramic Hydroxyapatite Type I*, 80 μm). The column was washed with 50 cm^3 of buffer C and eluted with a 200 cm^3 linear gradient of potassium phosphate (5 - 150 mM; pH 7.2) in buffer C (1 cm^3 min^{-1}). Two cm^3 fractions were collected and fractions with high activity were pooled and concentrated by ultrafiltration.

Gel filtration on *Sephacryl S-200*: The concentrated sample was applied to a *Sephacryl S-200* column (1.5 × 1 000 mm) equilibrated with buffer B and eluted with buffer B at 0.15 cm^3 min^{-1}.

Protein assay and electrophoresis: Protein concentrations were estimated by the Bradford method (Bradford 1976) using the *Bio-Rad* protein assay with BSA as standard. SDS-PAGE was performed in mini-gels (12 % polyacrylamide) according to Laemmli (1970). Gels were stained for 30 min in 0.2 % (m/v) Coomassie Brilliant Blue R-250 in methanol: acetic acid: water (40:10:50) and destained in this solution without dye.

Table 1. Purification of NNMT from leaves of *Glycine max* L.

Purification step	Total protein [mg]	Total activity [pkat]	Special activity [pkat mg^{-1}]	Purification [fold]	Recovery [%]
Crude extract	878.8	1028.2	1.17	1	100
Ammonium sulfate	204.0	963.6	3.15	4	93
DEAE-agarose	11.8	408.5	34.6	30	39.5
Adenosine-agarose	0.23	168.2	731.3	625	16.3
Hydroxyapatite	0.068	64.7	951.5	813	6.3
Sephacryl S-200	0.014	43.4	3100	2650	4.2

Results and discussion

Upmeier *et al*. (1988) partially characterized S-adenosyl-L-methionine: nicotinic acid-N-methyltransferase (EC 2.1.1.7) (NNMT) from crude extracts of soybean leaves. Utilizing a combination of precipitation, adsorption chromatography and electrophoretic analytical methods we have dramatically improved NNMT purification and recovery. NNMT was purified > 2600-fold to near homogeneity from leaves of soybean (Table 1). Adenosine agarose affinity chromatography was the key step that resulted in a 21-fold purification. Using DEAE fractionation, the NNMT peak eluted at approximately 60 mM NaCl (Fig. 1*A*.). The active fractions (*i.e*. eluted fractions 19 - 31) were pooled and subjected to subsequent purification steps. Hydroxyapatite fractionation (Fig. 1*B*.) removed the majority of protein from the activity peak. The active fractions (*i.e.* eluted fractions 9 - 17) were pooled and subjected to gel filtration using *Sephacryl S-200*. Based upon gel filtration, native NNMT enzyme had a migration rate corresponding to that of an 85 kDa protein. The purified enzyme preparation was analyzed by SDS-PAGE and two proteins (M_r 41.5 and 39.0 kDa) were identified (Fig. 2). The polypeptide M_r = 41.5 kDa is correlated with NNMT enzymatic activity. NNMT has been partially characterized from crude extracts of soybean leaves with a relative molecular mass of 90 kDa for the non-denatured enzyme (Upmeier *et al*. 1988). Our results demonstrate that native NNMT consists of a homodimer of 41.5 kDa subunits.

Like other SAM utilizing methyltransferases (Harvima *et al*. 1985, James *et al*. 1995), NNMT is unstable during purification (Upmeier *et al*. 1988). In order to enhance enzyme stability we purified NNMT in the presence of elevated concentrations of DDT (10 mM) and glycerol (20 %, v/v) during both purification and storage of the purified enzyme. The purified enzyme was stable (> 90 % activity) in buffer B (20 mM Tris-HCl, 10 mM DTT, 20 % glycerol, pH 8.0) at 4 °C and -20 °C for 3 and 14 d, respectively. Enzyme stored in buffer B without DTT lost all activity within 24 h at 4 °C. Enzyme activity was effectively inhibited by 0.1 mM para-hydroxymercuric benzoate and 1 mM Hg^{2+}, and the inhibition was reversed by 10 mM DTT. The optimum temperature was found to be between 40 and 45 °C with 50 % activity detected at 50 °C. Preincubation of NNMT in buffer B for 1 h at 40 and 45 °C did not decrease activity, while incubation of the purified enzyme in Tris buffer without DTT led to significant activity loss. These results suggest that added thiol groups are necessary for NNMT stability and activity.

TRG was clearly identified by paper chromatography and no methyl-nicotinamide was identified when using

nicotinamide instead of NiA. Absorbance (A_{264}) quantification of the purified TRG product (Cho *et al*. 1999) agreed with radiolabeled scintillation quantification confirming that purified NNMT catalyzed nicotinic acid methylation but not nicotinamide methylation. The K_m value for S-adenosyl-L-methionine (SAM) was 31 μM in the presence of 0.2 mM nicotinic acid. The K_m value for nicotinic acid was 12.5 μM in the presence of 0.3 mM SAM. The pH activity curve of NNMT was examined ranging from pH 4.13 to pH 8.8. NNMT demonstrated an optimum around pH 6.5 with 50 % activity at pH 5.4 and pH 7.5 (data not shown) that is in agreement with values reported by Joshi and Handler (1960).

Purified NNMT has low K_m for nicotinic acid (12.5 μM) and high substrate specificity, and only nicotinic acid was found to be the substrate from a wide selection of pyridine derivatives (Upmeier *et al*. 1988). We postulate that NNMT activity *in vivo* plays a key role in regulating flux through the nicotinamide metabolic pathway (Wood *et al*. 2000, Cho *et al*. 2001, Pfeiffer *et al*. 2001). Further research will characterize the 41.5 kDa protein, including determining the amino acid residue sequence, with the long-term goal of cloning the corresponding gene from soybean and other economically important crops.

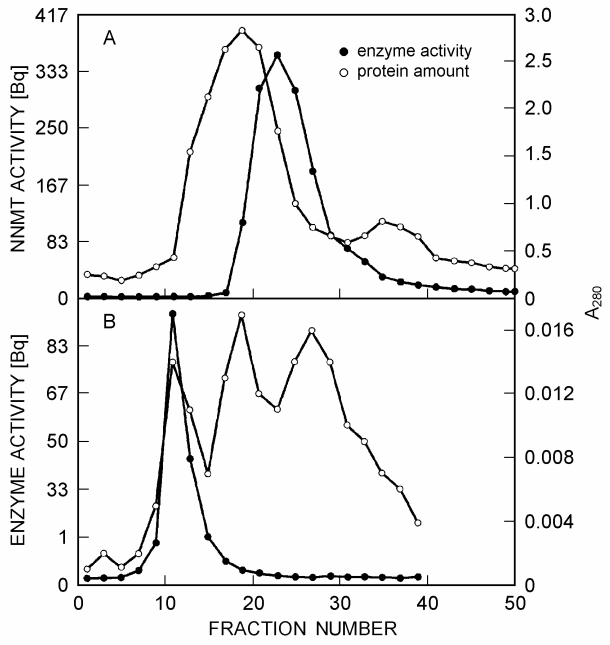

Fig. 1. Elution profile of *S*-adenosyl-L-methionine nicotinic acid-*N*-methyltransferase (NNMT) from DEAE-agarose (*A*) and hydroxyapatite (*B*) column chromatography. Either a linear potassium phosphate (*A*) or NaCl (*B*) gradient was utilized. Enzymatic activity and absorbance was determined as described in Materials and methods. *Open circles* represent protein amount (A_{280}) and closed circles represent enzyme activity [Bq]

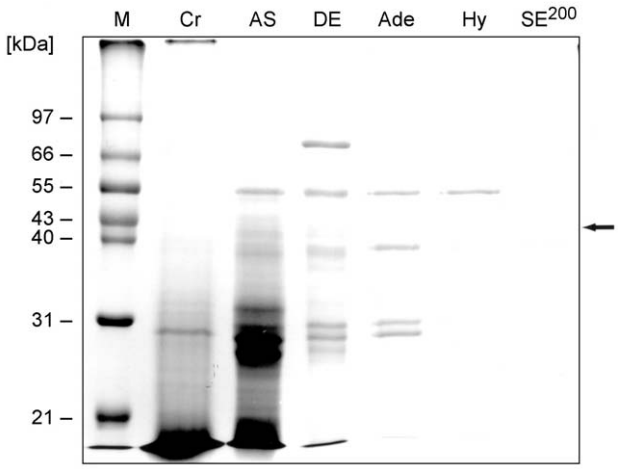

Fig. 2. SDS-PAGE analysis of protein at each stage of the purification process: M - protein standards, Cr - crude extract, AS - ammonium sulfate, DE - DEAE-agarose, Ade - adenosine-agarose, Hy - hydroxyapatite, SE^{200} - *Sephacryl S-200*. 0.02 cm^3 of each fraction was analyzed by SDS-PAGE (12 % gels) and stained with Coomassie Brilliant Blue.

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