Botanical Studies (2012) 53: 315-324.

Characterization of a novel Cyclophilin-type peptidylprolyl isomerase protein from sweet potato storage roots

Jung-Chun LIAO[1], Chuan-Sung CHIU[2], Hsien-Jung CHEN[3], Shyh-Shyun HUANG[1], Wen-Chi HOU[4], Wang-Ching LIN[1], Yaw-Huei LIN[5,*], and Guan-Jhong HUANG[6,*]

[1]*School of Pharmacy, China Medical University, Taichung 404, Taiwan*
[2]*Nursing Department, Hsin Sheng College of Medical Care and Management, Taoyuan 325, Taiwan*
[3]*Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung 804, Taiwan*
[4]*Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei 250, Taiwan*
[5]*Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115, Taiwan*
[6]*Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung 404, Taiwan*

(Received February 10, 2012; Accepted April 6, 2012)

ABSTRACT. An antioxidant protein of cyclophilin-type peptidylprolyl isomerase (SPPPI) from sweet potato (*Ipomoea batatas* (L.) Lam. 'Tainong 57') storage roots was isolated by differential display. The open reading frame of this cDNA encodes a pro-protein of 260 amino acids with a predicted molecular mass of 27,658 Da (pI 9.34). A comparison of the deduced amino acid sequence of SPPPI with precursor proteins indicates 65% identity to the *Arabidopsis thaliana* AraPPI sequence. Computer analysis of the deduced amino acid sequences of the conserved domain revealed that the protein belonged to the plant cyclophilin-type peptidylprolyl isomerase. Genomic Southern blot analyses using the full-length SPPPI cDNA probe revealed a multigene family in the sweet potato genome. Both the corresponding mRNA and protein level were found the highest in the storage roots, followed by that in sprout. Recombinant SPPPI overproduced in *E. coli* (M15) was purified by Ni^{2+}-chelated affinity chromatography. Both the peptidylprolyl isomerase and antioxidantive activity of active recombinant SPPPI were investigated. SPPPI and CP (calf thymus cytophilin, a positive control) displayed the highest ABTS (2, 2-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) scavenging ability (15.36 ± 0.80 and 17.79 ± 1.72%, respectively) at 100 μg/mL. In the DPPH (1, 1-diphenyl-2- picrylhydrazyl) assay, SPPPI and CP were found to have the highest radical- scavenging activity (5.78 ± 0.62 and 4.05± 0.80%, respectively) at 100 μg/mL. The Fe^{2+}-chelating ability of SPPPI and CP was found to be the highest (12.47± 2.37 and 14.57± 0.96%) at 100 μg/mL, respectively. It was suggested that SPPPI is an excellent candidate as a lead compound for the development of reductant agents.

Keywords: Gene expression; Peptidylprolyl isomerase protein; Recombinant protein; Sweet potato.

INTRODUCTION

Cyclophilin (Cyp) is a multifunctional protein family that was first identified from human as a cytosolic binding protein for the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984). It was subsequently identified as a peptidyl-prolyl *cis-trans* isomerase (EC 5.2.1.8) whose activity can be inhibited by CsA (Takahashi et al., 1989). Cyclophilins are ubiquitous proteins (Galat, 1999) present in all subcellular compartments, which are involved in a wide variety of processes including protein trafficking and maturation (Ferreira et al., 1996), receptor complex stabilization (Leverson and Ness, 1998), apoptosis (Lin and Lechleiter, 2002), receptor signaling (Brazin et al., 2002), RNA processing (Krzywicka et al., 2001), and spliceosome assembly (Horowitz et al., 2002). As an immunophilin, Cyp binds to CsA, and the Cyp-CsA complex suppresses the immune response through binding with the calcineurin. The Cyp-CsA complex inhibits the phosphatase activity of calcinuerin, which blocks the Ca^{2+}-dependent T-cell activation pathway by blocking the transcriptional activation of genes in the T cell response (Walsh et al., 1992). In a plant, the Cyp-CsA complex also plays a role as an inhibitor of calcinuerin, and blocks the Ca^{2+}-induced inactivation of K^+ channels in guard cells (Luan et al., 1993). This PPI activity of Cyps may accelerate folding and refolding of proteins such as procollagen I, rhodopsin, and carbonic anhydrase II (Kern et al., 1994). Cyps were isolated from all of the organisms that were

*Corresponding authors: Tel: 886-4-2205-3366 ext. 5508, Fax: 886-4-2208-3362, E-mail: gjhuang@mail.cmu.edu.tw (Guan-Jhong HUANG); Tel: 886-2-2787-1172, Fax: 886-2-2782-7954, E-mail: boyhlin@gate.sinica.edu.tw (Yaw-Huei LIN).

tested, and are known to be conserved (Rutherford and Zuker, 1994). They are abundant in cytosol, but some of them can either be secreted into the extracellular medium or transported into endoplasmic reticulum (ER), mitochondria, and chloroplast. As the number of genes that were characterized for Cyp increases, it becomes apparent that multiple genes encode Cyps, indicating diverse functions of Cyp (Lee et al., 2002).

Here, we report the cloning of the first SPPPI cDNA from sweet potato. We present the characterization of a peptidyl-prolyl *cis-trans* isomerase from sweet potato and demonstrate its antioxidant activity *in vitro* using recombinant SPPPI. The comparison of the sequence of our data with those published confirms the existence of a novel class of peptidyl-prolyl *cis-trans* isomerase with moderate but significant sequence conservation. We then expressed the gene in *Escherichia coli* and characterized enzymatic and antioxidant activities of SPPPI protein that we purified.

MATERIALS AND METHODS

Chemicals

Tris and electrophoretic reagents were purchased from E. Merck Inc. (Darmstadt, Germany); Seeblue prestained markers for SDS-PAGE including myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamate dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa) were from Invitrogen (Groningen, The Netherlands); Coomassie brilliant blue G-250, N-succ-Ala-Ala-Pro-Phe-*p*-nitroaniline, cyclophilin, other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant materials

Fresh storage roots of sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') were purchased from a local market. After cleaning with water, the roots were placed in a thermostated (28°C) growth chamber and sprayed with water twice a day. Sprouted plants were cultivated in the greenhouse to collect roots, stems, and full expanded green leaves for experiments.

PCR-based subtractive hybridization and RACE PCR

Total RNA was isolated separately from the storage roots and sprout of roots of sweet potato according to the method of Sambrook et al. (1989). Then, mRNA was purified with a purification kit (Promega) and used for the differentially-expressed first strand cDNA synthesis using a PCR-based subtractive hybridization kit (Clontech) following the protocol supplied by the manufacturer. The double-strand cDNAs of the storage roots were substracted by the sprout of roots, then ligated to the pGEM-T easy vector for *E. coli* DH5α competent cell transformation. Recombinant plasmids were isolated for DNA sequencing using ABI PRIZM 337 DNA Sequencer. Nucleotide sequence data were analyzed using the Genetics Computer Group (GCG) programs. Full-length cDNA clone was obtained by performing 5′ and 3′ RACE (5′ and 3′ rapid amplification of cDNA ends) using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. A gene-specific primer (5′-GGTGA ACTCC CAATT GTCT-3′) was designed for the 5′ and 3′ RACE reaction.

Expression of SPPPI in *E. coli*

Peptidylprolyl isomerase (PPI) was expressed in *E. coli*. The coding sequence was amplified from PPI cDNA using an oligonucleotide (5′-CTC TTG GAT CCA TGG CGC TTC CGT TTG C -3′), with a *Bam* HI site (underlined) at the putative initial Met residue, and an oligonucleotide (5′-G TGA ACT CCC AAT TGT CTA AGC TGC AGT -3′), with a *Pst* I site. The PCR fragment was subcloned in pGEM T-easy vector. And the plasmid was then digested with *Bam* HI and *Pst* I and subcloned in pQE30 expression vector (QIAexpress expression system, Qiagen). The resulting plasmid, termed pQE-PPI, was introduced into *E. coli* (M15). Cultures of the transformed *E. coli* (M15) overexpressed a protein of the expected molecular mass, which was purified by affinity chromatography in Ni-NTA columns (Qiagen), according to the manufacturer's instructions.

RNA isolation and northern blot analysis

Total RNA was extracted from different tissues of sweet potato with TRIzol reagents kit (Invitrogen) according to the manufacturer's instructions. For northern blotting, 10 μg of total RNA isolated from storage roots, sprouts, sprouted roots, veins, fully expanded green leaves, and flowers were applied to a formaldehyde denaturing gel, then transferred to an Amersham Hybond-N^+ nylon membrane after electrophoresis, according to Sambrook et al. (1989). The filter was hybridized sequentially with α-^{32}P-labelled PPI full-length cDNA. The procedures for hybridization and autoradiography were according to the Sambrook et al. (1989). Visualization of hybridization bands was carried out using X-ray film (Kodak).

Phylogenic analysis of SPPPI

Amino acid sequence alignment of SPPPI after GCG/Pileup comparison was used for phylogenic tree construction. The distances among entries were calculated with neighbor-joining method (Thompson et al., 1994). The internal support was evaluated by bootstrap analyses. In parsimony analysis, each of 100 bootstrap replicates was analyzed with the heuristic search option invoking one random addition replicate each, and not invoking the retention of multiple parsimonious trees. The phylogenic tree was drawn using NJ plot and redrawn by the graphic software of CLUSTALX 1.81 (Thompson et al., 1997).

DNA isolation and Southern blot analysis

Young leaves of sweet potato were harvested and ground in liquid N_2. The powder was transferred to a centrifuge tube, mixed gently and thoroughly with CTAB (cetyltrimethylammonium bromide) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 2% 2-mercaptoethanol, and 100 mM Tris-HCl pH 8.0), and kept in a water bath at 60°C for genomic DNA extraction according to the method of Huang et al. (2005). The total nucleic acid after precipitation with an equal volume of isopropanol was re-dissolved in sterile water, digested with various restriction enzymes and separated on 0.8% agarose gels. After electrophoresis, the DNA was transferred onto a Hybond-N^+ nylon membrane (Amersham) following the protocol of Molecular Cloning (Sambrook et al., 1989) for Southern blot hybridization.

Protein staining of SPPPI on 10% SDS-PAGE Gels

Samples were mixed with sample buffer, namely 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with 2-mercaptoethanol. Coomassie brilliant blue G-250 was used for protein staining (Huang et al., 2007a).

Production of polyclonal antibody and western blot hybridization

Expressed SPPPI protein was cut from the 10% polyacrylamide gel and eluted, mixed with appropriate amount of pH 7.5 phosphate buffer saline (PBS) containing 0.1% SDS. The eluted proteins were precipitated with acetone containing 10% trichloroacetic acid (TCA) at -20°C for 2 h. After centrifugation at 13,000 *g* for 20 min, the pellet was washed with acetone twice, then, dried at room temperature. The acetone powder was re-dissolved in a small amount of PBS containing 0.1% SDS and used as antigens for subcutaneous injections of rabbit to prepare the first antibody (Taiwan Bio-Pharm Inc.). The second antibody (goat against rabbit Fc portion of Ig) was a product of Sigma (USA). Polyclonal antibodies obtained from rabbit antiserum were utilized for western blot hybridization to study the gene expression of SPPPI, respectively, in different tissues of sweet potato (Huang et al., 2008a).

Protein extraction, electroblotting analysis of SPPPI

All steps were carried out at 4-8°C. Sweet potato full expanded green leaves, veins, sprouted roots, and storage roots were cleaned and air-dried and homogenized with 4 volumes (*v/w*) of 50 mM Tris-HCl buffer (pH 7.5) in a Polytron homogenizer (Luzern, Swiss). The homogenate was filtered through two layers of cheesecloth and then centrifuged in a Sorvall RC-2B with an SS-34 rotor at 10,000 *g* for 20 min. And the protein concentration of the supernatant was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA). The supernatant was saved for electroblotting. The crude extract was subjected to 15% SDS-PAGE according to Huang et al., (2008a). After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris-HCl, pH 8.3, 150 mM glycine and 10% (*w/v*) methanol). The separated proteins were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA) in transfer buffer at pH 8.3 for 1 h at 100 V. Membranes were blocked for 2 h at room temperature in 5% nonfat dry milk powder and then incubated with polyclonal antibodies as the primary antibody against SPPPI. After incubation, membranes were washed in Tris-buffer saline with 0.05% Tween (TBST) three times, 10 min each, then incubated with goat anti-rabbit Fc portion of IgG conjugated with alkaline phosphatase as secondary antibody, washed in TBST three times, 10 min each, and developed using NBT(nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Sigma, USA).

Measurement of SPPPI activity

The SPPPI activity was measured in a coupled reaction with α-chymotrypsin (Steinmann et al., 1991). PPIase activity was measured with the *cis-trans* isomerization of the alanine-proline peptide bond in the substrates. The *trans* form of the substrate is cleavable by α-chymotrypsin, which releases *p*-nitroaniline, while the cis form can not be cleaved. The *p*-nitroaniline is quantified spectrophotometrically at 390 nm. The assays were conducted at ambient temperature. The peptide substrate N-succ-Ala-Ala-Pro-Phe-p-nitroaniline was dissolved in trifluoroethanol with the addition of LiCl to increase the proportion of *cis* isomer. The reactions were started by peptide substrate addition to a mixture of protein sample and α-chymotrypsin (0.5 mg/mL) in 0.1 M Tricine pH 7.8. The change in absorbance at 390 nm for 5 min due to the release of *p*-nitroaniline was monitored (Beckman UV-Vis spectrophotometer, Model DU640B). Calf-thymus Cyp was used as a positive control.

Determination of antioxidant activity by $ABTS^+$ scavenging ability

The $ABTS^{\cdot+}$ (2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) scavenging ability was determined according to the method of Walker and Everette (2009). Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 h in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm spectrophotometrically after 1 min. The percentage of $ABTS^{\cdot+}$ decolorization by the samples were calculated according to the equation: % decolorization = $[1- (ABTS^{\cdot+}_{sample} / ABTS^{\cdot+}_{control})] \times 100$.

Determination of antioxidant activity by DPPH radical scavenging ability

The effect of SPPPI and the positive control (Calf-thy-

mus Cyp) on the DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical was estimated according to the method of Huang et al. (2004). An aliquot (20 μL) of each sample at various concentrations was mixed with 100 mM Tris-HCl buffer (80 μL, pH 7.4) and then with 100 μL of the DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution was measured spectrophotometrically. The percentages of DPPH decolorization by the samples were calculated according to the equation: % decolorization = [1- (ABS_{sample} /$ABS_{control}$)] ×100.

Determination of antioxidant activity by Fe^{2+}-chelating ability

The Fe^{2+}-chelating ability was determined according to the method of Huang et al. (2006). The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex at 562 nm. The SPPPI (0, 12.5, 25, 50, and 100 μg/mL) was mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 10 : 1 : 2. The mixture was shaken and left to stand at room temperature for 10 min. The absorbance of the resulting solution at 562 nm was measured. The lower the absorbance of the reaction mixture the higher the Fe^{2+}-chelating ability. The capability of the sample to chelate the ferrous iron was calculated using the following equation:

Scavenging effect (%) =[1-(ABS_{sample} /$ABS_{control}$)]×100

Statistical analysis

Means of triplicates were calculated. Student's *t* test was used for comparison between two treatments. All data (expressed as percent of control value) were means ± SE. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and nucleotide sequence of a cyclophilin-type peptidylprolyl isomerase cDNA clone from sweet potato storage roots

A SPPPI cDNA clone of sweet potato storage roots was isolated by differential display. We have completed the sequencing of the clone, which was named *SPPPI* (GenBank Accession Number FJ361763). The open reading frame of this cDNA encodes a pro-protein of 260 amino acids with a predicted molecular mass of 27,658 Da (pI 9.34). A comparison of the deduced amino acid sequence of SPPPI with precursor proteins indicates 65% identity to the *Arabidopsis thaliana* AraPPI sequence. SPPPI sequence was different from other PPI sequences in signal peptide portions. Figure 1A shows a multiple alignment of SPPPI protein and other homologous plant precursor PPI proteins available in the GenBank. Computer analysis of the deduced amino acid sequences of the conserved domain revealed that the protein belonged to the plant cyclophilin-type peptidylprolyl isomerase.

Moreover, the putative cleavage site on the N-terminal signals peptide for targeting to chloroplast was checked (http://www.cbs.dtu.dk/services/ChloroP/). SPPPI protein contains an N-terminal chloroplast transit peptide. In addition, two potential sites of N-glycosylation, 163NGTG166 and 200NGSQ203 (N-[^P]-S/T-[^P], where [^P] indicates non-proline residues), were found within the deduced amino acid sequences.

The PPIase activity of the SPPPI protein is suggested by the conservation of the three catalytic amino acids Arg-147, Phe-152, and His-218 and by the conservation of the amino acid Trp-213 as a residue required for CsA binding but not involved in PPI enzymatic activity (Figure 1A) (Zydowsky et al., 1992).

The phylogenic tree of SPPPI together with amino acid sequence of *Arabidopsis thaliana* AraPPI was constructed (Figure 1B).

Copy number of cyclophilin-type peptidylprolyl isomerase sequences in sweet potato

We performed Southern blot hybridization with *Eco RI* (E), *Hind III* (H) and *Pst I* (P) digests of sweet potato Tainong 57 DNA, using probe derived from 3′-noncoding sequence of the cDNAs to estimate the copy number of the gene. Tainong 57, an elite sweet potato cultivar derived from a cross between Tainong 27 and Nancy Hall, has a hexaploid number of chromosome (2n=6x=90). The results suggest that *SPPPI* belongs to a small multigene family in sweet potato (Figure 2).

Expression of cyclophilin-type peptidylprolyl isomerase in *E. coli*

SDS-PAGE analysis of SPPPI crude extracts from the transformed *E. coli* (M15) showed high amounts of a polypeptide with the expected molecular mass (ca. 28 kDa) (Figure 3A). The expressed protein had signal peptide. This polypeptide was found as a soluble protein in the supernatant (Figure 3A, lane 2), and was absent in protein extracts obtained from *E. coli* transformed with pQE-30 vector (Figure 3A, lane 1). The expressed protein was highly purified from crude extracts as His-tagged SPPPI (Figure 3A, lane 3).

Using the purified SPPPI protein, we performed PPI assays, which showed that SPPPI is an active PPIase that can catalyze the *cis-trans* interconversion of the Ala-Pro bond in the substrate compared to the level of spontaneous isomerization in the absence of SPPPI (Figure 3B). Also, the SPPPI activity was comparable to that of calf CP.

Both cyclophilin-type peptidylprolyl isomerase mRNA and protein levels were developmentally regulated

The presence and amounts of different sweet potato *SPPPI* mRNAs were examined in various organs and tissues by northern blot analysis. Figure 4A shows that *SPPPI* probe hybridized to mRNA species of approximately 1.0

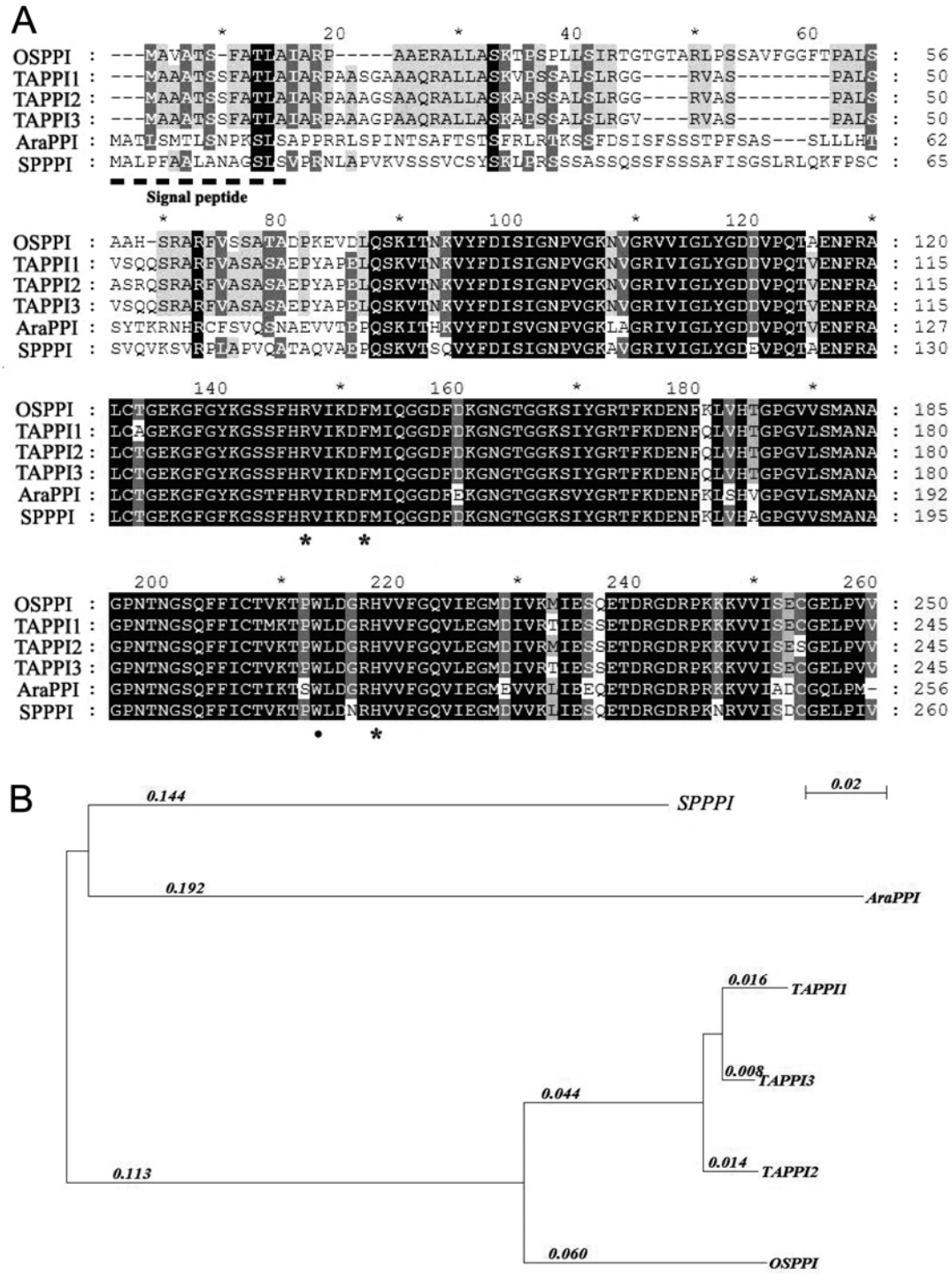

Figure 1. Multiple alignments of plant peptidylprolyl isomerase proteins. (A) The sequences are from sweet potato peptidylprolyl isomerase tuberous root SPPPI (FJ361763), *Oryza sativa* OSPPI (AAS88825); *Triticum aestivum* TAPPI1 (AAP44535); *Triticum aestivum* TAPPI2 (AAP44537); *Triticum aestivum* TAPPI3 (ABO37960); and *Arabidopsis thaliana* AraPPI (NP_196816). The regions corresponding to the signal peptide (black dashed line) are marked. The consensus amino acids for PPIase catalysis are marked with asterisks. The conserved amino acid W for CsA binding is marked with black dot. The proteins were aligned using the GCG program. Gray shading shows those amino acids with similar side-chain properties. The numbers above all sequences stand for the positions of the amino acids within individual proteins corresponding to the numbering system of sweet potato *SPPPI*, which is the longest among all sequences shown. The numbers at the end of the right hand side of each line stand for the cumulative total number of amino acids in each line of each preproprotein sequence; (B) Phylogenetic analysis of peptidylprolyl isomerases based on their amino acid sequences. The scale bar represents 0.02 units.

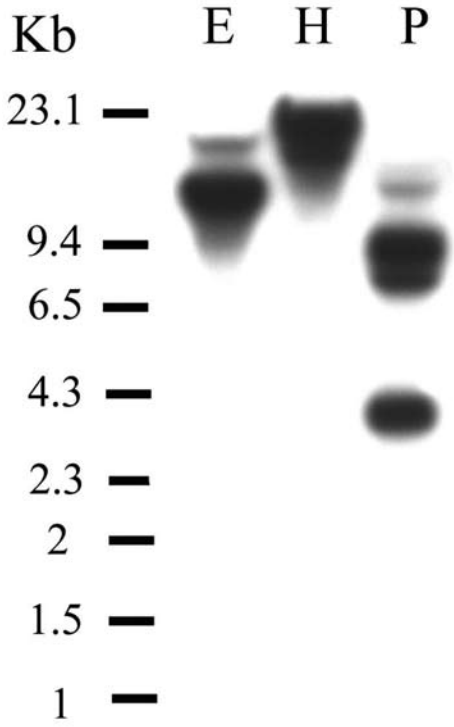

Figure 2. Southern blot detection of peptidylprolyl isomerase genomic sequences. Samples (10 μg) of genomic DNA from sweet potato Tainong 57 leaves were digested with *Eco RI* (E), *Hind III* (H) and *Pst I* (P). The DNA fragments were separated in 0.8 % agarose gels, transferred to a Hybond-N^+ nylon membrane, and hybridized with PCR-labeled cDNA probes. Molecular size markers were λ DNA/*Hind III* fragments. The experiments were done twice and a representative one is shown.

kb. *SPPPI* mRNA levels were the highest in the storage roots, followed by that in sprout; while were the lowest in full expanded green leaves.

Western blot hybridization using SPPPI polyclonal antibody from rabbit antiserum was used for the gene expression analysis of SPPPI in crude extracts from different sweet potato tissues (Figure 4B). SPPPI levels were the highest in the storage roots; followed by that in sprout, full expanded green leaves and sprouted roots. This result suggests that SPPPI may play an important role in storage roots.

Antioxidant activity estimated by ABTS assay

ABTS assay are often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Huang et al., 2007b). In this assay, ABTS radical monocation was generated directly in stable form from potassium peroxodisulfate. Generation of radical was allowed to form before the antioxidants were added, thus can prevent interference of compounds, which affected radical formation. This modification made the assay less susceptible to artifacts and prevented overestimation of antioxidant power (Sanchez-Moreno et al., 2002). The antioxidant sample was added to the reaction medium when the stable absorbance was obtained, and the antioxidant activity was measured in terms of decolorization.

Different amounts of SPPPI protein exhibited a dose-dependent ABTS scavenging ability within the applied concentrations (0, 12.5, 25, 50, 100 μg/mL). At the 100 μg/mL, SPPPI and CP (calf thymus cytophilin, positive control) displayed the highest ABTS scavenging ability (15.36 ± 0.80 and 17.79 ± 1.72%) (Figure 5A).

Scavenging activity against DPPH radical

The DPPH radical was widely used in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins

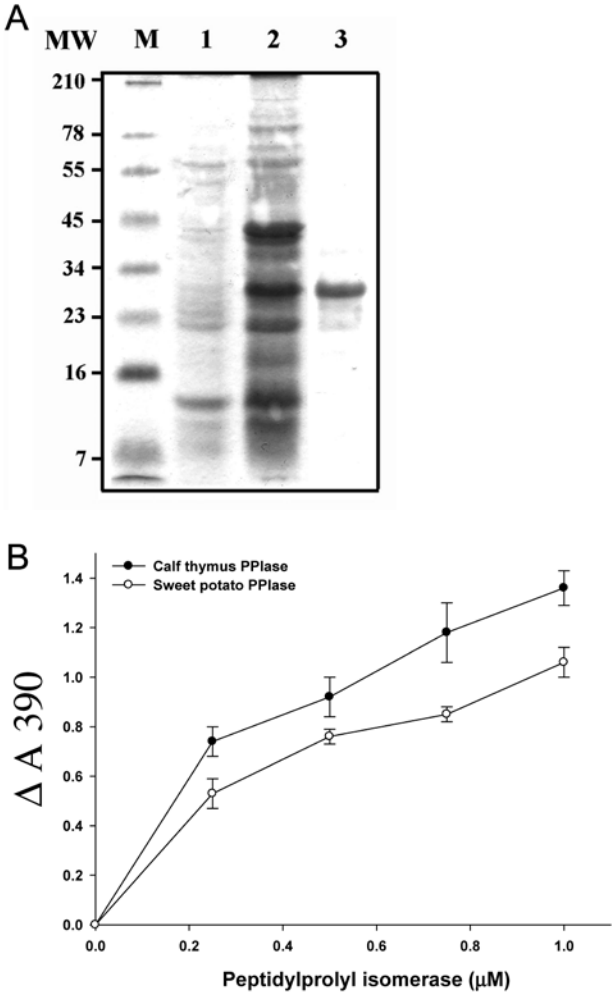

Figure 3. Purified recombinant sweet potato peptidylprolyl isomerase SPPPI. A, 10% SDS-PAGE analysis. Crude extracts (5 μg protein) from *E. coli* (M15) transformed with pQE30 (lane 1) or with pQE30- SPPPI (lane 2) were analyzed by 10% (*w/v*) SDS-PAGE, and then the gels were stained with Coomassie brilliant blue G-250. Molecular masses of standard proteins were indicated at the left of the figure. His-tagged SPPPI was purified by Ni^{2+}-chelated affinity chromatography (lane 3); B, Proteinase activity analysis. The experiments were done twice and a representative one is shown. "M" indicated the see Blue™ pre-stained markers for SDS-PAGE. "MW" indicated molecular weight.

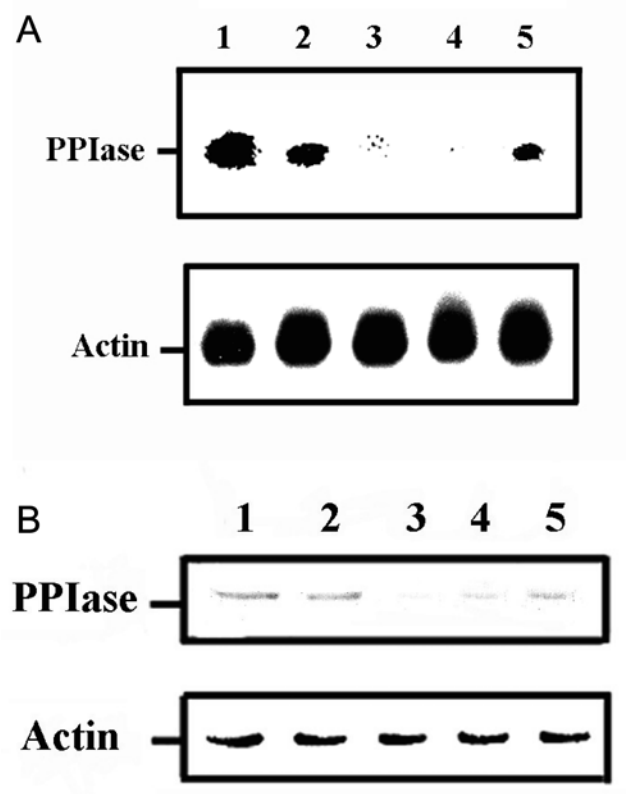

Figure 4. Northern and western blot detections of sweet potato peptidylprolyl isomerase SPPPI. A, Northern blot analysis. Samples (10 μg each) of total RNA were isolated from different tissues of sweet potato and actin (AY905538) was utilized as an internal control of mRNA from sweet potato. Blots were hybridized to α-^{32}P-labeled 3′ specific cDNA probes; B, Western blots analysis. Ten μg of crude extracted proteins from sweet potato were analyzed by 15% (*w/v*) SDS/PAGE, and then the gels were transferred onto PVDF membranes that were probed with a 1:1000 (*v/v*) dilution of rabbit antibodies raised against SPPPI as the primary antibody and goat-antirabbit IgG constant region conjugated with alkaline phosphatase as the second antibody. Lane 1: storage roots, lane 2: sprout, lane 3: veins, lane 4: sprouted roots, and lane 5: full expanded green leaves. The experiments were done twice and a representative one is shown.

or crude mixtures (Chang et al., 2007c). DPPH radical is scavenged by antioxidants through the donation of hydrogen forming the reduced DPPH-H. The color changed from purple to yellow after reduction, which could be quantified by its decrease of absorbance at wavelength 517 nm. Figure 5B shows the dose-response curve for the radical-scavenging activity of the different concentrations of SPPPI and CP using the DPPH coloring method. It was found that in the 100 μg/mL SPPPI and CP had the highest radical-scavenging activity (5.78 ± 0.62 and 4.05± 0.80%, respectively). Free cysteine residues in whey proteins (Allen et al., 1982) were reported to have antioxidant activity. These findings suggest that cysteine residues in sweet potato SPPPI may also participate in the antiradical activity.

Measurement of Fe^{2+}-chelating ability

The chelating capacity of SPPPI and CP was determined by assessing their ability to compete with ferrozine for the ferrous ions, which is shown in Figure 5C. The Fe^{2+}-chelating ability of SPPPI was lower than CP. The doses of 12.5, 25, 50, and 100 μg/mL of SPPPI exhibited 1.58±**0.61,** 5.02±**1.37**, 8.70±**1.73**, and 12.47±**2.37**% iron binding capacity, respectively. On the other hand, the doses of 12.5, 25, 50, and 100 μg/mL of CP have shown 1.45± **0.35,** 6.97±**1.20**, 13.58±**1.26**, and 14.57±**0.96**% chelating activity of iron, respectively. Values obtained from Figure 5C demonstrate that the action of SPPPI, as a peroxidation protector, may be more related to its iron-binding capacity (Huang et al., 2008b).

The cyclophilin CYP20-3 (also known as ''ROC4'') is the only member of this group located in the stroma (soluble phase) of chloroplasts. The *cyp20-3* mutant plants when grown under stress conditions were hypersensitive to various stress treatments, including high light that damages plants by producing reactive oxygen species (ROS) (Buchanan et al., 2005). CYP20-3 links photosynthetic electron transport and redox regulation, thereby enabling the cysteine-based thiol biosynthesis pathway to adjust to light and stress conditions. Both functions are regulated in dependence on the redox status of the active centre of

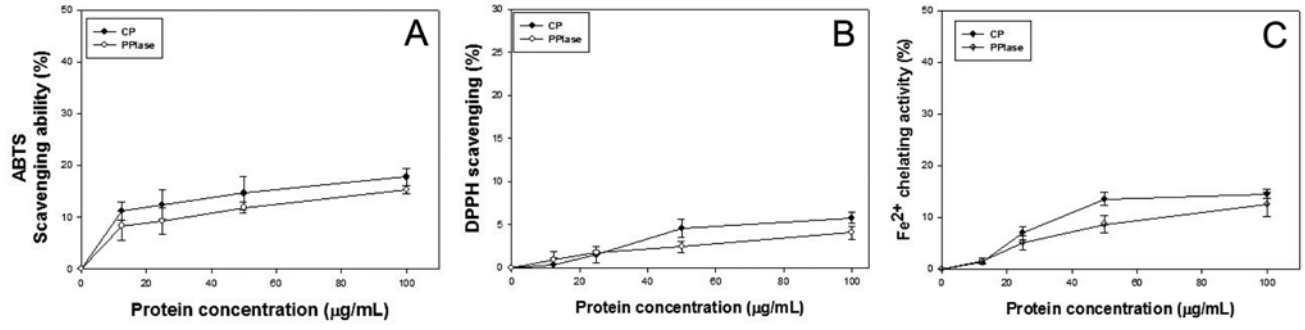

Figure 5. Antioxidant activities of recombinant peptidylprolyl isomerase SPPPI protein from sweet potato. (A) The inhibition of the $ABTS^{\cdot+}$; (B) DPPH radical scavenging activity; (C) Fe^{2+}-chelating ability. Concentration range includes 0, 12.5, 25, 50, and 100 μg/mL. CP (calf thymus cytophilin) was used as a control. Absorbance value represents average of triplicates of different samples analyzed.

CYP20-3. The best-known mechanism to modify Cyp activity is the binding of CsA, an immunosuppressive drug that specifically inhibits the Cyp PPI activity (Laxa et al., 2007). Subsequently, the CsA-Cyp complex binds to subunit B of the Ca^{2+}/calmodulin-dependent serine/threonine-phosphatase calcineurin leading to its inactivation and, finally, resulting in altered gene transcription in the nucleus (Dominguez-Solis et al., 2008). Thus the electron-donating property of CYP20-3 to 2-Cys Prx as observed in the DNA-protection assay does not necessarily indicate a major role of CYP20-3 in the antioxidant defence of the chloroplast, but does indicate a role in the redox signaling network adjusting the redox state and thereby the activity of CYP20-3.

We report here for the first time the isolation of the SP-PPI cDNA and its expression levels at different organ of sweet potato. However, the biochemical and physiological roles of SPPPI protein during plant development are still unclear. Our results suggest that recombinant SPPPI is an excellent candidate as a lead compound for the development of reductant agents.

Acknowledgement. The authors want to thank the financial supports from the National Science Council (NSC100-2313-B-039-004- and NSC 100-2320-B-039-033-), China Medical University (CMU) (CMU99-S-29, CCM-P99-RD-042, and CMU99-COL-10) and Taiwan Department of Heath Clinical Trial and Research Center of Excellence (DOH101-TD-B-111-004).

LITERATURE CITED

Allen, J.C. and W.L. Wrieden. 1982. Influence of milk proteins on lipid oxidation in aqueous emulsion I. Casein, whey protein and R-lactoalbumin. J. Dairy Res. **49:** 239-248.

Brazin, K.N., R.J. Mallis, D.B. Fulton, and A.H. Andreotti. 2002. Regulation of the tyrosine kinase Itk by the peptidyl-prolyl isomerase cyclophilin A. Proc. Natl. Acad. Sci. USA **99:** 1899-1904.

Buchanan, B.B. and S. Luan. 2005. Redox regulation in the chloroplast thylakoid lumen: a new frontier in photosynthesis research. J. Exp. Bot. **56:** 1439-1447.

Chang, H.Y., Y.L. Ho, M.J. Sheu, W.H. Pen, S.H. Wu, G.J. Huang, and Y.S. Chang. 2007. Antioxidants and free radical scavenging activity of *Phellinus merrillii* extracts. Bot. Stud. **48:** 407-417.

Dominguez-Solis, J.R., Z. He, A. Lima, J. Ting, B.B. Buchanan, and S.A. Luan. 2008. Cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. Proc. Natl. Acad. Sci. USA **105:** 16386-16391.

Ferreira, P.A., T.A. Nakayama, W.L. Pak, and G.H.Travis. 1996. Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. Nature **383:** 637-640.

Galat, A. 1999. Variations of sequences and amino acid compositions of proteins that sustain their biological functions: an analysis of the cyclophilin family of proteins. Arch. Biochem. Biophys. **371:** 149-162.

Handschumacher, R.E., M.W. Harding, J. Rice, R.J. Drugge, and D.W. Speicher. 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science **226:** 544-547.

Huang, D.J., H.J. Chen, W.C. Hou, C.D. Lin, and Y.H. Lin. 2004. Active recombinant Thioredoxin *h* protein with antioxidant activities from sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') storage roots. J. Agric. Food. Chem. **52:** 4720-4724.

Huang, D.J., H.J. Chen, W.C. Hou, and Y.H. Lin. 2005. Expression and localization of protein disulfide isomerase cDNA from sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') storage roots. Plant Sci. **169:** 776-784.

Huang, D.J., H.J. Chen, W.C. Hou, and Y.H. Lin. 2006. Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage roots mucilage with antioxidant activities *in vitro*. Food Chem. **98:** 774-781.

Huang, G.J., M.J. Sheu, H.J. Chen, Y.S. Chang, and Y.H. Lin. 2007a. Inhibition of reactive nitrogen species *in Vitro* and *ex Vivo* by trypsin inhibitor from sweet potato 'Tainong 57' storage roots. J. Agric. Food. Chem. **55:** 6000-6006**.**

Huang, D.J. H.J. Chen, W.C. Hou, and Y.H. Lin. 2007b. Sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') storage roots mucilage with antioxidant activities in vitro. Food Chem. **98:** 774-781.

Huang, G.J. H.J. Chen, Y.S. Chang, M.J. Sheu, and Y.H. Lin. 2007c. Recombinant sporamin and its synthesized peptides with antioxidant activities in *vitro*. Bot. Stud. **48:** 133-140.

Huang, G.J., H.C. Lai, Y.S. Chang, T.L. Lu, M.J. Sheu, H.Y. Chang, and Y.H. Lin. 2008a. Antimicrobial, dehydroascorbate reductase and monodehydroascorbate reductase activities of defensin from sweet potato (*Ipomoea batatas* [L.] Lam. 'Tainong 57') storage roots. J. Agric. Food. Chem. **56:** 2989-2995.

Huang, S S., G.J. Huang, Y.L. Ho, Y.H. Lin, H.J. Hung, T.N. Chang, M.J. Chan, J.J. Chen, and Y.S. Chang. 2008b. Antioxidant and antiproliferative activities of the four *Hydrocotyle* species from Taiwan. Bot. Stud. **49:** 311-322.

Horowitz, D.S., E.J. Lee, S.A. Mabon, and T. Misteli. 2002. A cyclophilin functions in pre-mRNA splicing. EMBO J. **21:** 470-480.

Kern, G., D. Kern, F.X. Schimid, and G. Fischer. 1994. Reassessment of the putative chaperone function of prolyl cis/trans-isomerase. FEBS Lett. **348:** 145-148.

Krzywicka, A., J. Beisson, A.M. Keller, J. Cohen, M. Jerka-Dziadosz, and C. Klotz. 2001. KIN241: a gene involved in cell morphogenesis in *Paramecium tetraurelia* reveals a novel protein family of cyclophilin-RNA interacting proteins (CRIPs) conserved from fission yeast to man. Mol. Microbiol. **42:** 257-267.

Laxa, M., J.K. Onig, K.J. Dietz, and A. Kandlbinder. 2007. Role of the cysteine residues in Arabidopsis thaliana cyclophilin CYP20-3 in peptidyl-prolyl *cis-trans* isomerase and redox-related functions. Biochem. J. **401:** 287-297.

Lee, Y.K., C.B. Hong, Y. Suh, and I.K. Lee. 2002. A cDNA Clone for Cyclophilin from *Griffithsia japonica* and Phylogenetic Analysis of Cyclophilins. Mol. Cells **13:** 12-20.

Leverson, J.D. and S.A. Ness. 1998. Point mutations in v-myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. Mol. Cell **1:** 203-211.

Lin, D.T. and J.D. Lechleiter. 2002. Mitochondrial targeted cyclophilin D protects cells from cell death by peptidyl prolyl isomerization. J. Biol. Chem. **277:** 31134-31141.

Luan, S., W. Li, F. Rusnak, S.M. Assmann, and S.L. Schreiber. 1993. Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. Proc. Natl. Acad. Sci. USA **90:** 2202-2206.

Rutherford, S.L. and C.S. Zuker. 1994. Protein folding and the regulation of signaling pathways. Cell **79:** 1129-1132.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual, 2nd, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Sanchez-Moreno, C. 2002. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci. Technol. Int. **8:** 121-137.

Steinmann, B., P. Brucker, and A. Superti-Furga. 1991. Cyclosporin A slows collagen triple-helix formation *in vivo*; indirect evidence for a physiologic role of peptidyl-prolyl *cis-trans* isomerase. J. Biol. Chem. **266:** 1299-1303.

Thompson, J.D., D.G. Higgins, T. Gibson, and J.W. Clustal. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4680.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:** 4876-4882.

Takahashi, N., T. Hayano, and M. Suzuki. 1989. Peptidylprolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. Nature **337:** 473-475.

Walker R.B. and J.D. Everette. 2009. Comparative reaction rates of various antioxidants with ABTS radical cation. J. Agric. Food. Chem. **57:** 1156-1161.

Walsh, C.T., L.D. Zydowsky, and F.D. McKeon. 1992. Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. J. Biol. Chem. **267:** 13115-13118.

Zydowsky, L.D., F.A. Etzkorn, H.Y. Chang, S.B. Ferguson, L.A. Stolz, S.I. Ho, and C.T. Walsh. 1992. Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. Protein Sci. **1:** 1092-1099.

甘藷塊根分離之親環蛋白型的脯胺基異構酶之特性

廖容君[1] 邱傳淞[2] 陳顯榮[3] 黃世勳[1] 侯文琪[4] 林旺慶[1] 林耀輝[5] 黃冠中[6]

[1] 中國醫藥大學 藥學系
[2] 新生醫護管理專科學校 護理科
[3] 中山大學 生命科學系
[4] 臺北醫學大學 生藥學研究所
[5] 中央研究院 植物暨微生物研究所
[6] 中國醫藥大學 中國藥學暨中藥資源系

利用差異表現法 (differential display) 分離甘藷 (*Ipomoea batatas* [L.] Lam. 'Tainong 57') 塊根之親環蛋白型的脯胺基異構酶 (cyclophilin-type peptidylprolyl isomerase; SPPPI)。SPPPI 之開放轉譯架構（open reading frame）轉譯成 260 個胺基酸，預測其分子量為 27,658 Da (pI 9.34)。SPPPI 胺基酸序列和阿拉伯芥之 AraPPI 胺基酸比對時，具有 65% 相似性。電腦分析其胺基酸序列中含有植物之親環蛋白型的脯胺基異構酶保守性特徵。藉由南方點墨法檢視 SPPPI 在甘藷中含有多套基因。藉由訊息核糖核酸和蛋白質表現發現 SPPPI 在甘藷中表現量最多的部位在塊根，其次為芽。在大腸桿菌 (M15) 中大量表現重組蛋白質 SPPPI，然後利用鎳離子螯合之親和性管柱純化，研究脯胺基異構酶和抗氧化的活性。SPPPI 和 CP (小牛胸腺 cytophilin, 正對照組) 在總抗氧化能力分析上，在 100 μg/mL 時可達最高的抗氧化活性 (15.36 ± 0.80 和 17.79 ± 1.72 %)。在 DPPH 分析法中，在 100 μg/mL 時可達最高的清除自由基活性 (5.78 ± 0.62 和 4.05 ± 0.80 %)。在亞鐵離子螯合能力中，在 100 μg/mL 時可達最高的螯合能力 (12.47 ± 2.37 和 14.57 ± 0.96 %) 。本實驗建議 SPPPI 是一個出色的蛋白質，可作為一個為發展還原劑的先導物。

關鍵詞：基因表現；脯胺基異構酶；重組蛋白質；甘藷。