Purification and characterization of cytosolic and microsomal cyclophilins from maize (*Zea mays*)

Philip S. SHELDON* and Michael A. VENIS

Cell Physiology Department, Horticulture Research International, Wellesbourne, Warwickshire, CV35 9EF, U.K.

Methods for the purification and separation of peptidyl prolyl *cis*–*trans* isomerase (PPI) from cytosolic and microsomal fractions of etiolated maize are described. On SDS/PAGE, the purified preparations appear as single polypeptides with molecular masses of 17.5 kDa and 17.7 kDa respectively. Instead of using immobilized cyclosporin A derivatives as affinity adsorbents, our methods employ conventional techniques enabling purification of the proteins on a much larger scale than previously described. An antiserum raised against the cytosolic PPI recognizes poly-

peptides of similar molecular mass from a wide range of plant species on an immunoblot. There is virtually no recognition of the microsomal PPI. The cytosolic and microsomal PPIs are inhibited by cyclosporin A ($K_i = 6$ nM in both cases), indicating that they are cyclophilins. The cytosolic enzyme is inactivated by 5 mM *N*-ethylmaleimide and 2 mM phenylglyoxal. N-terminal sequencing of the microsomal PPI indicates a high level of sequence similarity with the N-terminal sequence of mature animal s-cyclophilin (cyclophilin B).

INTRODUCTION

A potentially rate-limiting step of protein folding is the inter-conversion of peptide bonds between the *cis* and *trans* conformations at proline residues. An assay for this process was devised about 11 years ago and an enzyme which catalysed this isomerization was purified from porcine kidney [1]. Subsequently several forms of this enzyme, peptidyl prolyl *cis*–*trans* isomerase (PPI), have been purified from a wide range of organisms and the majority have been shown to be immunophilins, proteins which bind immunosuppressive drugs such as FK506, rapamycin or cyclosporin A (CsA) [2–6]. Immunosuppression is thought to occur as a result of formation of a ternary complex between immunosuppressant, immunophilin and protein phosphatase 2b (calcineurin), resulting in inactivation of the phosphatase [7–9]. CsA and FK506 bind to distinct immunophilins, respectively termed cyclophilins and FK506-binding proteins (FKBPs). Immunophilins can also interact with calcineurin in the absence of immunosuppressant drugs [10].

As well as a role in folding of newly translated proteins [11–13], particular PPIs may also take part in other cellular processes involving protein conformational change such as signal transduction. For example, the ryanodine and inositol-1,4,5-trisphosphate receptors associate with FKBP12, which has a role in modulating Ca^{2+} -channel activity [14–17]. A nuclear FKBP can form a ternary complex with casein kinase II and nucleolin [18]. Steroid hormone receptors form inactive high-molecular-mass complexes containing both FKBP and cyclophilin components. Activation of these complexes after hormone binding can be modulated by either class of immunosuppressants [19–22]. Transforming growth factor β type 1 receptors have been shown to interact with FKBP-12 by using the yeast two-hybrid system [23].

Plant immunophilins have been purified in small amounts by immunosuppressant affinity chromatography [24,25] and genes thought to encode cytosolic and plastidic cyclophilins have been cloned [26–32]. Immunophilins have been implicated in the regulation of potassium channels in guard cells [24]. However,

little is known about their normal functions. Here we describe convenient chromatographic methods which have enabled the purification and characterization of cytosolic and microsomal cyclophilins derived from etiolated maize seedlings.

EXPERIMENTAL

Materials

Phenylglyoxal, *N*-ethylmaleimide, α -chymotrypsin and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). The matrices Sephadex G25, CM-Sepharose CL-6B, phenyl-Sepharose and the columns containing phenyl-Superose, Superose 12 and Superdex 75 were from Pharmacia (St Albans, U.K.). The YM10 membrane and Centricon 10 concentration units were from Amicon Ltd (Stonehouse, U.K.). DEAE-BioGel and BioGel HT hydroxyapatite from Bio-Rad (Hemel Hempstead, U.K.), Coomassie and bicinchoninic acid (BCA) protein assay reagents from Pierce and Warriner (Chester, U.K.). CsA was a gift from Sandoz (Leeds, U.K.). A 16.7 mM stock solution was prepared by addition of 0.4 ml/ml Tween 80 to 28 mg/ml CsA in ethanol as suggested by the company. The drug remained in solution when this stock was diluted in water. Phenylglyoxal was freshly prepared as a stock solution (1 M in acetone) immediately before use.

Enzyme assay

The assay for PPI was carried out at 10 °C, essentially as described previously [1]. The assay mixture (1 ml) contained PPI, 35 mM Hepes/Na⁺, pH 7.8, 22 μ M succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The reaction was monitored by the increase in absorbance at 390 nm measured on a Uvikon spectrophotometer (Kontron, Watford, U.K.) and was initiated by the addition of α -chymotrypsin to a final concentration of 0.53 mg/ml. The first-

Abbreviations used: PPI, peptidyl prolyl *cis*–*trans* isomerase; CsA, cyclosporin A; DTT, dithiothreitol; FKBP, FK506-binding protein; BCA, bicinchoninic acid.

* To whom correspondence should be addressed.

order rate constant (k) was calculated by non-linear regression using the Sigmaplot program (Jandel Corporation) in Microsoft Windows. There was a linear relationship between the calculated value of k and the amount of enzyme used up to a limit of $k \approx 0.08 \text{ s}^{-1}$. An enzyme unit is defined as the amount of enzyme which would be required to give a theoretical rate constant of 1 s^{-1} in the assay mixture after subtraction of the non-enzymic rate constant (0.0079 s^{-1}). Values of k_{cat}/K_m were calculated on the basis of protein concentrations determined by the dye binding assay and molecular masses determined by SDS/PAGE. An inhibition constant for CsA was determined as the gradient of the line of best fit from a plot of $[\text{CsA}]/(1 - k/k_0)$ against k/k_0 where k is the rate constant at any given CsA concentration and k_0 is the rate constant in the absence of CsA [33].

Purification of cytosolic PPI

All operations except FPLC were carried out at 0–4 °C. Shoots of etiolated maize (*Zea mays* cv Clipper, 200 g), grown for 5 days at 25 °C in darkness, were ground in a mortar and pestle in 50 mM Tris/acetate, pH 8.0, 1 mM EDTA, 0.1 mM MgCl_2 , 10% (w/v) sucrose, 1 mM PMSF (500 ml) and filtered through a 100 μm nylon mesh. After centrifugation (3000 g, 20 min), the pellet was discarded and the supernatant was centrifuged again (33000 g, 45 min). The microsomal pellet produced from the second centrifugation could be frozen and stored at –70 °C for subsequent use in purification of microsomal PPI.

Cytosolic PPI was purified from the 33000 g supernatant. Ammonium sulphate (390 g/l) was added to give a 60% saturated solution. After centrifugation (20000 g, 20 min), the pellet was retained and dissolved in 45 ml of buffer. The extract was desalted by passage through a column of Sephadex G25 (2.2 cm $\times$ 90 cm), previously equilibrated in buffer A [10 mM sodium phosphate, pH 6.7, 1 mM EDTA, 1 mM dithiothreitol (DTT)] and applied to a column (14 cm $\times$ 1.3 cm) of CM-Sepharose CL-6B which had been pre-equilibrated in buffer A. After washing, the column was eluted with a gradient of 0–0.2 M NaCl over 120 ml. Fractions containing the enzyme, eluting at approx. 0.1 M NaCl, were pooled and ammonium sulphate was added to give a final concentration of 1.0 M. The extract was applied to a column (8 cm $\times$ 0.9 cm) of phenyl-Sepharose pre-equilibrated in buffer A containing 1.0 M $(\text{NH}_4)_2\text{SO}_4$ and, after washing, the column was eluted with a gradient to buffer A over 120 ml. Pooled fractions containing activity, eluting at approx. 0.25 M $(\text{NH}_4)_2\text{SO}_4$, were concentrated using a stirred pressure cell with a YM10 membrane and a Centricon 10 concentrating unit. The final step was FPLC gel filtration on Superose 12. Extract (200 μl) was injected on to the column (10/30 series) which was run in 10 mM sodium phosphate, pH 6.8, 150 mM NaCl, 1 mM DTT at a flow rate of 0.5 ml/min.

Purification of microsomal PPI

The unwashed microsomal fraction prepared from 370 g of 5-day-old etiolated maize shoots was extracted by sonication after the addition of 40 ml of buffer B (5 mM sodium phosphate, pH 7.4, 1 mM DTT). A 10% solution of Triton X-100 was added to give a final concentration of 1%. Following centrifugation (33000 g, 45 min) the supernatant was retained and the pellet re-extracted with buffer B containing 1% Triton X-100 as described above. The supernatants were pooled and desalted using a Sephadex G25 column (2.2 cm $\times$ 90 cm) equilibrated in buffer B. The extract was then applied to a column (14 cm $\times$ 1.3 cm) of DEAE-BioGel which had been pre-equilibrated in buffer B. The non-adsorbed fraction, containing the enzyme activity, was brought to a final concentration of

10 μM CaCl_2 and applied to a column (1.4 cm $\times$ 11 cm) of BioGel HT hydroxyapatite equilibrated in buffer B containing 10 μM CaCl_2 . The column was washed extensively with buffer to remove Triton X-100, then eluted with a linear gradient of buffer B containing 10 μM CaCl_2 to 0.4 M potassium phosphate, pH 7.4, 1 mM DTT, 10 μM CaCl_2 over 200 ml. Two discrete peaks of enzyme activity were resolved. Fractions containing the activity eluting at higher salt concentrations were pooled and EDTA was added to give a final concentration of 1 mM. Solid ammonium sulphate was added to give a final concentration of 1.5 M. Following centrifugation (20000 g, 15 min) to remove any precipitate, the supernatant was applied to a phenyl-Superose 5/5 series column equilibrated in 10 mM sodium phosphate, pH 7.4, 1.5 M $(\text{NH}_4)_2\text{SO}_4$, 1 mM DTT, 1 mM EDTA. The column was run on a Pharmacia FPLC system at 0.5 ml/min. After washing, the column was eluted with a linear gradient from equilibration buffer to 10 mM sodium phosphate, pH 7.4, 1 mM DTT over 30 ml. Fractions containing activity, eluting at approx. 0.7 M $(\text{NH}_4)_2\text{SO}_4$, were concentrated using a Centricon 10 unit. In the final step, gel filtration in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM DTT using a Superdex 75 column (10/30 series) run at 1 ml/min on the FPLC system; the sample was applied via a 200 μl injection loop.

Protein characterization

Protein concentration was generally measured using the dye binding assay [34]. Samples containing Triton X-100 were precipitated in 10% trichloroacetic acid and assayed using the BCA reagent [35]. BSA was used as a standard. SDS/PAGE and electrophoretic transfer to nitrocellulose were carried out by the methods of Laemmli [36] and Towbin et al. [37]. Immunoreactive proteins were visualized by incubation of the blots with primary antiserum (1:500 dilution), followed by biotinylated anti-(rabbit IgG) (1:2000 dilution) and avidin-peroxidase (1 $\mu\text{g}/\text{ml}$) as described previously [38].

Amino acid sequencing

Microsomal PPI was prepared for sequencing by SDS/PAGE on a system designed to minimize N-terminal blocking while maintaining high resolution [39]. Both microsomal and cytosolic PPIs were electroblotted in 10 mM 3-(cyclohexylamino)propane-1-sulphonic acid/ Na^+ , pH 11.0, 10% methanol (7 V/cm, 100 min) on to a polyvinylidene difluoride (PVDF) membrane. The protein was visualized on the blot by staining with Coomassie Blue R250 and excised prior to sequencing on an Applied Biosystems Model 470A protein sequencer, carried out by Pat Barker (Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, U.K.).

Production of antiserum

A New Zealand White rabbit was immunized using three intraperitoneal injections at approx. 4-week intervals, each containing 150 μg of purified cytosolic PPI in Freund's adjuvant. The first injection was in complete adjuvant. Bleeds were taken around 10 days after each injection.

RESULTS AND DISCUSSION

Purification and properties of cytosolic PPI

The cytosolic PPI from etiolated maize has been purified from an initial extract by ammonium sulphate precipitation followed by sequential chromatography on cation-exchange, hydrophobic-interaction and gel-filtration matrices. The procedure, sum-

Table 1 Purification of cytosolic PPI

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Supernatant	530	223	2311	10.4	100	1
0–60% (NH ₄) ₂ SO ₄	65	143	2450	17.1	106	1.6
CM-Sepharose CL-6B	35.5	3.7	1782	478	77	46
Phenyl-Sepharose	58	1.6	1421	875	61	84
Superose 12	2	0.58	355	610	15	59

Figure 1 Purification of cytosolic PPI by Superose 12 chromatography

The extract had previously been purified by ammonium sulphate fractionation, CM-Sepharose chromatography and phenyl-Sepharose chromatography (see the Experimental section). Pooled fractions containing PPI activity are indicated by the bar. Elution volumes of the following molecular mass markers are indicated: 1, Blue dextran (2000 kDa); 2, apoferritin (443 kDa); 3, β -amylase (200 kDa); 4, alcohol dehydrogenase (150 kDa); 5, BSA (66 kDa); 6, carbonic anhydrase (29 kDa); 7, cytochrome *c* (12.4 kDa); 8, azide (42 Da).

marized in Table 1, gave an overall yield of 15% and a 59-fold purification. The reduction in specific activity at the final step probably arises from inactivation during the two concentration steps after phenyl-Sepharose chromatography. SDS/PAGE of the latter fractions (results not shown) indicated that further purification was needed. In the final gel-filtration step shown in Figure 1, the enzyme activity eluted at the same position as the major 280 nm peak corresponding to a molecular mass in the region of 11000 Da. Gel filtration on other matrices indicated the following range of molecular masses (Sephadex G75, 16 kDa; BioGel P60, 11 kDa; Superdex G75, 13 kDa). Increasing the NaCl concentration to 0.5 M did not affect the enzyme's elution volume on Superose 12, suggesting that no significant ionic interactions were occurring.

Analysis of the purified protein by SDS/PAGE indicated that it consisted of a single polypeptide of molecular mass 17.5 kDa (Figure 2, lane 1). The apparent molecular masses obtained from size-exclusion chromatography suggest that either the protein is monomeric with a relatively low partial specific volume for its size, or that it interacts with the matrices.

The value of k_{cat}/K_m was estimated to be $11 \mu\text{M}^{-1} \cdot \text{s}^{-1}$. The enzymic activity of the PPI was inhibited by CsA with an inhibition constant estimated to be 6 nM (Figure 3), indicating that the enzyme is a cyclophilin. In other cyclophilins the value k_{cat}/K_m ranges from 2 to $10 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ at 10 °C, $16.6 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ at 0 °C, $19 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ at 25 °C and K_i for CsA ranges from 2 to 200 nM [3,40–44].

The N-terminus of the purified enzyme appeared to be blocked. Sequencing of polypeptides generated by cyanogen bromide

Figure 2 Analysis of cytosolic and microsomal PPI by SDS/PAGE

Samples were separated on SDS/15% polyacrylamide gels and either stained with Coomassie Blue R250 (lanes 1–3) or transferred to nitrocellulose and probed with the H92 antiserum raised against cytosolic PPI (lanes 4–6). Lanes 1 and 4, purified cytosolic PPI (3 μg); lanes 2 and 5, purified microsomal PPI (3 μg); lanes 3 and 6, a sample (5 μg) from the first peak of PPI activity, elution volume 250 ml from hydroxyapatite chromatography (Figure 5A, thought to represent contamination of the microsomes by cytosolic PPI; see the text).

Figure 3 Inhibition of cytosolic and microsomal PPI by CsA

The main part of the Figure shows apparent first-order rate constant (k) plotted against CsA concentration: ■, cytosolic enzyme; ×, microsomal enzyme. Also shown are theoretical curves plotted using parameters derived from the fitted data. Inset are plots of linear transformations giving K_i (slope) and enzyme concentration (y -axis intercept).

cleavage of the protein (results not shown) indicated that the enzyme is closely related to the product of a previously cloned gene encoding a 172-amino-acid polypeptide from maize [27].

When the enzyme was incubated in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA with 5 mM *N*-ethylmaleimide, a reagent

Figure 4 Immunoactivity of H92 antiserum with cytosolic PPI from a range of species

100 000 g supernatants were prepared from 6-day-old etiolated seedling shoots following a homogenization procedure similar to that described for the purification of maize cytosolic PPI (see the Experimental section). Proteins were precipitated with 10% trichloroacetic acid, redissolved in sample buffer and separated on a 15% polyacrylamide gel. Each track contains 60 µg of protein. Lane 1, oat (*Avena sativa* cv. Bulwark); lane 2, barnyard grass (*Echinochloa crusgalli*); lane 3, wheat (*Triticum aestivum*); lane 4, barley (*Hordeum vulgare*); lane 5, rye (*Secale cereale*); lane 6, maize (*Zea mays*); lane 7, zucchini (*Cucurbita pepo*); lane 8, sorghum (*Sorghum bicolor*); lane 9, oilseed rape (*Brassica napus*); lane 10, soybean (*Glycine max*); lane 11, field bean (*Vicia faba* cv. Minor); lane 12, *Vicia lucerne*.

which reacts predominantly with cysteine and occasionally with lysine and histidine residues, 90 % of the starting activity was lost after 40 min. Inclusion of 100 nM CsA did not prevent inactivation (results not shown). The mammalian enzyme was also inactivated slowly by this reagent [1]. The enzyme is also inactivated by 2 mM phenylglyoxal (in 50 mM sodium bicarbonate, pH 8.3, 0.5 mM EDTA, 1 mM DTT) with 85 % activity lost after 15 min, suggesting that arginine residues may be required for activity (results not shown).

An antiserum, H92, was raised in a rabbit against the purified native protein. On immunoblots, the serum recognized the 17.5 kDa polypeptide with a high degree of reactivity (Figure 2, lane 4). A 36 kDa polypeptide was also recognized. This is probably a dimeric aggregate, since on re-electrophoresis of this excised band, both 17.5 kDa (minor) and 36 kDa (major) bands were seen (results not shown). Polypeptides of similar molecular masses were recognized with a high degree of specificity in a wide variety of plant species tested, including both monocots and dicots (Figure 4). No reaction was found against extracts from three animal sources (pig liver, goat spleen and rabbit brain; results not shown).

Purification and characterization of microsomal PPI

Extracts from thoroughly washed microsomal fractions did not appear to contain polypeptides giving a specific reaction with the

Figure 5 Purification of microsomal PPI

Pooled fractions containing PPI activity are represented by bars. (A) Hydroxyapatite chromatography. The dotted line indicates elution with a 5–400 mM phosphate gradient. The peak eluting at the higher salt concentration was then purified by phenyl-Superose chromatography (see the Experimental section) before the final step. (B) Superdex 75 chromatography. The elution volumes of the following molecular-mass markers are shown: 1, Blue dextran (2000 kDa); 2, BSA (66 kDa); 3, ovalbumin (45 kDa); 4, carbonic anhydrase (29 kDa); 5, myoglobin (17 kDa); 6, cytochrome *c* (12.4 kDa).

H92 antiserum raised against the cytosolic enzyme (results not shown). However, PPI activity released from maize microsomes by treatment with Triton X-100 could be purified by a protocol similar to that used for the cytosolic enzyme. Since activity tended to bind with a low yield to CM-Sepharose CL-6B, an alternative protocol was developed and is summarized in Table 2. In the first step, DEAE-agarose chromatography, the enzyme did not bind to the anion-exchange matrix. The enzyme activity could be bound to hydroxyapatite, enabling removal of Triton X-100 and resolution of two peaks of activity by the phosphate gradient used for elution (Figure 5A). It was suspected that the first activity peak represented contaminating cytosolic PPI activity (see below). The enzyme contained in the second activity peak was then purified by sequential chromatography on phenyl-Superose and Superdex 75 (Figure 5B). In common with cytosolic

Table 2 Purification of microsomal PPI

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Sephadex G25	98	200 ^a	452	2.3	100	1
DEAE-BioGel	135	35 ^a	380	10.9	84.1	4.8
Hydroxyapatite	37	1.23	251	204	56	90
Phenyl-Superose (concentrate)	0.2	0.114	163	1430	36	633
Superdex 75	1.5	0.063	89	1413	20	625

^a Protein measured using BCA reagent.

[illegible]

Figure 6 N-terminal sequence of microsomal PPI

The sequence is aligned and compared with the N-terminal sequences of mature s-cyclophilin from chick [47], human [46,48], mouse [48] and bovine microsomes [45]. Asterisks indicate residues identical with the maize sequence; underlining indicates conservative substitutions scoring 4 or more in the system of Feng et al. [55].

PPI, the enzyme had an elution volume on size-exclusion chromatography slightly larger than that of cytochrome *c*. About 80 % of activity was lost on freezing the purified enzyme in liquid N₂ and thawing.

On analysis by SDS/PAGE, the purified microsomal enzyme appeared as a single 17.7 kDa polypeptide (Figure 2, lane 2). On an immunoblot it was virtually unrecognized by the H92 antiserum raised against the cytosolic enzyme (Figure 2, lane 5) and is therefore immunologically distinct. The first activity peak eluted from the hydroxyapatite column (elution volume of 250 ml, Figure 5a) contained a 17.5 kDa polypeptide which gave a positive reaction with the H92 antiserum (Figure 2, lanes 3 and 6). This is therefore likely to represent contamination of the microsomal fraction by the cytosolic enzyme. CsA inhibited the activity of the microsomal enzyme with a K_i estimated to be 6 nM (Figure 3) showing that it is also a cyclophilin. It has a value of k_{cat}/K_m estimated to be $25 \mu\text{M}^{-1}\cdot\text{s}^{-1}$ compared with values of 3–14 $\mu\text{M}^{-1}\cdot\text{s}^{-1}$ for cyclophilin B from mammals [45,46].

The sequence of the first 30 amino acids from the N-terminus of the microsomal enzyme was determined. It shows a high level of sequence similarity (~60% identity) with the sequences of mature s-cyclophilin (also known as cyclophilin B) from chick [47], human [46,48], mouse [48] and bovine [45] microsomes (Figure 6). At residue 15, about equal levels of valine and isoleucine are present, suggesting that the purified enzyme contains the products of two different genes or alleles. On an immunoblot (results not shown), the plant microsomal enzyme was not recognized by either MAC 256 or 2E7 monoclonal antibodies [49], indicating that in common with its mammalian homologue, cyclophilin B, it does not contain either a KDEL or HDEL endoplasmic reticulum retention sequence at its C-terminus. Nevertheless the localization of cyclophilin B from vertebrates inside the lumen of intracellular vesicles suggests that a retention mechanism exists [50] and immunocytological studies of mammalian cells have indicated that cyclophilin B co-localizes with calreticulin in a specialized intracellular compartment associated with Ca^{2+} signalling, the calciosome [51]. In the latter context, it may also be significant that cyclophilin B can interact in the yeast two-hybrid system with another protein (calcium-signal modulating cyclophilin ligand, CAML) which has been implicated in Ca^{2+} signalling [52]. In some circumstances, cyclophilin B appears to be secreted, into milk [53], or when expressed at high levels in mammalian [54] or insect cells [47].

Cyclophilins from animals are involved in a wide range of

roles and interactions. The availability of the purified cyclophilins and the new specific antiserum described here should facilitate an understanding of the roles of these proteins in plants.

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