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ISOLATION AND CHARACTERIZATION OF A NITRIC OXIDE SYNTHASE (NOS)-LIKE PROTEIN OF PEA (*PISUM SATIVUM L.*)

[1]Wong Mui-Yun,[*] Huang Jeng-Sheng and [2]Eric L. Davis

[1]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Malaysia
[2]Department of Plant Pathology, North Carolina State University, Raleigh, NC27695-7616, USA

Abstrak: Aktiviti nitrik oksida sintase (NOS) berdasarkan pembentukan asal cerakin sitrulina yang digunakan dalam sistem mamalia, dapat dikesan dalam ekstrak *Pisum Sativum L.* (kacang manis). Protein seakan-NOS dalam kacang manis diekstrak paling efisien menggunakan penimbal ekstrak yang ditambah penghalang protease [ethylene bis (oxyethylenenitrilo)tetraacetic acid (EGTA) dan leupeptin)] dan dalam keadaan alkali (pH 8.5–9.0) berbanding keadaan neutral dalam sistem mamalia. Pemendakan protein menggunakan pelbagai kepekatan garam ammonium sulfat, natrium sitrat dan natrium klorida menyebabkan kehilangan aktiviti NOS, berbeza dengan sistem mamalia, dan ia tidak dimendakkan oleh pelarut organik (aseton atau polietilena glikol, PEG). Protein seakan-NOS dalam kacang manis telah diasingkan dengan jayanya menggunakan turus pertukaran-ion, tetapi ia tidak terikat kepada turus β-nicotinamide adenine dinucleotide phosphate (NADPH) dan kalmodulin yang menunjukkan kekurangan tapak untuk mengikat kofaktor NADPH dan kalmodulin yang diperlukan oleh aktiviti NOS dalam sel mamalia. Keputusan ini menunjukkan bahawa protein seakan-NOS dalam kacang manis adalah berbeza daripada NOS mamalia dari segi struktur.

Abstract: Nitric oxide synthase (NOS) activity based on citrulline formation assay, which was used in mammalian system, was detected in *Pisum Sativum* L. (pea) extracts. The pea NOS-like protein was most efficiently extracted with the addition of protease inhibitors (ethylene bis (oxyethylenenitrilo)tetraacetic acid (EGTA) and leupeptin) in the extraction buffer and under alkaline condition (pH 8.5–9.0) as compared to neutral condition in mammalian system. The precipitation of this protein with various concentrations of ammonium sulfate, sodium citrate and sodium chloride caused rapid loss of NOS activity, in contrast to that in the mammalian system, and the protein was not precipitated by organic solvents (acetone or polyethylene glycol, PEG). The pea NOS-like protein was successfully isolated using ion-exchange column, but did not bind to β-nicotinamide adenine dinucleotide phosphate (NADPH) and calmodulin affinity columns suggesting that it lacked binding sites for the cofactors NADPH and calmodulin that were required for NOS activity in mammalian cells. The results indicated that the pea NOS like protein was significantly different in structure from mammalian NOS.

Keywords: Nitric Oxide Synthase, NOS, Pea

[*]Corresponding author: muiyun@putra.upm.edu.my

INTRODUCTION

Nitric oxide (NO) is a multifunctional signal molecule in both animal and plant cells. In mammalian cells, NO acts as a signal molecule in neurotransmission in the nervous system; displays antimicrobial, antitumor and cytotoxicity properties in the immune system; and a regulator of blood pressure and gatekeeper of blood flow to different organs in the cardiovascular system (Bredt & Snyder 1994). In plant cells, NO is involved in growth and developmental processes such as leaf-expansion and regulation of stress responses (Leshem & Haramaty 1996), root elongation (Gouvea *et al*. 1997), and maturation and senescence (Leshem *et al*. 1998). It is also involved in defense responses such as induction of hypersensitive response (HR), expression of defense genes and accumulation of phytoalexin (Durner *et al*. 1998; Noritake *et al*. 1996).

In mammalian cells, the production of NO is catalyzed by nitric oxide synthase (NOS) which occur in three isoforms and named on the basis of the tissue source from which they were originally extracted: neuronal NOS (nNOS), also known as Type I, inducible NOS in macrophages (iNOS), Type II and endothelial NOS (eNOS), Type III (Nathan & Xie 1994). All NOS isoforms show 50%–60% identity in their amino acid sequences (Wendehenne *et al*. 2001). Each NOS is a bi-domain enzyme consisting of an N-terminal oxygenase and a C-terminal reductase (Alderton *et al*. 2001). The oxygenase domain contains a cytochrome P-450 type heme center and a binding site for the cofactor tetrahydrobiopterin (BH_4). The reductase domain contains NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) binding sites. Both oxygenase and reductase domains are connected by a calmodulin (CaM) binding site in the middle of the enzyme. Each NOS has a different N-terminal extension determining the intracellular localization of the enzyme. In the active form, all NOS enzymes are homodimers.

A key step in purification of the three isoforms of NOS includes 2',5'-ADP affinity chromatography eluted with NADPH (Bredt & Snyder 1990; Forstermann *et al*. 1991; Stuehr *et al*. 1991). nNOS, eNOS and iNOS have a molecular mass of approximately 160, 135 and 130 kDa respectively. Both nNOS and eNOS require Ca^{2+}/CaM, NADPH and BH_4 for their activity. On the contrary, iNOS requires NADPH, BH_4, FAD, FMN but not Ca^{2+}/CaM for its activity. Other than mammalian cells, NOS proteins have also been isolated and purified from the bacteria *Nocardia* sp. (Chen & Rosazza 1995), insects (Regulski & Tully 1995), snails (Huang *et al*. 1997), the fungus *Flammulina velutipes* (Song *et al*. 2000) and the slime mold (*Physarum polycephalum*) (Golderer *et al*. 2001) using various types of liquid chromatography such as ion-exchange, affinity, gel filtration and chromatofocusing.

Although NOS activity has been documented in plants (Delledonne *et al*. 1998), the process of NO synthesis in plants is not well understood. Isolation of a NOS protein and/or cloning of the corresponding gene will greatly facilitate the understanding of NO synthesis and its role in plant defense against pathogen infection. However, these efforts have remained a challenge until recently when one plant protein demonstrating NOS activity was identified, known as AtNOS1 (Guo *et al*. 2003). The constitutively expressed AtNOS1 protein from *Arabidopsis*

thaliana has sequence similarity to the protein implicated in NO synthesis in the snail *(Helix pomatia)* (Huang *et al*. 1997) and was related to NO production in hormonal response in plants. The gene encoding AtNOS1, however, has no significant similarity to any gene encoding mammalian NOS.

There may be multiple forms of NOS-like protein in plants that differ from their counterparts in animal cells. Isolation of a NOS-like protein in pea will provide another source for comparison of the characteristics of NOS-like protein in plants. In addition, isolation of a NOS-like protein may be necessary in order to successfully clone the corresponding genes since the nucleotide sequence of NOS in plants has limited similarity to animal NOS. The objective of this study was to isolate and characterize a NOS-like protein in pea as pea tissues demonstrated the highest NOS activity as compared to soybean, tobacco, tomato, pepper, corn or *Arabidopsis thaliana* (Huang, pers. comm.).

MATERIALS AND METHODS

Materials

Pisum Sativum L., cultivar Alaska (pea) seeds were obtained from Floridata Marketplace (Tallahassee, USA). Tris-HCl, ethylene diamine tetraacetic acid (EDTA), ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), leupeptin, phenylmethylsulfonyl fluoride (PMSF), polyvinylpolypyrrolidone (PVPP), glycerol, bromophenol blue, FAD, FMN, BH_4, NADPH, CaM, calcium dichloride ($CaCl_2$), nonradio-labeled arginine, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), dithiothreitol (DTT), 2-mercaptoethanol, ρ-Aminobenzamidine and ε-Amino-n-caproic acid, ammonium sulfate, sodium citrate, sodium chloride, acetone, polyethylene glycol (PEG), Phosphodiesterase 3',5'-Cyclic Nucleotide Activator Agarose, sodium dodecyl sulphate (SDS), glycerol, and Coomassie brilliant blue R-250 were obtained from Sigma Chemical Co. (St. Louis, USA). Protein assay reagent was obtained from Bio-Rad Laboratories (Hercules, USA) and bovine serum albumin (BSA) from Merck & Co. (Whitehouse Station, USA). Dowex AG50W X-8 (Na^+ form) and liquid scintillation cocktail were obtained from Fluka (Seelze, Germany). Radio-labeled L-[U-^{14}C]arginine, Diethylaminoethyl (DEAE)-Sepharose Fast Flow, 2', 5' Adenine diphosphate (ADP) Sepharose 4B and ARG-Sepharose 4B were obtained from Amersham Biosciences (Piscataway, USA).

Effects of pH, Protease Inhibitors and Chelator on NOS Activity

Total protein was extracted from one gram of healthy leaf tissues of *Pisum Sativum*, cv Alaska (pea) in extraction buffer which contained 50 mM Tris-HCl at either pH 7.0, 7.5, 8.0, 8.5, or 9.0. Buffer at the pH optimum for NOS activity was used subsequently to homogenize one gram of leaf tissues in one of the following protein extraction treatments that included: dithiothreithol (DTT, 1 mM) and 2-mercaptomethanol (10 μl/10ml) as reducing agents; EDTA (1 mM) and EGTA (10 mM) as metallo-protease inhibitors; ρ-Aminobenzamidine (1 mM), ε-Amino-n-caproic acid (5 mM), leupeptin (1 μM) and PMSF (1 mM) as protease inhibitors, and polyvinylpolypyrrolidone [PVPP, 1% (w/v)] as phenolic chelator. NOS activity

in the supernatant of crude protein extract from each treatment was determined as described below.

Protein Extraction and NOS Activity Assay

Leaf tissues (0.5–1.0 g) in each experiment were ground in liquid nitrogen and homogenized in two volumes (1–2 ml) of modified extraction buffer which contained 50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 1 µM leupeptin, 1 mM PMSF and 1% (w/v) PVPP at pH 8.5 and perform at 23ºC–25ºC. The homogenate was filtered through one layer of Miracloth and centrifuged at 4°C for 30 minutes at 20,000 g. Protein concentration of supernatant from the crude extracts was determined using protein assay reagent and BSA as standard. NOS activity in all preparations was determined by the conversion of L-[U-^{14}C]arginine to L–[U-^{14}C]citrulline using the method of Bredt and Snyder (1990) with modifications (Wong *et al*. 2006). Specific activity was expressed as picomole of L–[U-^{14}C]citrulline min^{-1} mg^{-1} protein after subtracting the background value, that is the value of similarly prepared reaction mixture terminated immediately after adding protein sample. Activity detection of each sample was replicated three times.

Precipitation of NOS-Like Protein of Pea

Total protein was extracted from healthy leaf tissues as described above at pH 8.5 and precipitated using both salts and organic solvents. Concentrations of 0.5, 1.0, 1.5 and 2.0 M of ammonium sulfate, sodium citrate and sodium chloride; 10, 20, 30, 40 and 50% (v/v) acetone; and 10, 20 and 30% (w/v) PEG were added separately in each sample and centrifuged at 4°C for 30 minutes at 20,000 g. Pellets were re-suspended in 200 µl of protein extraction buffer. NOS activity in the supernatants, re-suspended pellets and resuspended pellets added with small molecular weight (SMW) filtrate (<8 kDa) were determined. SMW filtrate was prepared from supernatants of crude leaf extracts filtered through Centricon® Plus-20 cartridges (Amicon, Millipore Corp., Bedford, MA) and included as a treatment because it significantly enhanced NOS activity in previous assays (Huang, pers. comm.). The experiment was conducted with two replicates.

Liquid Chromatography

Total protein was extracted as described above from 70 g of leaf tissues, and the supernatant was passed through an ion-exchange column consisting DEAE-Sepharose Fast Flow packed into a Pharmacia column (2.6 × 10 cm) according to the manufacturer's protocol at a flow rate of 66 ml/h and equilibrated with equilibration buffer [50 mM Tris-HCl (pH 8.5), 10 mM EGTA, 1 µM leupeptin, and 1 mM PMSF]. The protein was eluted with a linear gradient of 0 to 0.3 M NaCl in equilibration buffer. Fractions of 3.4 ml were collected and each fraction was assayed for NOS activity and total protein content as described above. Fractions with NOS activity eluded from the DEAE column were pooled and concentrated in Centricon® Plus-20 cartridges to half the original volume. The pooled, concentrated sample containing NOS-like protein was subjected to NADPH affinity chromatography consisting 2',5'-ADP-Sepharose 4B packed according to the manufacturer's protocol at a flow rate of 1.8 ml/h, equilibrated in binding

buffer containing 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 10 mM EGTA and 1 μM leupeptin, and recirculated through the column 5–7 times. The protein was eluted with binding buffer containing 10 mM NADPH. Fractions of 3.4 ml were collected and each fraction was assayed for NOS activity and total protein content as described above. In addition to NADPH affinity column, eluted, pooled and concentrated protein samples with NOS activity obtained from DEAE chromatography were passed through either an ARG-Sepharose 4B affinity column (for the isolation of proteins with arginine binding sites) or a Phosphodiesterase 3',5'-Cyclic Nucleotide Activator Agarose affinity column (for the isolation of proteins with calmodulin binding sites) according to the manufacturer's protocol and recommendations. All fractions (3.4 ml) collected were assayed for NOS activity and total protein content as described above. All isolation steps were carried out in a chromatography chamber maintained at 4°C to 8°C. The isolation was repeated at least three times and the best result was presented in this report.

SDS-PAGE

Samples of total proteins extracted from leaf tissues as described above were separated by 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEAN II slab cell (Bio-Rad, Hercules, CA). Loading samples (100 μg) were prepared in 0.5 M Tris-HCl (pH 6.8), containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-Mercaptoethanol and 0.125% (w/v) Bromophenol Blue, and were heated at 95°C for 4 minutes. Gel was run at 180 V for 45 minutes and visualized by staining with 0.1% (w/v) Coomassie brilliant blue R-250. The experiment was repeated twice.

Experimental Design and Statistical Analysis

All treatments with quantitative dependent variables were replicated three times in a completely randomized experimental design. Data was analyzed using SAS program version 9.1 (SAS Institute, Cary, NC) and General Linear Models (GLM) procedure. Analysis of variance (ANOVA) was determined using the Least Squared Means and Waller-Duncan k-ratio t test procedures at P = 0.05 level.

RESULTS

NOS activity detected was significantly higher when crude protein extracts were assayed at pH 8.5 and 9.0 (8.5 and 8.6 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein, respectively) [Fig. 1(a)] as compared to those at pH 8.0, 7.5 and 7.0 (7.0, 5.9 and 5.9 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein, respectively). In subsequent experiments, pH 8.5 was selected to be used due to its optimum NOS activity. At pH 8.5, NOS activity was increased significantly by the addition of EGTA or leupeptin to the homogenization buffer (10.5 and 10.8 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein, respectively) compared to other treatments including the control where no chemical was added (6.0 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein) [Fig. 1(b)]. In contrast, the addition of 2-Mercaptoethanol or ρ-Aminobenzamidine and ε-Amino-n-caproic acid suppressed NOS activity (4.2 and

5.0 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein, respectively) as compared to the control [Fig. 1(b)].

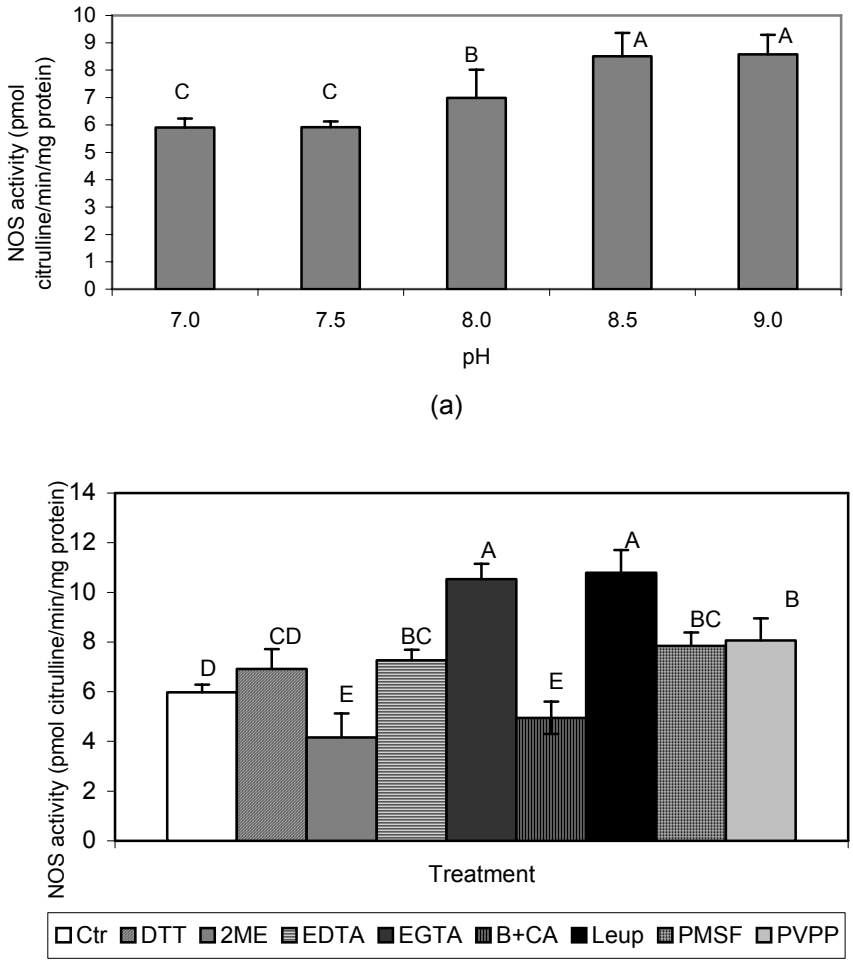
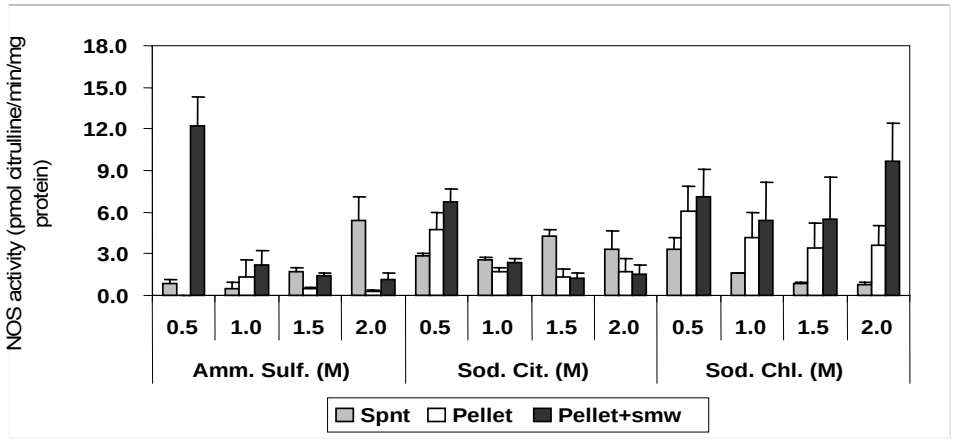


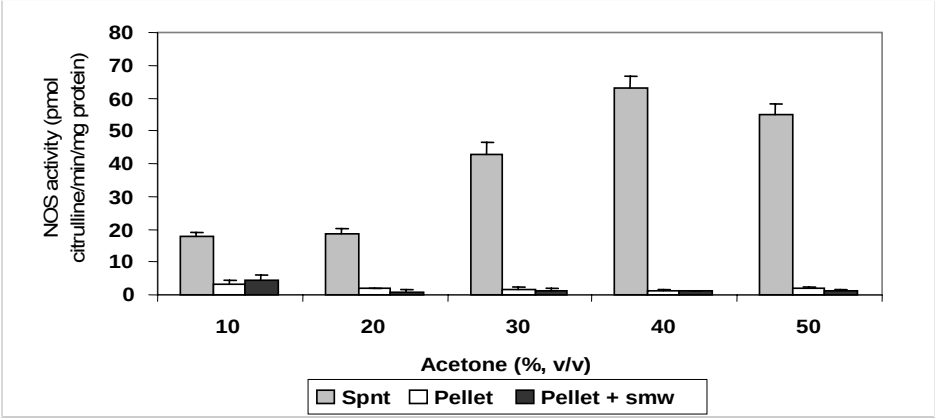
Figure 1: Effects of (a) pH (b) reducing agents, metal chelators, protease inhibitors and phenolic chelator on NOS activity during protein extraction at 23ºC–25ºC. Error bars indicate standard deviation (n = 3). Means with the same letter are not significantly different at $P < 0.05$. Ctr, control; 2ME, 2-mercaptomethanol; EDTA; EGTA; B+CA, ρ-Aminobenzamidine and ε-Amino-n-caproic acid; Leup, leupeptin; PVPP.

Precipitation of crude protein extracts with ammonium sulfate, sodium citrate or sodium chloride at concentrations of 0.5, 1.0, 1.5 and 2.0 M caused substantial loss of NOS activity as compared to that in crude extract [Fig. 2(a)]. Precipitation with various concentrations of ammonium sulfate, sodium citrate and sodium chloride increased NOS activity by 37%–77%, 30%–31%, and 16%–63%, respectively, after adding SMW filtrate to the pellets. In contrast to precipitation with salts, precipitation of crude protein extracts with various concentrations of acetone and PEG resulted in NOS activity being detected mostly in the supernatant and minimally in the pellets [Fig. 2(b) & 2(c)]. Precipitation with 40% (v/v) acetone resulted in the highest increase in NOS activity (63.2 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein or 4.7-fold) in the supernatant as compared to control (13.5 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein).

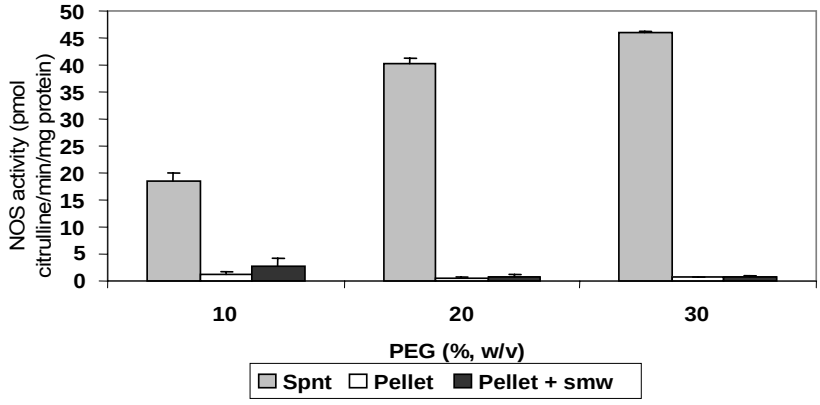


(a)

Figure 2: Precipitation of pea NOS-like protein with various concentrations of (a) salts, (b) acetone and (c) PEG. NOS activity for crude extract in experiment A was 18.5 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein, and for experiments B and C were 13.5 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein. Error bars indicate standard deviation (n = 2). Spnt: supernatant; smw: small molecular weight filtrate. *(Continue on next page)*



(b)



(c)

Figure 2: *(continued)*

NOS activity increased from 16 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein in the crude extracts to 255 pmol L-[U-^{14}C]citrulline min^{-1} mg^{-1} protein in the DEAE-Sepharose eluded fractions that represented purification of 16-fold (Table 1). Treatment with acetone was substituted with ethanol due to the acetone-sensitivity of Centricon® Plus-20 cartridges in the concentration process. Treatment with 40% (v/v) ethanol gave a similar result in NOS activity as that with 40% (v/v) acetone (data not shown). Two protein peaks were observed in the DEAE-Sepharose elution profile, but NOS activity was detected only in the first peak (Fig. 3). Fractions with NOS activity were eluded at between 0.135 to 0.20 M NaCl. Pea NOS-like protein did not bind to any of the affinity column tested (2',5'-ADP-Sepharose 4B, ARG-Sepharose 4B and Phosphodiesterase 3',5'-Cyclic Nucleotide Activator Agarose) as all NOS activity was detected in the flow-

through (data not shown). SDS-PAGE revealed the presence of more than 20 proteins, including NOS-like protein, in the DEAE-Sepharose eluded fractions (data not shown).

Table 1: Partial purification of pea NOS-like protein, repeated at least three times and the best result was presented here.

Step	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol/mg/min)	Recovery (%)	Purification (-fold)
Crude extract	689	11024	16	100	1
Treatment with 40% ethanol	246	7626	31	69	2
DEAE-Sepharose	136	6231	255	56	16

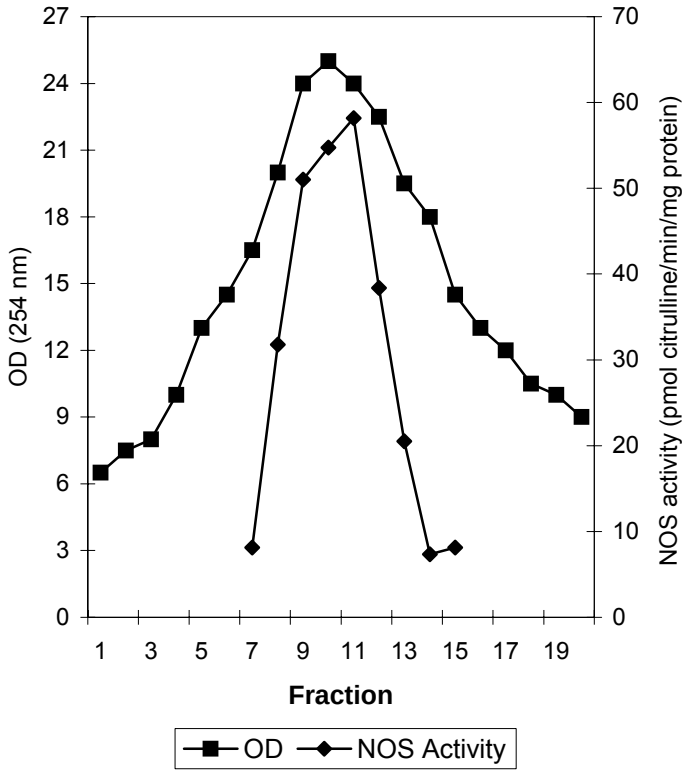


Figure 3: Isolation of pea NOS-like protein using DEAE-Sepharose liquid chromatography.

Note:
OD: Optical Density

DISCUSSIONS

In this report, NOS activity was optimal when protein extractions were performed at pH 8.5 and 9.0, and at 23°C–25°C. These conditions differed from those in the fungus *Flammulina velutipes* (pH 8.0 at 50°C), the bacterium *Norcadia* sp. (pH 7.0–7.5 at 30°C), and mammals (pH 7.5–7.8 at 37°C) (Song *et al*. 2000; Chen & Rosazza 1995; Hevel *et al*. 1991; Stuehr *et al*. 1991). The findings indicated that different species of NOS protein performed optimally at different conditions. NOS-like protein of pea was efficiently extracted under alkaline condition.

NOS protein extractions from various organisms typically include a cocktail of protease inhibitors, metal ion chelators, reducing agents, and phenolic chelators (Bredt & Snyder 1990; Chen & Rosazza 1995; Song *et al*. 2000; Golderer *et al*. 2001). These chemicals are important components in the isolation process and they protect the protein of interest from proteolysis and oxidation (van Renswoude & Kempf 1984). In this work, adding 10 mM EGTA and 1 mM EDTA (metal ion chelator and metalloprotease inhibitor respectively), 1 μM leupeptin and 1 mM PMSF (serine and thiol protease inhibitors respectively) and 1% (w/v) PVPP (phenolic chelator) in the extraction buffer retained NOS activity significantly. This was especially important in the isolation of NOS-like protein of pea since the protein lost its activity rapidly in each step of the isolation process. Using high doses of EGTA (10 mM) also helped to disrupt and extract membrane-associated proteins (van Renswoude & Kempf 1984).

The precipitation of the NOS-like protein of pea with various concentrations of salts (ammonium sulfate, sodium citrate, and sodium chloride) was either minimal or the salts caused the precipitated NOS-like protein to lose activity rapidly. The protein did not seem to be present in the supernatant because NOS activity was only 2.5%–29% of that in crude extract. On the contrary, NOS proteins of *Physarum polycephalum*, *Flammulina velutipes*, and animals were successfully precipitated with 30%–75% of ammonium sulfate and with minimal loss in NOS activity (Golderer *et al*. 2001; Song *et al*. 2000; Hevel *et al*. 1991; Stuehr *et al*. 1991).

Substantial increase in NOS activity in the supernatant was possibly due to the release of NOS-like protein bound to the membranes when a high dose of EGTA (10 mM) was used during protein extraction. Minimal activity in the pellets after precipitation with organic solvents (acetone and PEG) suggested that NOS-like protein of pea was membrane-bounded. Proteins that are not precipitated by organic solvents are categorized as hydrophobic proteins, particularly those that are located in the cellular membranes (Bollag *et al*. 1996). The result indicated that the NOS-like protein of pea might be located within cellular membranes and further work need to be done to verify this indication. In mammals, nNOS is typically localized in neurons in both soluble and particulate forms, iNOS in macrophages in soluble form, while eNOS in endothelial cells in particulate form (Bredt & Snyder 1990; Pollock *et al*. 1991; Stuehr *et al*. 1991). An isoform of NOS localized in the inner membrane of rat liver mitochondria was also reported (Tatoyan & Giulivi 1998). Barroso *et al*. (1999) demonstrated that a NOS-like protein of pea was localized in the matrix of peroxisomes (designated as perNOS) and chloroplasts using electron microscopic examination of

immunogold-labeling with antibody against murine iNOS. Another NOS-like protein was detected either in the cytosol of cells in the division zone or nucleus of cells in the elongation zone of maize root tips (depending on the growth phase of cells) using antibody against mouse macrophage NOS labeled with fluorescein isothiocyanate (FITC) (Ribeiro *et al*. 1999).

The purification of mammalian NOS has been conducted with relative ease using 2',5'-ADP-Sepharose affinity chromatography after crude sample preparation with ammonium sulfate precipitation or ion-exchange chromatography (Tatoyan & Giulivi 1998; Bredt & Snyder 1990; Pollock *et al*. 1991; Stuehr *et al*. 1991). NOS proteins from other species (*Nocardia* sp., *Flammulina velutipes* and *Physarum polycephalum*) have been purified using a combination of chromatography techniques. Song *et al*. (2000) reported that NOS of *Flammulina velutipes* did not bind to an anion exchanger and purification of *Nocardia* NOS was more successful using hydroxylapatite chromatography than anion-exchange or gel filtration chromatography as a final purification step, in addition to 2',5'-ADP-agarose chromatography (Chen & Rosazza 1995). In this work, the NOS-like protein of pea bound to DEAE-Sepharose ion-exchange column and was eluted successfully with a linear gradient of 0.135 to 0.20 M NaCl. However, the protein did not bind to any of the affinity column tested, i.e., 2',5'-ADP-Sepharose, ARG-Sepharose or calmodulin-agarose. Similarly, Lo *et al*. (2000) found that homogenates of 3 day-old mung bean roots did not bind to 2',5'-ADP-Sepharose or ARG-Sepharose. The fact that NOS-like protein of pea did not bind to 2',5'-ADP-Sepharose and calmodulin-agarose indicated that the protein lacked binding sites for cofactors NADPH and CaM. The findings suggested that pea NOS-like protein may be significantly different in structure from mammalian NOS. Such difference was also reported in a NOS protein identified in the model plant *Arabidopsis thaliana* (AtNOS1). AtNOS1 was constitutively expressed and its activity was dependent on the presence of NADPH, Ca^{2+} and CaM, but not H_4B, FAD, FMN or heme (Guo *et al*. 2003). The cDNA sequence has homology to a sequence in the *Helix pomatia* (snail) that encodes a protein implicated in NO synthesis and contains ATP/GTP binding domains (Huang *et al*. 1997). However, the gene encoding AtNOS1 has no significant similarity to any gene encoding mammalian NOS.

The challenge of isolating a NOS protein from plant is increasingly evident as shown in a couple of reports. Although perNOS could be detected in the peroxisomes and chloroplasts of pea (Barroso *et al*. 1999), to date neither its purification nor the identification of the gene encoding the protein has been reported. In addition, although a NOS gene has been identified in *Arabidopsis* through screening of mutants (Guo *et al*. 2003), the protein itself was not purified from crude extracts. Using isolation methods employed in animal system was not helpful in isolating a NOS protein from plant and alternative techniques need to be explored.

Another challenge in isolating a pea NOS-like protein was the rapid loss of activity observed during the isolation process. This may be due to the disruption of protein configuration or the dissociation of some interacting proteins or unidentified cofactors. In this report, adding the SMW filtrate of pea leaf extracts to the proteins pelleted after precipitation by various concentrations of

salts restored NOS activity at various degree of significance. The SMW filtrates might contain unidentified cofactors required for NOS activity which were dissociated from the pea NOS-like protein during the isolation process. Huang *et al*. (1997) also reported that the activity of a NOS protein isolated from the *Helix pomatia* (snail) might require interactions with other associated proteins. In mammalian cells, NOS activity was affected by the interactions of the enzyme with more than 20 interacting proteins (Nedvetsky *et al*. 2002). For example, CaM binding was required for electron transfer (Bredt & Snyder 1990), NOSTRIN (NOS3 traffic inducer) binding was important for intracellular trafficking of NOS3 (Zimmermann *et al*. 2002) and Dynamin-2, Porin and protein kinase B/Akt were required for activation (Sun & Liao 2002; Cao *et al*. 2001; Dimmeler *et al*. 1999). The stability of NOS homodimers was also affected by protein-protein interactions. For example, the binding of PIN (a protein inhibitor of NOS1) to NOS1 or NAP110 (a protein inhibitor of NOS2) to NOS2 prevented dimerization of these enzymes, a configuration required for activation (Ratovitski *et al*. 1999; Jaffrey & Snyder 1996).

In summary, the pea NOS-like protein was efficiently extracted from leaf tissues at pH 8.5 and 9.0. The addition of two protease inhibitors, EGTA and leupeptin, to the homogenization buffer significantly increased NOS activity at pH 8.5. Precipitation of the pea NOS-like protein with various concentrations of salts caused rapid loss of NOS activity and the protein was not precipitated by organic solvents. The pea NOS-like protein was purified 16-fold and the partial purification steps included treatment of crude extracts with 40% ethanol, and passing the supernatant of the treated sample through an ion exchange DEAE-Sepharose column and eluting bound proteins (including pea NOS-like protein) with a linear gradient of 0.135–0.2 M NaCl. The pea NOS-like protein did not bind to any affinity columns tested which were 2',5'-ADP-Sepharose, ARG-Sepharose and calmodulin-agarose, suggesting that the protein lacked binding sites for cofactors NADPH and CaM.

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REFERENCES

Alderton W K, Cooper C E and Knowles R G. (2001). Nitric oxide synthases: Structure, function and inhibition. *Biochem. J.* 357: 593–615.

Barroso J B, Corpas F J, Carreras A, Sandalio L M, Valderrama R, Palma J M, Lupianez J A and del Rio L A. (1999). Localization of nitric-oxide synthase in plant peroxisomes. *J. Biol. Chem.* 274: 36729–36733.

Bollag D M, Rozycki M D and Edelstein S J. (1996). *Protein methods.* 2nd Ed. New York: Wiley-Liss, 7–24.

Bredt D S and Snyder S H. (1990). Isolation of nitric oxide synthethase, a calmodulin-requiring enzyme. *Proc. Nat. Acad. Sci.* USA 87: 682–685.

Bredt D S and Snyder S H. (1994). Nitric oxide: A physiologic messenger molecule. *Annu. Rev. Biochem.* 63: 175–195.

Cao S, Yao J, McCabe T J, Yao Q, Katusic Z S, Sessa W C and Shah V. (2001). Direct interaction between endothelial nitric-oxide synthase and Dynamin-2. *J. Biol. Chem.* 276: 14249–14256.

Chen Y and Rosazza J P N. (1995). Purification and characterization of nitric oxide synthase (NOS_{NOC}) from a *Nocardia* species. *J. Bacteriol*. 177: 5122–5128.

Delledonne M, Xia Y, Dixons R A and Lamb C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* 394: 585–588.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R and Zeiher A M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601–605.

Durner J, Wendehenne D and Klessig D F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Nat. Acad. Sci.* USA 95: 10328–10333.

Forstermann U, Pollock J S, Schmidt H H, Heller M and Murad F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cell. *Proc. Nat. Acad. Sci.* USA 88: 1788–1792.

Golderer G, Werner E R, Leitner S, Grobner P and Werner-Felmayer G. (2001). Nitric oxide synthase is induced in sporulation of *Physarum polycephalum*. Genes & Dev. 15: 1299–1309.

Gouvea C M C P, Souza J F and Magalhaes M I S. (1997). NO-releasing substances that induce growth elongation in maize root segments. *Plant Growth Reg*. 21: 183–187.

Guo F, Okamoto M and Crawford N M. (2003). Identification of a plant Nitric Oxide Synthase gene involved in hormonal signaling. *Science* 302: 100–103.

Hevel J M, White K A and Marletta M A. (1991). Purification of the inducible murine macrophage nitric oxide synthase. *J. Biol. Chem*. 266: 22789–22791.

Huang S, Kerschbaum H H, Engel E and Hermann A. (1997). Biochemical characterization and histochemical localization of nitric oxide synthase in the nervous system of the snail, *Helix pomatia. J. Neurochem*. 69: 2516–2528.

Jaffrey S R and Snyder S H. (1996). PIN: An associated protein inhibitor of neuronal Nitric Oxide Synthase. *Science* 274: 774–777.

Leshem Y Y and Haramaty E. (1996). The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn. Foliage. *J. Plant Physiol*. 148: 258–263.

Leshem Y Y, Wills R B H and Veng-Va Ku V. (1998). Evidence for the function of the free radical gas-nitric oxide (NO) as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem*. 36: 825–833.

Lo S C L, Cutt Y K and Tam M F. (2000). Characterization of plant NOS isolated from mung bean. *Nitric Oxide* 4: 207 (Abstract).

Nathan C and Xie Q W. (1994). Nitric oxide synthases: Roles, tools and controls. *Cell* 79: 915–918.

Nedvetsky P I, Sessa W C and Schmidt H H H W. (2002). There's NO binding like NOS binding: Protein-protein interactions in NO/cGMP signaling. *Proc. Nat. Acad. Sci. (PNAS)* 99: 16510–16512.

Noritake T, Kawakita K and Doke N. (1996). Nitric oxide induces phytoalexin accumulation in potato tuber tissue. *Plant Cell Physiol*. 37: 113–116.

Pollock J S, Forstermann U, Mitchell J A, Warner T D, Schmidt H H H W, Nakane M and Murad F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Nat. Acad. Sci.* USA 88: 10480–10484.

Ratovitski E A, Bao C, Quick R A, McMillan A, Kozlovsky C and Lowenstein C J. (1999). An inducible nitric-oxide synthase (NOS)-associated protein inhibits NOS dimerization and activity. *J. Biol. Chem*. 274: 30250–30257.

Regulski M and Tully T. (1995). Molecular and biochemical characterization of dNOS: A *Drosophila* Ca^{2+}/calmodulin-dependent nitric oxide synthase. *Proc. Nat. Acad. Sci*. USA 92: 9072–9076.

Ribeiro E A, Cunha F Q, Tamashiro W M S C and Martins I S. (1999). Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS* 445: 283–286.

Song N K, Jeong C S and Choi H S. (2000). Identification of nitric oxide synthase in *Flammulina velutipes*. *Mycologia* 92: 1027–1032.

Stuehr D J, Cho H J, Kwon N S, Weise M F and Nathan C F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein. *Proc. Nat. Acad. Sci*. USA. 88: 7773–7777.

Sun J and Liao J K. (2002). Functional interaction of endothelial nitric oxide synthase with a voltage-dependent anion channel. *Proc. Nat. Acad. Sci*. USA 99: 13108–13113.

Tatoyan A and Giulivi C. (1998). Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. *J. Biol. Chem*. 273: 11044–11048.

van Renswoude J and Kempf C. (1984). Purification of integral membrane proteins. *Methods Enzymol*. 104: 329–339.

Wendehenne D, Pugin A, Klessig D F and Durner J. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci*. 6: 177–183.

Wong Mui-Yun, Huang Jeng-Sheng and Davis E L. (2006). Physiological and immunoblot analysis of a nitric oxide synthase (NOS)-like protein of pea (*Pisum sativum* L.). *Jurnal Biosains* 17: 77–87.

Zimmermann K, Opitz N, Dedio J, Renne C, Muller-Esterl W and Oess S. (2002). NOSTRIN: A protein modulating nitric oxide release and subcellular distribution of endothelial nitric oxide synthase. *Proc. Nat. Acad. Sci. (PNAS)* 99: 17167–17172.