# A New Cyclophilin Inhibitor from *Ganoderma lucidum*: Purification and Characterization

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A new inhibitor for peptidylprolyl cis-trans isomerase (PPIase) has been isolated from Ganoderma lucidum and purified to homogeneous state by organic solvent extraction. The purified PPIase inhibitor (GPI) is assumed to be a membrane-associated glycoprotein. GPI inhibits specifically the bovine brain PPIase, a cyclophilin, and has no effect on the FKBP activity. The results of our chemical modification study of GPI indicate the presence of Lys residue(s) at or near its binding site. Like CsA-cyclophilin complex, GPI-bovine brain PPIase complex strongly inhibits the calcineurin activity in vitro, suggesting the possible involvement of GPI in immunomodulating pathway by the formation of PPIase-inhibitor-calcineurin complex.

**Key Words :** Cyclophilin inhibitor, PPIase inhibition, Immunosuppression, *Ganoderma lucidum*

## Introduction

Peptidylprolyl *cis-trans* isomerases (PPIase, EC. 5.2.1.8)[1-3] have been known as the intracellular receptors for cyclosporin A (CsA),[4-6] FK506[7,8] and rapamycin,[9] all clinically important immunosuppressive drugs. Naturally, studies on the biological function and mechanism of PPIase have been largely pursued using these immunosuppressant molecules.[10] Cyclophilin and FKBP are two major classes of this enzyme and their fates in the immunosuppression pathway have been studied in detail. Cyclophilins are the predominant binding proteins of CsA,[5,11] and FK506 binds to FK506-binding protein (FKBP) in calf thymus, human spleen and T-cell line Jurkat cell. [7,8,12] Rapamycin, which is similar to FK506 in both structure and potency of immunosuppressive effect, also binds to FKBP, perhaps competitively with respect to FK506.[9,13] In addition to cyclophilin and FKBP, the third, minor PPIase family has been reported in prokaryotes.[14,15] Cyclophilin-CsA and FKBP-FK506 complexes bind to the third protein, calcineurin, to form a triple complex, and by inhibiting the phosphatase activity of calcineurin, block somewhere in the pathway of T-cell activation.[16-18] Although rapamycin binds to FKBP, the rapamycin-FKBP does not interact with calcineurin.[19] Instead, the rapamycin-FKBP complex inhibits the PI kinase activity of FRAP or mTOR to exert the immunosuppressive effect.[20, 21]

Although the accumulated experimental results from studies with various ligand molecules imply that PPIase could be involved in various physiological functions, the general essential physiological role of PPIase is not clearly understood. The kinetic and mechanistic studies of immunosuppressants reveal that only a small fraction of the abundant cyclophilin and FKBP could be inhibited by effective drugs, suggesting that there should be more general cellular function for the PPIase.[22-24] Furthermore, not all the PPIase inhibitors are known to be inhibitors of T-cell activation. Some CsA and FK506 analogs,[13,25,26] which strongly inhibit respective PPIase, were found to exert no immunosuppressive effect even at high concentration. PPIases, those are not even inhibited by CsA or FKBP, were also identified in prokaryotes.[27,28] Investigation of different kinds of PPIase inhibitors, including endogenous inhibitors, could be a promising way to unveil the physiological function of the PPIase.

In the present study, we have purified from the fruit body of *Ganoderma lucidum*[29-33] a new PPIase inhibitor specific for cyclophilin. This cyclophilin inhibitor seems to be a membrane-associated glycopeptide, which inhibits strongly the bovine brain PPIase catalyzed reaction in vitro, and its complex with PPIase inhibits the phosphatase activity of calcineurin. The general chemical and biochemical properties of this potential nontoxic immunomodulating agent were studied.

## Experimental Section

**Materials.**     N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide, $\alpha$-chymotrypsin, trypsin, trizma base, acrylamide, N,N'-methylenebisacrylamide, maleic anhydride, 2,3-butandione, $NaIO_4$ were purchased from Sigma (St. Louis, USA). Trifluorethanol (TFE) and tetrahydrofuran (THF) were obtained from Aldrich Chemicals (Milwaukee, USA). FK506 and CsA were donated by Dr. E.S Lee, courtesy of Denver Swedish Hospital (Denver, USA). Sephadex G-75 and CM-Trisacryl M were purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Bovine brain and porcine spleen for the enzyme source and 6 year-old dried Korean-grown *Ganoderma lucidum* were purchased in Seoul, Korea.

**Purification of PPIase inhibitor from *Ganoderma lucidum* (GPI).** 30 g dry weight of *Ganoderma lucidum*

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were homogenized in 120 mL absolute ethanol. More ethanol was added to the mixture to get a total volume of 800 mL, which was extracted in a shaking incubator at 30 $^o$C for 7 days. Insoluble debris was removed by centrifugation. To 100 mL of the supernatant solution, 10 mL of 4M alcoholic NaOH were added, and the resulting reddish brown precipitate was centrifuged and dissolved in 20 mL of distilled water. Ammonium sulfate was added to this solution to get 30% saturation, and the resulting precipitate was washed 3 times with absolute ethanol, dissolved in a small amount of distilled water and lyophilized to dryness. The purity of the isolated GPI was confirmed by the single peak in the reversed phase HPLC, using a Hi-Pore Reverse Phase $C_{18}$ column (0.46 × 25 cm, Bio-Rad, USA). Isocratic elution in a mobile phase of acetonitrile in 0.1% trifluoro-acetic acid at a flow rate of 0.5 mL/min was monitored at 280 nm with a Waters 490 Programmable Wavelength Detector.

**Electrophoresis.** Gel electrophoresis in the presence of SDS was carried out in 15% polyacrylamide slab gel (HSE 250 minigel, Hoefer Scientific Instrument) according to the method of Laemmli[34] and in the absence of SDS as demonstrated by Davis.[35] Polyacrylamide gels were stained with Coomassie Brilliant Blue for the protein bands. To identify the glycopeptide in the gel, the stained protein band was de-stained completely in the 25% isopropanol/10% acetic acid solution and counterstained by immersing the gel consecutively in 0.2% thymol (v/v) and 80% sulfuric acid/ 20% ethanol solution (v/v). [36]

**Enzyme purification.** PPIases were isolated and partially purified by the method of Kofron *et al.*[37] with the modification as described elsewhere[38] from porcine spleen and bovine brain. The enzyme preparations were purified about 250 fold as compared to the ammonium sulfate precipitates.

**Assay of enzyme activity.** PPIase activity was determined by using PPIase-chymotrypsin coupled assay method originally suggested by Fischer *et al.*[1] Standard PPIase assay mixture contained 50 mM Tris-HCl buffer, pH 8.0, 1.6 mg (90 U) $\alpha$-chymotrypsin, 0.11 mM synthetic peptide substrate, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, 2% (v/v) TFE, 9.4 mM LiCl and PPIase in total a volume of 0.25 mL. Reaction was initiated by adding substrate to the equilibrated reaction mixture, and changes in absorption at 390 nm were measured using an Ultrospec 4000 uv/visible spectrophoto-meter (Pharmacia Biotech). The first order rate constants were determined by exponential curve fit to the reaction progress curves. The effects of inhibitors and nonenzymic spontaneous isomerization rate constants were determined in a similar manner.

Calcineurin activity was determined by the method of Webb[39] using the "Biomol Green" calcineurin assay kit, AK-804 (Biomol), according to the manufacturers instructuion. Each well of the 96-well microliter plate contained, 50 mM Tris buffer pH 7.5, 0.25 $\mu$M calmodulin, 40U calcineurin, 0.15 mM synthetic substrate RII peptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Val-pSer-Val-Ala-Ala-

Gly), 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.025% NP-40 and 0.5 mM $CaCl_2$ in total volume of 50 $\mu$L. Reactions were run for 10 minutes after addition of substrate and terminated by the addition of 100 $\mu$L of Biomol Green reagent. The liberated inorganic phosphates were determined by measuring the absorption at 620 nm.

**Chemical modification studies.** Arg residues of GPI were chemically modified by the method of Mahley *et al.* with a slight modification.[40,41] GPI was incubated with 100-fold molar concentration of 2,3-butanedione in 0.2 M sodium borate buffer of pH 8.0, and aliquots were removed at intervals and assayed for PPIase inhibitory activity. Lys residues were modified by incubating 1.7 $\mu$M GPI with 0.85 mM maleic anhydride in 50 mM phosphate buffer of pH 8.0 at room temperature.[42] Oxidation of carbohydrate was carried out by reaction of 1.7 $\mu$M GPI with 1.7 $\mu$M $NaIO_4$ at 37 $^o$C.[43]

**Molecular weight determination by MALDI analysis.** MALDI-TOF spectra were obtained using a Voyagen-DE STR Biospectrometry Biosystem (USA). Saturated sinapinic acid was used as the matrix.

**Amino acid composition and sugar composition analysis.** Dried GPI was hydrolyzed in constant boiling HCl for 24 hours at 110 $^o$C. For determination of total cysteine, the protein was oxidized with performic acid before hydrolysis, and for Trp analysis, GPI was hydrolyzed with methane-sulfonic acid. The hydrolyzed amino acid mixture was derivatized with PITC and dried completely. Samples were dissolved into solvent (A) for HPLC (1.4 mM NaOAc, 0.1% TEA, 6% $CH_3CN$, pH 6.1), applied to the HPLC (Hewlett Packard, 1100 series), using Phenomenex luna C-18 column (4.6 × 250 mm, 5 $\mu$m), and eluted with linear gradient of 60% $CH_3CN$ (Solvent (B), 0-100%). Amino acid content was analyzed from the peak area of HPLC chromatogram by comparison with standards. The carbohydrate composition of the isolated GPI was analyzed by the method of Damon and Pettitt.[44] GPI was hydrolyzed 4 hours in 6 N HCl at 100 $^o$C and then another 4 hours in 2 M trifluoroacetic acid at 100 $^o$C. Carbohydrates were analyzed by chromatography in Bio-LC DX600 system, using CarboPac, PA1 column (2 × 250 mm, Dionex, Sunnyvale, USA)

**Protein and sugar determination.** Protein concentration was determined by the method of Bradford[45] with bovine serum albumin as a standard. The carbohydrate content of the GPI preparation was determined by the phenol-sulfuric acid method of Dubois *et al.*[46] using D-mannose as a standard.

## Results and Discussion

**Purification of PPIase inhibitor from *Ganoderma lucidum* (GPI).** Not all efforts to isolate PPIase inhibitor from the soluble fraction of *Ganoderma lucidium* in this study were successful. As a matter of fact, only by mere accident were we able to measure the significant PPIase inhibitory activity from the ethanol solution containing *Ganoderma lucidium*, which had been laid aside on the shelf
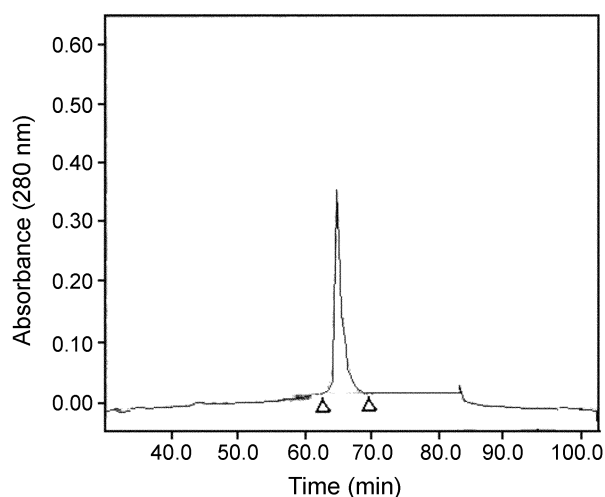
**Figure 1**. Reversed-phase HPLC chromatography of PPIase inhibitor purified from *Ganoderma lucidum.*
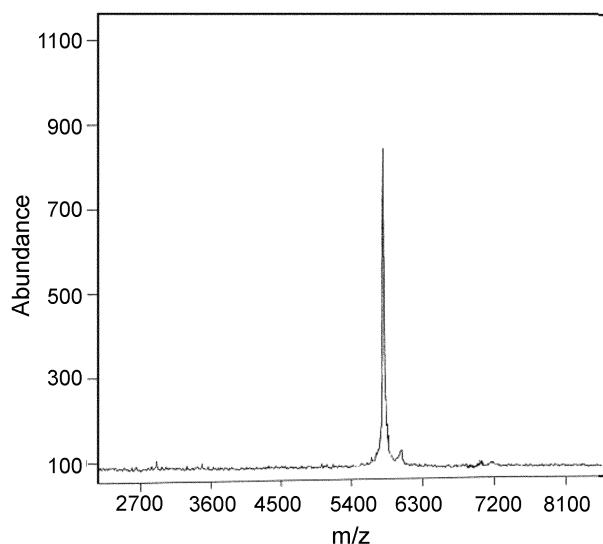


**Figure 2**. MALDI-mass spectral analysis of purified PPIase inhibitor purified from *Ganoderma lucidum*. The purified inhibitor in 0.1% tricholoroacetic aicd was added to the matrix (saturated sinapinic acid in formic acid:water:2-propanol), so that the final inhibitor concentration of the mixture was less than 0.1 mg/mL. 0.5 $\mu$L aliquot of matrix/inhibitor was then applied to a metal probe tip.

**Table 1**. Summary of purification of PPIase Inhibitor from *Ganoderma lucidum*

| | Protein (mg) | $k$ (sec$^{-1}$) | Specific activity (/sec.mg) $\times 10^3$ | Purification fold |
|---|---|---|---|---|
| Ethanol extraction | 66.9 | 0.083 | 1.2 | 1 |
| NaOH ppt. | 23.7 | 0.067 | 2.8 | 1.3 |
| (NH$_4$)$_2$SO$_4$ ppt. | 1.9 | 0.056 | 29 | 24.2 |
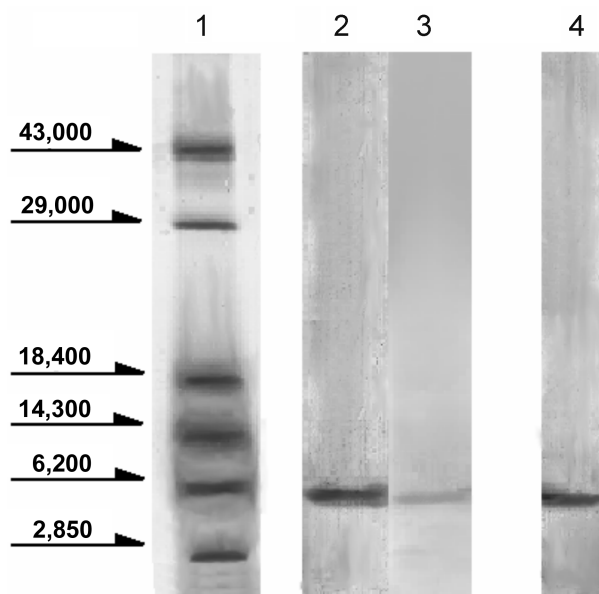


**Figure 3**. Electrophoresis of purified GPI. SDS-polyacrylamide gel electrophoresis of GPI is shown in lane 2 and 3 with molecular weight markers (lane 1): ovalbumin (43,000) carbonic anhydrase (29,000), $\beta$-lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6,200), insulin B chain (2,850). Lane 4 represents the electrophoresis of GPI in non-reducing state. 25 $\mu$g of GPI were loaded for each lane. Lane 2 was stained by Coommassie blue to get the protein band and lane 3 was stained by thymol-sulfuric acid in order to identify the carbohydrate component in GPI.

for about 2-3 months. The alkaline precipitation followed fractional precipitation with ammonium sulfate, and extensive washing with ethanol resulted in the pure glycopeptide fraction with PPIase inhibitor activity as shown in HPLC chromatogram (Figure 1). PPIase inhibitor isolated from *Ganoderma lucidium* was readily soluble in water even though absolute ethanol was required for the primary extraction. This may indicate that the inhibitor is imbedded at least in part in the cellular membranous structure. The molecular weight of GPI was estimated as 5,760 Da, according to MALDI-TOF analysis (Figure 2). In Table 1, the summary of the purification of GPI is shown.

**Electrophoresis of GPI.** Figure 3 shows the single peptide band in both native gel electrophoresis (lane 4) and SDS-PAGE (lane 2 and 3) of the purified GPI. Lane 2 was stained with Coomassie blue to identify the peptide band, and in lane 3, the peptide band was completely de-stained before counterstaining by thymol-sulfuric acid to identify

**Table 2**. Amino acid composition of purified GPI

| Amino acid | Mole % | Amino acid | Mole % |
|---|---|---|---|
| Cya$^*$ | 3.50 | Pro | 6.29 |
| Asx$^{**}$ | 6.51 | Tyr | 1.32 |
| Glx$^{***}$ | 6.88 | Val | 6.31 |
| Ser | 4.22 | Met | 0.96 |
| His | 1.27 | Ile | 4.21 |
| Gly | 17.31 | Leu | 8.10 |
| Arg | 4.21 | Phe | 4.33 |
| Thr | 2.55 | Trp | 2.27 |
| Ala | 10.42 | Lys | 9.34 |

$^*$cysteine & cystine. $^{**}$asparagine & aspartic acid. $^{***}$glutamine & glutamic acid.

**Table 3**. Carbohydrate composition of purified GPI

| Sugars | Content (%) |
|---|---|
| Glucose | 72 |
| Mannose | 11.6 |
| Galactose | 10.4 |
| Galactosamine | 5.1 |
| Fucose | 0.6 |
| Glucosamine | 0.3 |

the carbohydrate component of the GPI (pink colored). This result identifies the existence of carbohydrate component in the GPI.

**Amino acid composition of GPI.** To determine the amino acid composition, the GPI fraction purified by organic extraction and precipitation was further purified by reversed phase HPLC. As shown in Table 2, GPI consisted of relatively large amounts of Gly, Ala, Leu and Lys and
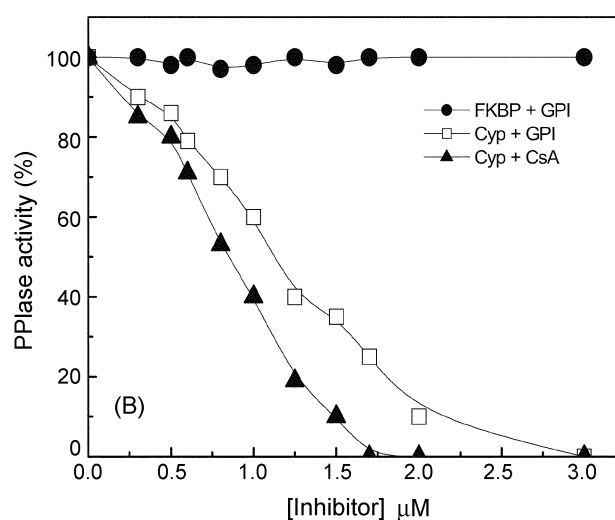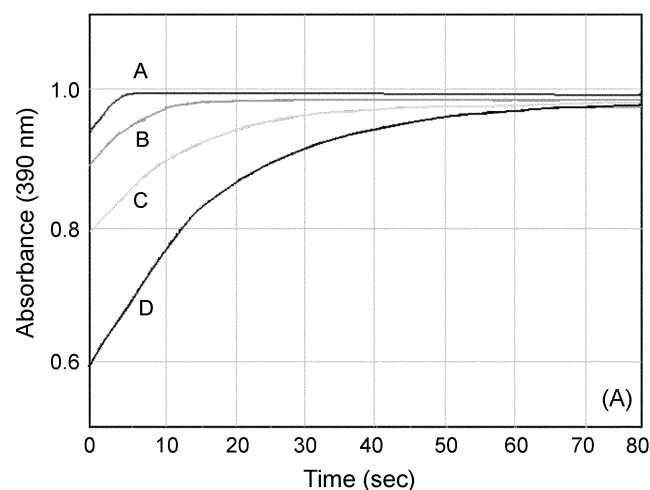




**Figure 4**. PPIase inhibitory activity of purified GPI. (A). Effect of GPI on the progress curves of bovine brain PPIase with various concentration of GPI: A; 0, B; 1.3 $\mu$M, C; 2.1 $\mu$M D; 3.0 $\mu$M. (B). Enzyme specificity of GPI: ● porcine spleen PPIase (FKBP) + GPI; □ bovine brain PPIase (cyclophilin) + GPI; ▲ bovine brain PPIase + CsA.

relatively low contents of aromatic amino acids. N-terminal amino acid could not be identified using either native or deblocking aminopeptidase treated GPI, suggesting that GPI may be a cyclic form, but no further information about its cyclic structure was available at the time of our study.
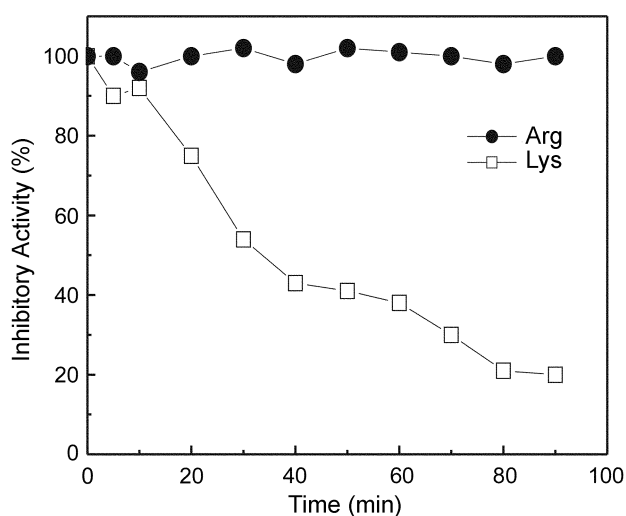
**Sugar analysis of GPI.** The purified GPI comprised about equal amount of peptide and carbohydrate in mass basis (peptide : carbohydrate = 50.2% : 49.8%). The major sugar component is glucose (72% of mass), and mannose, galactose, galactosamine, fucose and glucosamine together composed about 28% of the total mass of carbohydrate (Table 3).

**Enzyme inhibitory activity of GPI.** Figure 4(A) shows the progress curves of the bovine brain PPIase with N-Suc-Ala-Ala-Pro-Phe-pNA as substrate in the PPIase-chymotrypsin coupled reaction. PPIase activity is inhibited by GPI with concentration dependence. In Figure 4(B), the enzyme specificity of GPI is shown. GPI inhibits the activity of PPIase isolated from bovine brain but not from porcine spleen. The bovine brain PPIase is a cyclophilin that is inhibited by cyclosporin A (CsA) but not by FK-506, and the porcine spleen enzyme is found to be a FKBP, which is inhibited specifically by FK-506.[38] These results indicate that GPI is an inhibitor specific for the cyclophilin family of PPIase. In this respect, GPI is similar to CsA and the inhibitory activities of these two inhibitors are comparable.

**Binding site of GPI.** The PPIase inhibitory activity of GPI was not affected by the oxidation of the carbohydrate portion of the GPI by NaIO$_4$. As shown in Figure 5, upon oxidation of 75% of the carbohydrate component of GPI by NaIO$_4$, PPIase inhibitory activity remained unaffected, suggesting that the binding site of the GPI might be located in the peptide region of the molecule and the carbohydrate moiety would not be involved in the inhibitory mechanism. In considering its solubility and high carbohydrate contents, we assume that GPI is a membrane-associated glycopeptide
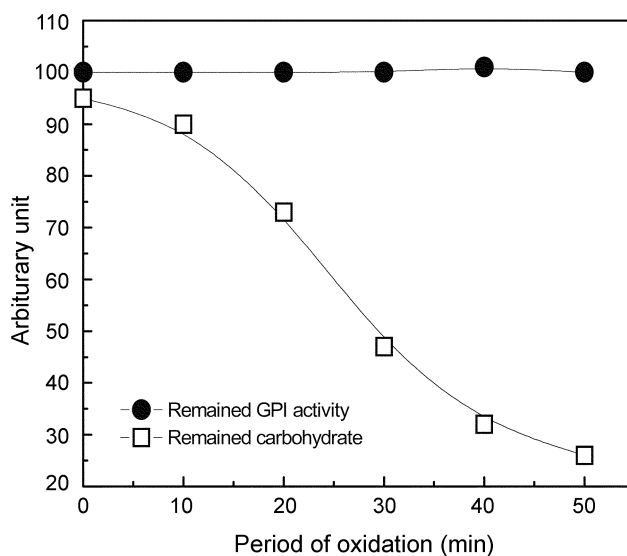


**Figure 5**. Effect of oxidation of carbohydrate of GPI by sodium periodate on its inhibitory activity: ● remained GPI activity; □ remained carbohydrate content.

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**Figure 6**. The effect of the chemical modifications of Arg and Lys residues of GPI on its inhibitory activity; ● Arg modified; □ Lys modified.

whose carbohydrate moiety is exposed at the surface of the membrane and may provide the recognition sites for many extra-cellular compounds and that the binding to cyclophilin is carried out by the polar peptide region exposed to intracellular soluble media. The binding site of the GPI was further studied by the chemical modification. In our preliminary experiments, it was found that GPI lost its inhibitory activity by trypsin digestion and this inhibition by trypsin was protected by prior formation of PPIase-GPI complex, showing that PPIase binds to GPI site in competition with trypsin (data not shown). Figure 6 shows the effect of chemical modifications of Lys residues and Arg residues of GPI by maleic anhydride and 2,3-butanedione, respectively, on its inhibitory activity. By modification of Lys residues, GPI loses most of its inhibitory activity. Meanwhile, the modification of Arg residues of GPI had no effect on its activity. These results and others together suggest that GPI contains Lys residue(s) at or near its binding site to react with PPIase, possibly by electrostatic

**Table 4**. Effect of GPI on the calcineurin activity

| Experi-ments | Composition | [Inorganic phosphate] (nmole) | CnA Inhibition (%) |
|---|---|---|---|
| 1 | Control[*] | 0.435 | none |
| 2 | + PPIase | 0.432 | none |
| 3 | + GPI (0.8 $\mu$M) | 0.447 | none |
| 4 | + GPI (0.8 $\mu$M) + PPIase | 0.229 | 47.4 |
| 5 | + GPI (1.6 $\mu$M) + PPIase | 0.1 | 77.1 |
| 6 | + CsA (0.56 $\mu$M) + PPIase | 0.269 | 38.2 |
| 7 | + GPI (0.8 $\mu$M, Lys modified) + PPIase | 0.342 | 21.4 |

[*]Standard assay mixture contained in 50 mM Tris buffer pH 7.5, 0.25 $\mu$M calmodulin, 40U calcineurin, 0.15 mM synthetic substrate RII peptide, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.025% NP-40 and 0.5 mM $CaCl_2$

interaction between PPIase and GPI.

**Calcineurin inhibitory activity of PPIase-GPI complex.** It has been known that in the process of immunosupression mediated by FK506 or CsA, each of these drugs associates with two enzymes, PPIase and calcineurin.[47] To find out whether PPIase-GPI complex could be involved in the immunomodulating activity or not, the PPIase-GPI complex's effect on the clacineurin activity was studied (Table 4). *In vitro*, neither GPI nor PPIase by itself changes calcineurin activity. Only in the presence of both of PPIase and GPI, calcineurin phosphatase activity was strongly inhibited. This inhibition was partially protected by modification of Lys of GPI. This was readily anticipated because by blocking the Lys residue of the binding site of GPI, it could not form PPIase-GPI complex to react with calcineurin (Figure 6). The results of the present study indicate that GPI inhibits the calcineurin activity by forming PPIase-inhibitor-calcineurin complex. The findings have well elucidated that cyclophilin-CsA-clacineurin complex blocks the proliferation of T-cells during the immunosupression process. Aside from the fact that both of GPI and CsA contain a peptide structure, no other close resemblance is recognized in the structures of these two inhibitors. CsA is a fungal cyclic undecapeptide containing hydrophobic amino acids, which are mostly unusual amino acid, including methylleucine, MeBmt, and D-alanine. On the other hand, GPI, isolated from the medicinal mushroom, is a glycopeptide of which the peptide portion contains various common amino acids of which Gly and Ala are the most abundant, and carbohydrate accounts for up to 50% in mass. The results from this study, neverthe-less, reveal that GPI represents a remarkable similarity to CsA in enzyme specificities. They both inhibit cyclophilin type PPIase at ìmolar concentration, and both of the respec-tive complexes they form with PPIases inhibit calcineurin catalyzed reaction. Recently Zenke *et al*.[48] reported that Sangliefehrin A (SFA), a novel cyclophillin binding macro-lide from Actinomyces, reacted as an immunosuppressor by binding to cyclophillin. In contrast to CsA, SFA-PPIase complex does not bind to calcineurin phosphatase. It is an example of a different mechanism of immunosuppression from well known cyclophillin-drug-calcineurin mechanism. Both rapamycin and FK-506 bind to FKBP, but the two FKBP-drug complexes proceed by different mechanisms in the immunosuppressive process.[19] GPI satisfies the two important prerequisites as a CsA type immunosupressor, at least *in vitro*. This does not mean that GPI would inhibit both PPIase and calcineurin *in vivo*, too, and modulate the immune process within mammalian cells. However, it is certain that GPI can be used to study and modulate at least some parts in the signal transduction of immunosuppression pathway. To our knowledge, the present study is the first to introduce a plant originated cyclophilin inhibitor that corresponds to the microbial originated small molecular weight inhibitors. There have been numerous reports about the physiological and pharmacological efficacy of *Ganoderma lucidum* including iimmunomodulating activity and anti-tumor activity,[49-51] but a real, active chemical component

operating as immunosuppressor had never been elucidated. Further study of this novel, nontoxic, potentially immuno-modulating substance might enlighten about the biological role of PPIase and also open the way to the development of a novel type of immunosuppressor. The function of GPI within the plant cell is not known. It would be interesting to find out whether this substance is involved in some signal transduction in the defense mechanism of the host plant.

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