Expression and purification of pheophorbidase, an enzyme catalyzing the formation of pyropheophorbide during chlorophyll degradation: Comparison with the native enzyme

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Abbreviations used: Chl, chlorophyll; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; Pheid, pheophorbide; PyroPheid, pyropheophorbide; PPD, pheophorbidase; PVDF, polyvinylidene difluoride.

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Abstract

Formation of pyropheophorbide (PyroPheid) during chlorophyll metabolism in some higher plants has been shown to involve the enzyme pheophorbidase (PPD). This enzyme catalyzes the conversion of pheophorbide (Pheid) *a* to a precursor of PyroPheid, C-13^2-carboxylPyroPheid *a*, by demethylation, and then the precursor is decarboxylated non-enzymatically to yield PyroPheid *a*. In this study, expression, purification, and biochemical characterization of recombinant PPD from radish (*Raphanus sativus* L.) were performed, and its properties were compared with those of highly purified native PPD. Recombinant PPD was produced using a glutathione *S*-transferase (GST) fusion system. The PPD and GST genes were fused to a pGEX-2T vector and expressed in *Escherichia coli* under the control of a T7 promoter as a fusion protein. The recombinant PPD-GST was expressed as a 55 kDa protein as measured by SDS-PAGE and purified by single-step affinity chromatography through a GSTrap FF column. PPD-GST was purified to homogeneity with a yield of 0.42 mg/L of culture. The protein purified by this method was confirmed to be PPD by measuring its activity. The purified PPD-GST fusion protein revealed potent catalytic activity for demethylation of the methoxycarbonyl group of Pheid *a* and showed a pH optimum, substrate specificity, and thermal stability quite similar to the native enzyme purified from radish, except for the K_m values toward Pheid *a*: 95.5 μM for PPD-GST and about 15 μM for native PPDs.

1. Introduction

The pathway for the breakdown of chlorophyll (Chl) consists of several reaction steps,[1-6] and the pathway is operationally divided into three stages based on the modes of reaction and structures of the product formed (Fig. 1). The early stage includes release and modification of the side chain of tetrapyrrole, macro- and isocyclic rings. The middle stage involves cleavage of the macrocyclic ring by pheophorbide (Pheid) *a* oxygenase and its successive modifications, including reduction of red Chl catabolite.[7-9] The last stage is the subsequent degradation of the open tetrapyrrole to smaller carbon- and nitrogen-containing fragments, like organic acids, via monopyrroles.[10, 11]

The first committed step in the early stage of Chl *a* degradation is hydrolysis of the phytyl ester linkage catalyzed by chlorophyllase (EC 3.1.1.14), which forms chlorophyllide *a* and phytol. Molecular cloning of the chlorophyllase gene from *Chenopodium album*[12] and *Citrus* fruit[13] was performed recently. At the same time, the coronatine-induced gene *ATHCOR1* was determined to be the gene encoding chlorophyllase by homology searches of sequences and expression of its gene from *Arabidopsis thaliana*.[12] Subsequently, magnesium is released from the macrocyclic ring and Pheid *a* is formed. Previously, we demonstrated that an activity catalyzing this reaction is not due to a protein, but to a low-molecular-mass, heat-stable substance.[14] The highly purified substance from *C. album* was specific not only for Mg^{2+}, but also for several divalent cations, and therefore we named it "metal-chelating substance".[15] Recent studies surveying the entire Mg-dechelatase activity in extracts from *C. album* using a native substrate confirmed these facts.[16, 17] The metal-chelating substance is thus a possible candidate for the catalyst of the Mg-dechelating reaction. The formed Pheid is shown to be converted into red Chl catabolite by Pheid *a* oxygenase and metabolized further into small compounds in the main Chl degradation

pathway.[5, 10] In some restricted orders of higher plants and algae, an auxiliary step of macrocyclic ring modification occurs further when the formed Pheid *a* is converted to pyropheophorbide (PyroPheid) *a*.[18-20] Two kinds of enzymes that catalyze alternative reactions in the formation of PyroPheid *a* were found. One enzyme, called pheophorbidase (PPD), is an esterase that catalyzes demethylation of the methoxycarbonyl group of Pheid *a* to a precursor of PyroPheid *a*, identified as C-13^2-carboxylPyroPheid *a*; next, non-enzymatic decarboxylation of the precursor occurs, leading to PyroPheid *a*. The other enzyme, termed Pheid demethoxycarbonylase, was purified from the Chl *b*-less mutant NL-105 of *Chlamydomonas reinhardtii*.[21] This enzyme produced no intermediate, unlike the PPD reaction, indicating that Pheid *a* is converted directly to PyroPheid *a*, probably by the acetyl transferase reaction.

Recently, we purified two types of PPD from senescent cotyledons of radish (*Raphunus sativus* L.) to homogeneity and determined the biochemical and molecular properties, including cDNA cloning.[22] The enzyme activity was separated into two peaks by chromatography on a DEAE-Toyopearl column. These enzymes, termed type 1 and type 2 according to their order of elution in the chromatography, correspond to senescence-induced and constitutive enzymes, respectively. Two types of PPD were found in radish, but only one PPD gene, named *PPD*, was obtained in spite of several screenings of radish cDNAs. The deduced polypeptide has a lipase domain that was presumed to be the active site of the esterase, as in the case of chlorophyllase.[12] These enzymes are considered to be involved in the formation of PyroPheid because of their extremely specific substrate specificity for Pheids, and possibly to be responsible for the production of PyroPheid, especially in the anaerobic state,[21] in which the next oxygen-requiring reaction catalyzed by Pheid *a* oxygenase in the main Chl degradation

pathway does not occur due to oxygen limitation. The significance of this auxiliary step of macrocyclic ring modification in the strategy of degradation of photoreactive chlorophylls is, however, still unknown.

In the course of purification, we found fragmentation of the enzyme protein when it was subjected to heat treatment for gel electrophoresis under denatured conditions. Under non-heated conditions, one major band, which size was about 61-77 kDa, appeared depending on the polyacrylamide concentration used, and it was considered to be a dimer from the size of the deduced amino acid sequence. Upon heat treatment of the enzyme even for only 1 min, PPD was separated into three bands with molecular masses of 16.8, 15.9, and 11.8 kDa. It is, however, unclear why PPD is so fragile in heat treatment and the relationship to its protein structure.

In the present study, we found that the GST-fusion PPD expressed by *E. coli* had comparable enzymatic activity and also stability against thermal fragmentation of the protein. In this report, the expression, purification, and biochemical characterization of recombinant fusion PPD are described. In addition, the characteristics of both the recombinant and native enzymes are compared.

2. Materials and methods

Plant materials and chlorophylls

Greening cotyledons of radish (*R. sativus* L.) were purchased from a local market. Senescence was induced according to the method described previously.[10] Briefly, shoots were excised and placed in 50-ml flasks containing 15 ml distilled water. The cotyledons were allowed to reach senescence in complete darkness at 25°C and a constant humidity of 75%.

Chls *a* and *b*, chlorophyllide *a*, Pheid *b*, and pheophytin *a* were prepared as

described previously.[22] Pheid *a* and PyroPheid *a* were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Enzyme assay

The PPD activity was assayed basically according to the methods described by Shioi *et al*.[18] The reaction mixture consisted of 20 mM phosphate buffer (pH 7.0), 160 μM Pheid *a* in acetone (final 20%, v/v), and PPD in a total volume of 100 μl. After incubation in darkness for 10 min at 30°C, the reaction was terminated by adding 200 μl acetone. The amount of C-13^2-carboxylPyroPheid *a* formed was assayed using an HPLC system (Shimadzu, Kyoto, Japan). HPLC was performed using a Cosmosil 5C_{18}-MS-II column (4.6 x 250 mm) (Nacalai Tesque, Kyoto, Japan). Pigments were eluted isocratically with methanol-2 M ammonium acetate (95/5, v/v) at a flow rate of 1.0 ml per min at 30°C. The pigments were monitored spectrophotometrically at 410 nm and quantified by a Chromatopak integrator (Shimadzu). The concentration of C-13^2-carboxylPyroPheid *a* was tentatively calculated using the standard curve for Pheid *a* because isolated C-13^2-carboxylPyroPheid *a* rapidly and spontaneously changed to PyroPheid *a*.[18]

Expression of PPD-glutathione *S*-transferase (GST) fusion protein

Expression and induction of recombinant PPD protein was carried out using plasmid pGEX-2T-*PPD* to transform the *E. coli* BL21(DE3)pLysS strain according to the previous report.[22]

Purification of enzymes

Purification of native enzymes from radish

Native PPDs were purified from greening cotyledons of radish, in which senescence was induced by one day in the dark, as described previously.[22]

Purification of PPD-GST fusion protein

For purification, transformed cells from 2 L culture medium were collected by centrifugation at 5,500 x *g* for 5 min. The cells (8.65 g) were suspended in 20 ml binding buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.3) and disrupted by ultrasonication. The lysate was centrifuged at 12,000 x *g* for 20 min at 4°C, and the resulting solution was clarified again by centrifugation at 160,000 x *g* for 1 h at 4°C. The supernatant was applied to a GSTrap FF column (1 ml) (GE Healthcare Bio-Sciences), washed with 10 ml binding buffer, and then the fusion protein was eluted with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at a flow rate of 1.0 ml/min using the template program for GSTrap on the ÄKTA*prime* liquid chromatography system (GE Healthcare Bio-Sciences), according to manufacturer's protocol.

In order to release the tag-free protein from PPD-GST, the fusion protein was digested with thrombin after concentration of the protein solution to about 0.1 mg protein/ml by a Centricon YM-10 (Millipore). The digestion was carried out with thrombin (5 units/mg proteins) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3) at 20°C. To remove thrombin and the GST tag, the reaction mixture was applied to a TSKgel G3000SW column (7.5 x 300 mm) (Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and eluted with the same buffer used for HPLC. The protein fractions were further separated by SDS-PAGE, and PPD was extracted from the gel after it was cut out. A polyclonal antiserum was prepared by the standard protocol and used without further

purification as described previously.[22]

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed by the method of Laemmli[23] using 15% or 12% (w/v) polyacrylamide gel under reducing conditions. Heat treatment of recombinant PPD was performed with a sample buffer containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol at about 95°C for 5 min. Fixing and staining were done with aqueous methanol (25%, v/v) containing acetic acid (7.5%, v/v) and Coomassie Brilliant Blue (CBB) R-250 (0.25%, w/v), respectively.

For Western blots, proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P; GE Healthcare Bio-Sciences). The blots were incubated with a monoclonal anti-GST antibody or polyclonal antiserum raised against PPD as the primary antibody, and then with a peroxidase-conjugated secondary antibody. Color was developed using diaminobenzidine (DAB) and H_2O_2.

Protein determination

Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, IL, USA) or Bio-Rad Protein Assay (Bio-Rad Lab., CA, USA) with bovine serum albumin as a standard. For column chromatographic fractions, proteins were assayed spectrophotometrically by measuring absorbance at 280 nm at room temperature.

3. Results

Expression and hydrolysis activity of fusion protein

PPD is an esterase that catalyzes demethylation of the methoxycarbonyl group

of Pheid.[18-20, 22] In radish cotyledons, PPD activity increases sharply at one-day senescence. The cDNA coding for PPD, *PPD*, was cloned with the template synthesized from radish RNA.[22] In order to subclone *PPD* into an expression vector, pGEX-2T, we designed a primer set in which *Sma*I and *Eco*RI restriction enzyme sites were appended to forward and reverse primers, respectively, and then amplified *PPD* by PCR. The *PPD* obtained from radish was cloned into pGEX-2T. The recombinant PPD was expressed in the *E. coli* BL21(DE3)pLysS strain using the GST fusion system. When the protein expression was induced by addition of IPTG, *E. coli* cells transformed with the vector containing *PPD* showed a band of 55 kDa (arrowhead in Fig. 2) on SDS-PAGE, while it was not observed with the pGEX-2T vector alone or without induction by IPTG (Fig. 2A). Western blot analysis showed that an anti-PPD antiserum specifically recognized the 55 kDa peptide including a 44 kDa protein and a band of a smaller size (33 kDa) (Fig. 2B). Further, the anti-GST-tag antibody also reacted with the 55 kDa peptide as well as with GST (26 kDa) and the 44 kDa protein (data not shown, *cf.* Fig. 2B, lane 3), demonstrating, together with the result of the calculated size of the protein, that the induced protein was PPD-GST. The 44 kDa protein thus appears to be a degraded GST-PPD that contains both antibody recognition sites. As shown in Fig. 2B (lane 2), the 33 kDa band was also detected from *E. coli* without IPTG induction (Fig. 2B, lane 2), but not detected by the anti-GST-tag antibody, suggesting that the 33 kDa protein is not related to PPD-GST. When the cells of transformed *E. coli* were cultured under favorable expression conditions, such as at 37˚C, almost all PPD was found in the pellet after centrifugation of disrupted cells, suggesting that it is aggregated by forming inclusion bodies. Abundant induction of PPD occurred when the recombinant strain was grown at a relatively low temperature and low IPTG concentration. Recombinant PPD found in the soluble fraction showed

activity reasonably comparable to that of the native purified PPD in the formation of C-13^2-carboxylPyroPheid *a* from Pheid *a*, confirming that the cloned gene was *PPD* (Table 2).

Purification of PPD-GST fusion protein

The PPD-GST fusion protein was purified from the bacterial lysate using a glutathione-Sepharose column (Table 1, Fig. 3). The PPD-GST was effectively recovered in a soluble fraction from the recombinant cells grown for 2 h at 27˚C after addition of 0.1 mM IPTG at the cell concentration at which $A_{600} \simeq 0.6$. The fusion protein was extracted from bacterial cells collected from 2 L culture medium and purified by a GSTrap FF column with an ÄKTA*prime* system according to the manufacturer's protocol. Most of the protein was eluted as one major peak by this one-step purification, and approximately 96% of proteins were removed, including a flow-through fraction. The PPD-GST was purified 104-fold, and the final recovery of the recombinant protein after chromatography was 77.6%; however, 20.0% of the total activity was found in the flow-through fraction of the glutathione-Sepharose column (Table 1). On SDS-PAGE analyses (Fig. 3), PPD-GST emerged as a clear main band with a molecular mass of 55 kDa in the lane of the eluted fraction, with some minor bands (Fig. 3, lane 3).

Removal of GST tag from fusion protein

The plasmid pGEX-2T was designed to allow the removal of GST from the expressed fusion proteins; thus, the PPD-GST fusion protein had a thrombin cleavage site between PPD and GST. Thrombin recognizes the specific sequence X_1-X_2-Pro-Arg/Lys-Y_1-Y_2 (X: hydrophobic amino acid, Y: any except an acidic amino

acid) and truncates it at the C-side of Arg/Lys. The PPD band on SDS-PAGE (Fig. 4A) and the activity (Fig. 4B) both decreased time-dependently when thrombin was added at the concentration of 20 units/mg protein at 25˚C. The PPD-GST band had disappeared by approximately 6 h after the start of incubation, whereas the PPD band was observed from 2 h incubation to about 16 h (Fig. 4A). Concomitantly, PPD activity decreased time-dependently, reaching the 40% level at 6 h, and continued to decline slowly (Fig. 4B). These findings show that a time-dependent decrease, rather than an increase, in activity was caused by removal of the GST tag. The decrease in PPD protein could be partly ameliorated by lowering the concentration of thrombin to 5 units/mg protein and the temperature to 20˚C, suggesting that the decrease is at least partly due to thrombin digestion. In fact, an unknown digestion by thrombin was previously reported by Ayrapetov *et al*. (2003).[24]

To examine the effect of removal of the GST-tag on PPD activity, the expression vector was changed from pGEX-2T to pGEX-6P to change the recognition sequence of thrombin to that of PreScission, which has the least protease-digestion effect on the targeted protein. In the case of PreScission digestion, a decrease in PPD activity was also observed due to thrombin by separating the GST tag at several concentrations tested, although PPD was detected at a similar level as GST by SDS-PAGE and CBB staining (data not shown). This finding suggests that PPD activity is probably decreased by the decreased stability owing to the removal of the GST tag from PPD-GST rather than by partial digestion of PPD itself by protease.

After the digestion of PPD-GST by thrombin, we attempted to remove thrombin from the reaction mixture to stop the reaction by using a HiTrap Benzamidine FF column, which traps thrombin. However, PPD was not recovered due to its adsorption onto the resin. Accordingly, we removed the thrombin and purified the PPD by HPLC

using gel filtration instead of the affinity column. The tag-free PPD purified by HPLC showed fairly low activity, although it was detectable with a CBB stain. The purified protein was used as an antigen to raise a polyclonal antiserum in guinea pig. This polyclonal antibody recognized PPD-GST, tag-free PPD, and native PPD, indicating that the purified protein is PPD.

Comparison between biochemical properties of recombinant PPD-GST and native PPDs

The PPD-GST fusion protein, tag-free PPD, and native PPD were assayed by immunoblotting (Fig. 5). Removal of the GST tag from PPD-GST fusion protein was examined by using anti-PPD antiserum. The soluble fusion protein showed one major band with a molecular mass of 55 kDa and two smaller size bands, one of which, a 44 kDa protein, is probably due to degraded PPD-GST as described above (see Fig. 2B), and the other is an unknown 33 kDa protein (Fig. 5, lane 1). The thrombin-treated, tag-free recombinant PPD showed a molecular mass of 31 kDa in addition to the non-digested PPD-GST (55 kDa) and the unknown 33 kDa protein (Fig. 5, lane 2). The 33 kDa protein emerged only in the expression system using *E. coli*, and it may be due to non-specific binding of the polyclonal antiserum for PPD to protein derived from *E. coli*. In the non-heated conditions, the native PPD showed a molecular mass of 77 kDa with a slightly cleaved band (Fig. 5, lane 3), while PPD-GST had a main activity peak at a molecular mass of 138 kDa and also 309 kDa, but not at 55 kDa after gel filtration (data not shown). The weaker staining of this band is probably due to weak reactivity to antiserum. When the native protein was heat-treated in a sample buffer containing SDS and 2-mercaptoethanol, the band was cleaved into three fragments (16.8, 15.9, and 11.8 kDa).[22] In the present immunoblot analysis, two bands corresponding to

17 kDa and 16 kDa appeared, but the band corresponding to 12 kDa was not found, probably because it was unreactive to the anti-PPD antiserum (Fig. 5, lane 4). These results reveal that the SDS-PAGE profiles, especially those of tag-free PPD (Fig. 5, lane 2) and native PPD (Fig. 5, lane 4), are clearly different.

Some biochemical properties of PPD-GST and native PPDs from radish are compared in Table 2. In this study, we characterized the recombinant PPD-GST because there was no increase, but rather a decrease in the activity, after removal of the GST tag, as described above (see Fig. 4). To determine the effect of pH on the enzyme activity, 60 mM MES-HEPES-tricine buffer was used as an incubation medium in the pH range of 5.5 to 9.0. PPD-GST and the native PPDs, especially type 1, had very similar pH optima of 7.0-7.5 (see Table 2).

Activation energy was calculated from the slope of Arrhenius plots at temperatures from 20˚ to 60˚C to examine the difference between the protein structures of recombinant and native PPD. After 10-min incubation of a reaction mixture including the enzyme solution at the indicated temperatures, the reaction was stopped immediately, and the activity was determined by the standard assay method as described in Materials and methods. The value of PPD-GST was within the ranges of native PPDs. The thermal stability of the enzyme activities was also determined in the temperature range of 30˚ to 80˚C. In this case, residual activity of the enzyme was assayed in the standard conditions after incubation at the indicated temperatures for 10 min in the absence of substrate. All PPDs showed relatively similar activation energies and heat stabilities. Substrate specificity for Chl species was examined with six species of Chls and their derivatives (Chls *a*, *b*, chlorophyllide *a*, Pheids *a*, *b*, and pheophytin *a*). PPD-GST and native PPDs both had substrate specificity only for Pheids *a* and *b*, but not for others, indicating that they have the same substrate

specificity. However, the K_m values for Pheid *a* of the native and recombinant PPDs were different (Table 2). The K_m values calculated for a concentration range of 1 μM to 200 μM of Pheid *a* were 95.5 μM for PPD-GST and 14.1μM and 15.1 μM for native PPD types 1 and 2, respectively. These results indicate that PPD-GST has about 6-fold lower affinity for Pheid *a* than native PPDs, although we did not examine the affinity of tag-free PPD due to its low activity.

4. Discussion

The accumulation of Pheid *a* derivatives in a variety of photosynthetic organisms has been reported.[5, 6] PPD is the enzyme responsible for the formation of PyroPheid as an auxiliary reaction in the early stage of the Chl degradation pathway. PPD has previously been purified from *C. album*[19] and radish,[20] and recently we characterized the enzymatic and molecular properties, including the cDNA, of radish PPD.[22]

In this study, we constructed a recombinant plasmid to produce a fusion protein consisting of GST and PPD in *E. coli*. When grown at 37˚C, the cells expressed a large amount of recombinant protein, but almost all of it existed in inclusion bodies when grown under the conditions described in the usual protocol. By lowering the cultivation temperature, however, the recombinant protein was effectively obtained from a soluble fraction without denaturing or unfolding. The soluble recombinant PPD-GST produced by *E. coli* was confirmed to be PPD by measurement of the Pheid *a* demethylation activity. In addition, except for the K_m value for Pheid *a*, several properties and the substrate specificity of PPD-GST were very similar to those of native PPD. Among the properties compared, the recombinant PPD-GST showed a difference only in the affinity for Pheid *a*. This lower affinity may be partly due to a

disturbance of substrate binding to the active site by the attached fusion protein molecule. However, removal of the GST tag produced no increase, but rather a decrease in the activity (Fig. 4). We therefore could not determine the properties of tag-free recombinant PPD.

The most characteristic difference between tag-free recombinant and native PPDs was in the SDS-PAGE profiles with heat treatment. Native PPD was cleaved into at least three peptides by heat treatment, while the recombinant PPD produced by *E. coli* retained only one peptide, although an unidentified non-specific protein exists. This indicates that the PPD from a prokaryotic expression system is structurally different from the native one obtained from an endogenous expression system, and therefore has reduced enzyme activity, as described above.

In the recombinant PPD, there was also an obvious difference between tag-free and tag-fused PPD. The tag-fused PPD showed enzyme activity comparable to the native enzyme, while the tag-free PPD was unstable and easily lost its activity, suggesting that the GST-tag might be necessary for the enzyme activity, probably by keeping a structure of the enzyme protein in favorable folding condition. This may be related to the subunit structure of native PPD, which forms a tight dimer. Interestingly, it is reported that GST has a similar molecular mass of 26-28 kDa to PPD and also forms a dimer.[25] It is thus speculated that the formation of a subunit structure such as a chimera tetramer may participate to keep the enzyme structure and therefore maintain the enzyme activity, while it is difficult for tag-free PPD to form an active subunit structure automatically after the removal of GST, and therefore the activity is lost due to its instability. The results of the molecular mass determination may support this idea. After gel filtration of PPD-GST by Superdex 200, PPD activity was found in the 138 kDa and 309 kDa fractions, but not in the 55 kDa fraction, suggesting at least that a

chimera dimer has no activity. Furthermore, these findings do not contradict our speculation. We previously proposed a three-dimensional domain-swapping model based on the strong dimer formation and the fragile primary structure.[22] However, the significance of this tertiary structure of PPD itself or in relation to the activity of this enzyme is not clear yet.

5. Conclusion

We constructed an expression system of PPD fused with a GST tag in *E. coli* and produced a recombinant PPD-GST that has activity comparable to that of the native enzyme. The recombinant enzyme showed quite similar biochemical properties to the native enzymes purified from radish. After removal of the GST tag, tag-free PPD showed decreased enzyme activity, probably due to an inability to maintain its higher structure derived from the formation of a tetramer composed of two tight dimers. Our system was adequate to produce the polyclonal antibody, but probably not appropriate for analysis to determine the tertiary structure. To solve this problem, a plant or endogenous cell expression system of PPD will be necessary.

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Figure legends

Fig. 1. Two routes of PyroPheid *a* formation in the degradation pathway of Chl *a*. PPD catalyzes a demethylation reaction from the methoxycarbonyl group of Pheid *a* to a precursor, C-13^2-carboxylPyroPheid *a* (open arrow: left), followed by spontaneous conversion of the precursor to PyroPheid *a* (right). PDC, pheophorbide demethoxycarbonylase, the enzyme catalyzing PyroPheid *a* formation by the alternative reaction.

Fig. 2. SDS-PAGE (A) and Western blot (B) analyses of proteins during expression. Proteins were extracted from *E. coli* cells that had been transformed with pGET-2T or pGEX-2T-*PPD*. Expression of the fusion protein was induced by addition of 0.1 mM IPTG at 37˚C. Proteins were analyzed by SDS-PAGE on 12% polyacrylamide gel and stained by CBB R-250. For Western blotting, peptides were transferred onto a PVDF membrane using a specific anti-PPD antiserum. Numbers on the left denote the molecular masses of the marker proteins. Lanes: M, Molecular markers; 1, Vector + IPTG; 2, *PPD* gene; 3, *PPD* gene + IPTG.

Fig. 3. Purification of GST-PPD fusion protein (for summary, see Table 1). Proteins were separated by SDS-PAGE on 12% polyacrylamide gel and stained by CBB R-250. Lanes: M, Molecular markers; 1, Soluble lysate; 2, Flow-through fraction; 3, PPD-GST fraction. PPD-GST fusion protein is indicated by a solid arrow.

Fig. 4. Sequential changes in thrombin digestion analyzed by SDS-PAGE (A) and enzymatic assay (B). Proteins were separated by SDS-PAGE on 12% polyacrylamide

gel and stained by CBB R-250. Lane M, Prestained molecular markers. Changes in PPD activity during thrombin digestion were determined as described in the text. The activity is expressed relative to non-incubation as the control (100%).

Fig. 5. Comparison of recombinant PPD with native PPD by Western blot analysis. Purified recombinant and native PPDs were transferred onto a PVDF membrane for immunoblotting using a specific anti-PPD antiserum. Proteins were separated by SDS-PAGE on 15% polyacrylamide gel. Numbers under kDa denote the molecular masses of marker proteins. Lanes: 1, Heated recombinant PPD-GST, - Thrombin; 2, Heated recombinant PPD-GST, + Thrombin; 3, Non-heated native PPD; 4, Heated native PPD.

Table 1

Purification of PPD-GST fusion protein

Step	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min /mg protein)	Yield (%)	Purification (-fold)
Lysate	113	7.49	0.0660	100	1
GSTrap FF flow-through	100	1.50	0.0150	20.0	0.227
GSTrap FF	0.843	5.81	6.89	77.6	104

Purification was started with 8.65 g wet weight cells from 2 L culture medium.

Table 2

Comparison of biochemical properties of PPD-GST fusion protein with native PPDs

Property	PPD-GST (Type 2)	Native PPD	
		Type 1	Type 2
K_m (pheophorbide *a*)	95.5 μM	14.1 μM	15.1 μM
pH optimum	pH 7.0-7.5	pH 7.0-7.5	pH 6.5
Activation energy	13.6 kcal/mol	15.9 kcal/mol	12.3 kcal/mol
Thermal stability (50% loss)	65.0˚C	67.5˚C	67.5˚C

Fig. 1

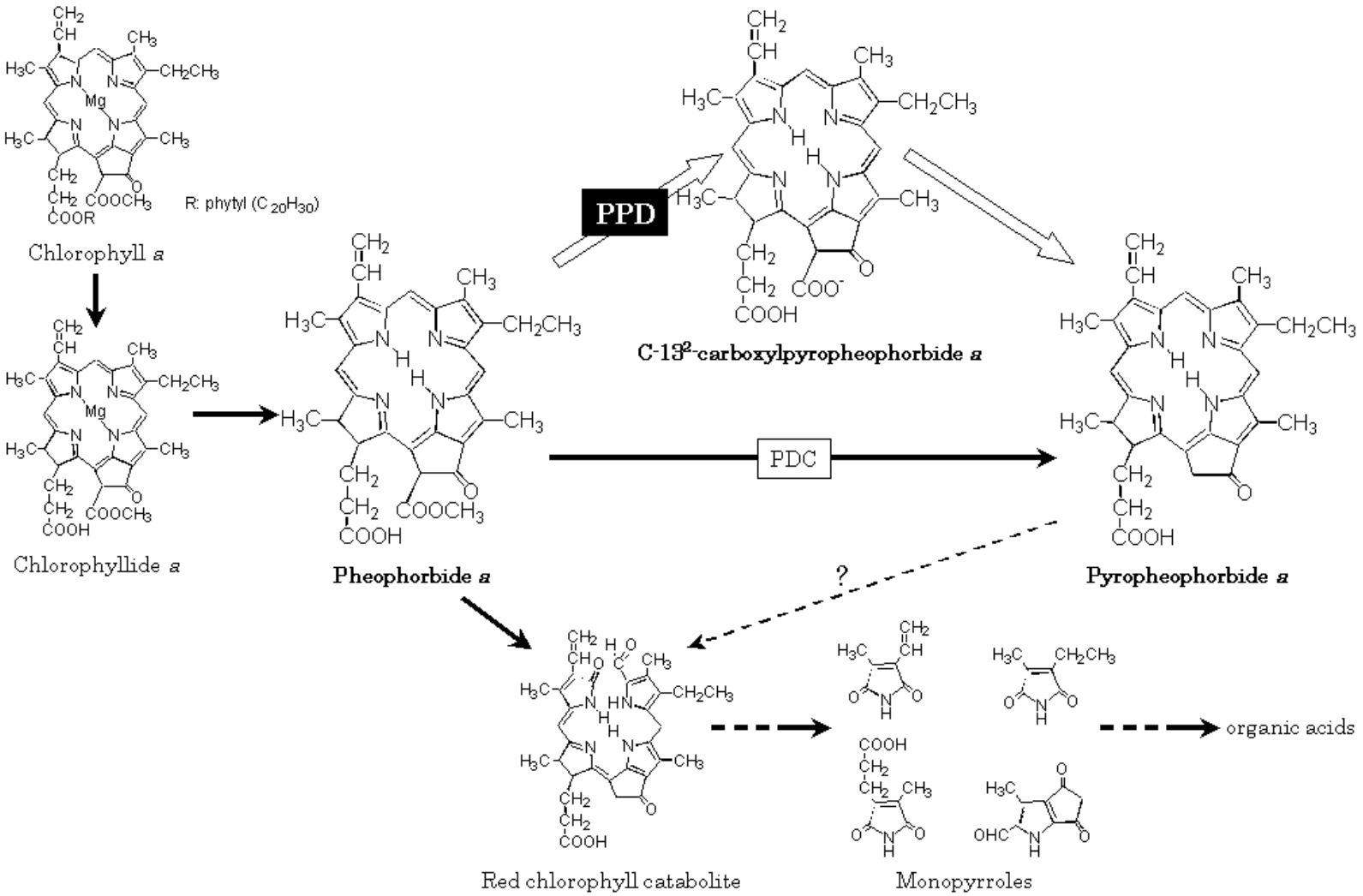

R: phytyl ($C_{20}H_{30}$)
Chlorophyll *a*
Chlorophyllide *a*
PPD
C-13²-carboxylpyropheophorbide *a*
Pheophorbide *a*
PDC
Pyropheophorbide *a*
?
Red chlorophyll catabolite
Monopyrroles
organic acids

Fig. 2

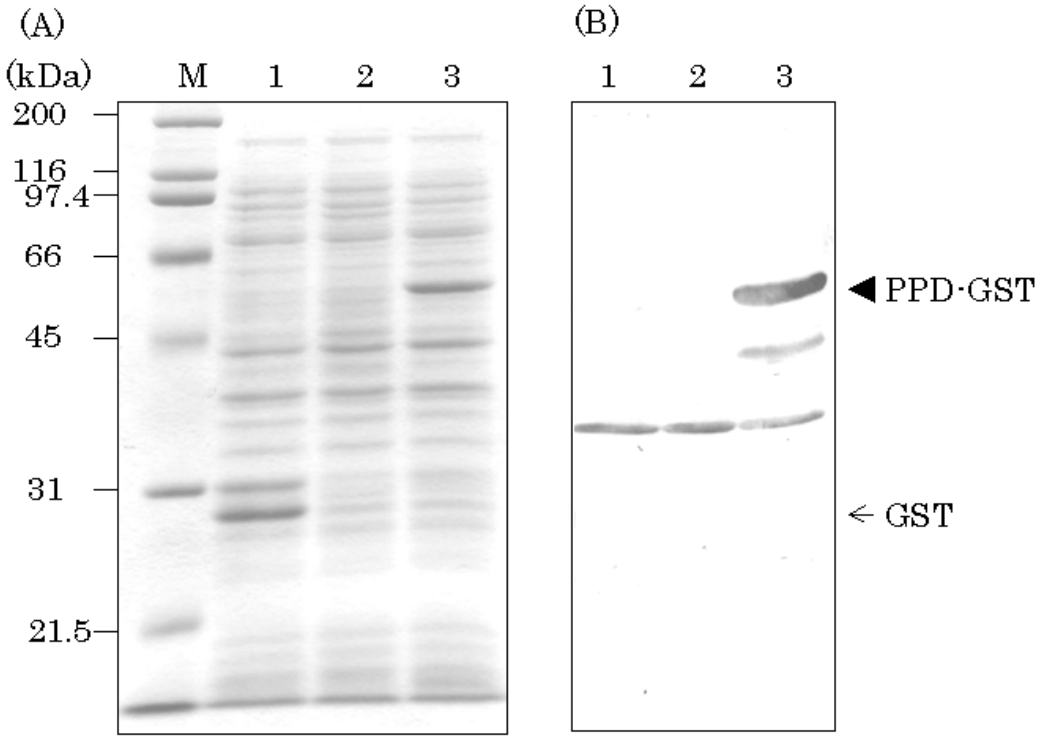


Fig. 3

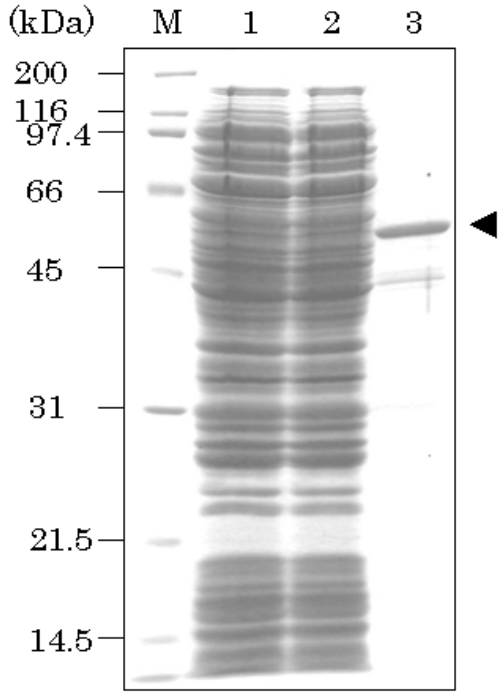


Fig. 4

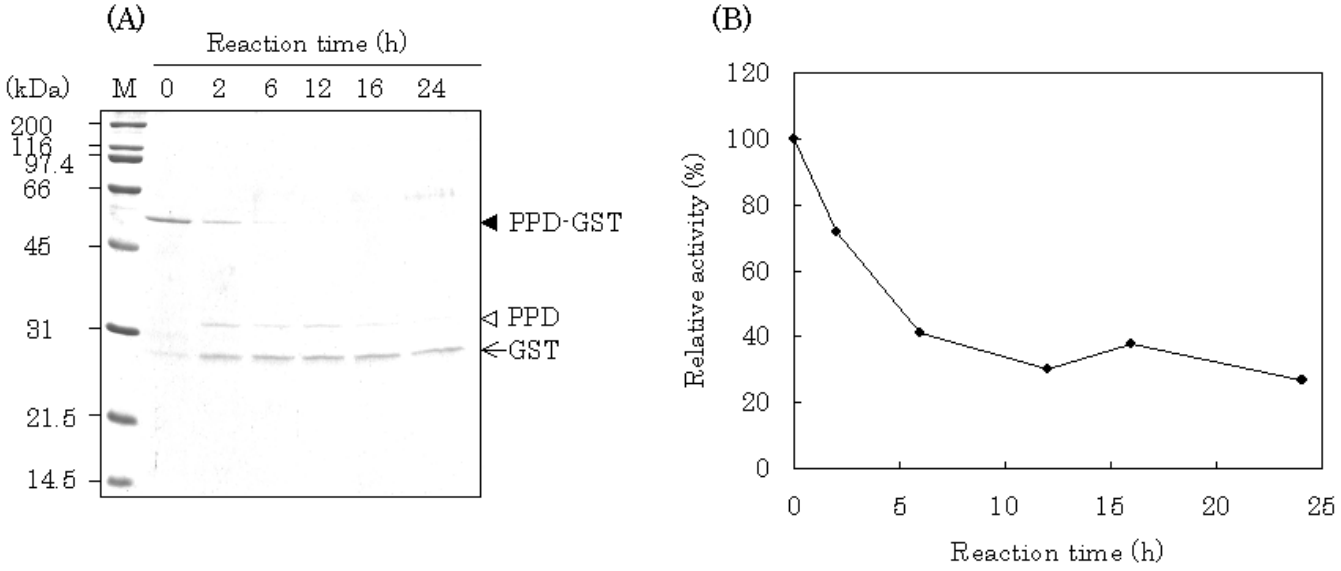
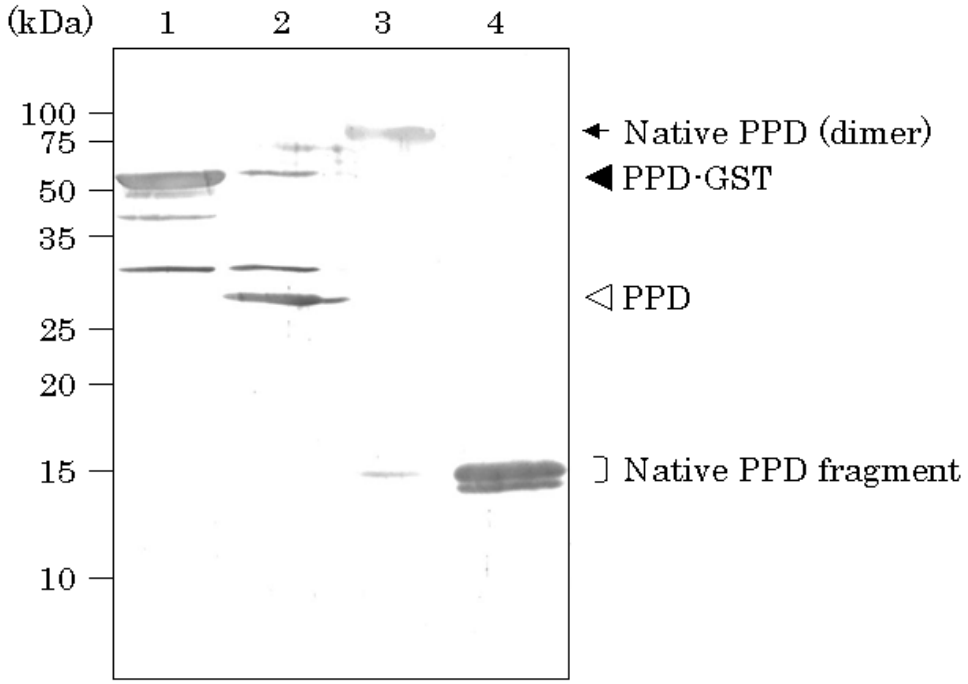

(A)
Reaction time (h)
(kDa)
M
0
2
6
12
16
24
200
116
97.4
66
45
31
21.5
14.5
PPD-GST
PPD
GST
(B)
Relative activity (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
Reaction time (h)

Fig. 5


(kDa)
1
2
3
4
100
75
50
35
25
20
15
10
Native PPD (dimer)
PPD·GST
PPD
Native PPD fragment