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Cyclosporin A Binding Protein Type-19 kDa Peptidyl-Prolyl *Cis/Trans* Isomerase from *Euglena gracilis*

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Abstract Cyclosporin A binding protein type-19 kDa peptidyl-prolyl *cis/trans* isomerase (PPIases, EC 5.2.1.8) of *Euglena gracilis* was purified and some of its biochemical characters were elucidated. Purification of the PPIase was achieved by employing a series of steps involving ammonium sulfate precipitation, Superdex G-75 gel filtration chromatography, Mono-Q anion and Mono-S cation exchange chromatographies, and Superdex S-200 gel filtration chromatography on FPLC. Purified PPIase had a specific activity of 8,250 units/mg, showing a 27-fold increase compared with that of cell-free extract of *Euglena gracilis*. The enzyme consisted of a single polypeptide chain with a molecular mass of 19 kDa. It showed high substrate specificity to succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, and k_{cat}/K_m for this substrate was found to be 61.19×10^5/sec. The isomer distributions were investigated at an equilibrium of seven different peptide substrates, varying Xaa in Suc-Ala-Xaa-Pro-Phe-p-nitroanilide in dimethylsulfoxide. The *cis/trans* equilibrium constants were estimated to be from 0.14 (Ile) to 0.63 (Gly), which correspond to 12.00% to 38.52% of the *cis* population, respectively, under experimental condition. The enzyme was highly sensitive to the immunosuppressive ligand cyclosporin A, but not to other immunosuppressants such as FK506 and rapamycin. Thus, it appears to belong to the class of cyclophilin.

Key words: Peptidyl-prolyl *cis/trans* isomerase, cyclosporin A, cyclophilin, *Euglena gracilis*

Peptidyl-prolyl *cis/trans* isomerases (PPIase), which were first discovered by Fisher *et al.* [6], accelerate the conformational change of the proline-peptide bond from *cis* to *trans* (rotamase activity). Proline-containing oligopeptides and proteins in the unfolded form usually exhibit two slowly interconverting conformational states with respect to prolyl peptide bonds [7]. The reason of why high-energy barriers for the *cis/trans* interconversion exist may be found in the partial double-binding character of the prolyl peptide bond. The PPIase can considerably accelerate this interconversion in both directions. The existence of three families of PPIase has been confirmed by numerous studies [4, 12]. The families were named as cyclophilins (Cyps) [5, 21, 25, 32–34], FK506-binding proteins (FKBPs) [9, 14, 28], and parvulins [26]. Despite the fact that the amino acid sequences of Cyps and FKBPs do not show noticeable homology to each other, both classes are able to ligate immunosuppressive peptide derivatives. Cyps form complexes with the cyclic undecapeptide cyclosporin A, and FKBPs are able to bind FK506 as well as rapamycin [17]. The number of the PPIase family can be grouped, depending on their binding specificity for their competitive inhibitor, such as rapamycin, FK506, and cyclosporin A. However, elucidation of PPIase functions *in vivo* may not be successful with these inhibitors, because active site competition simultaneously affects both complex formation and catalysis [8]. Furthermore, the significance of enzyme activity to cell signaling cannot be dissected from inhibitory effects because of the additional bioactivity associated with the PPIase-inhibitor complexes themselves [1, 23, 30].

Euglena is a single-celled microorganism that has both animal and plant characteristics [3]: *Euglena* exhibits a photosynthetic property and requires vitamin B_1 and B_{12} as growth factors, and like animal cells, they have a long whip-like flagellum used for locomotion. The body of *Euglena* is typically elongated and somewhat spindle-shaped and is enclosed by a flexible, striated pericle made chiefly of proteins [3]. The front end of the body forms a flask-shaped cavity, or reservoir. Extending from the reservoir is the flagellum. A red eyespot, or stigma, is located near one wall of the reservoir at about the same level as a swelling on the flagellum. In addition to the numerous chloroplasts, there are reserves of carbohydrate in the cell. *Euglena* has been used for the production of sulfur-rich

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single-cell proteins, and the single-cell proteins are very useful for materials in various fields including the food and feed industries. Therefore, the formation of proper proteins in the cell is of major interest. We found a cyclosporin A binding protein-type of PPIase in cell-free extract *of E. gracilis*, but the question of why *Euglena* has the cyclopilin-type PPIase remains to be answered.

The objectives of this research were to purify and to characterize PPIase to mediate protein folding from *E. gracilis* containing rich protein. These activities may be involved in the correct folding of proteins in the cell. Binding study using various immunosuppressive ligands was also employed to classify PPIase in *E. gracilis*.

MATERIALS AND METHODS

Materials

Ethylenediamine-tetraacetic acid (EDTA), DL-dithiothreitol (DTT), and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, U.S.A.). Substrates for the assay of PPIase were products of Bachem, Bubendorf, Switzerland. Standard proteins (ovalbumin, anhydrase, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin) as molecular mass markers were obtained from Gibco (Gaithersburg, MD, U.S.A.). Superdex G-75, Mono-Q, Mono-S, and Superdex S-200 were obtained from Pharmacia Biotech (Uppsala, Sweden). All other chemicals had the highest purities commercially available.

Growth of Organism

E. gracilis Z (wild-type) was obtained from Osaka Prefecture University, and the strain was maintained on CM (Cramer-Myers) broth: $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; EDTA-2Na, 5 mg; $Fe_2(SO_4) \cdot 7H_2O$, 3 mg; $MnCl_2 \cdot 4H_2O$, 1.8 mg; $CoSO_4 \cdot 7H_2O$, 1.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.4 mg; $NaMoO_4 \cdot 2H_2O$, 0.2 mg; $CuSO_4 \cdot 5H_2O$, 0.02 mg; vitamin B_1, 2.5 mg; and vitamin B_{12}, 0.005 mg per liter of distilled water. After the pH was adjusted to 3.5 with HCl, the CM broth was autoclaved at 121°C for 20 min. For the main cultures, 4 l of CM broth was inoculated with 200 ml of preculture (3.2×10^5 cells/ml) in a Jar-fermenter (Korea Fermentor Co, Korea). The culture was maintained with stirring at 100 rpm with continuous addition of 5% CO_2 and illumination at 1,000 lux (27°C). The cells were harvested by centrifugation (3,000 rpm, 10 min) 4 days after inoculation.

Enzyme Assay

The activity of PPIase was determined according to the method described by Fischer *et al.* [5]. The peptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was used as a substrate. The assay mixture contained 100 mM Tris-HCl (pH 8.0) buffer, 2 μl of the test peptide (1.25 mg/ml DMSO), and PPIase in a final volume of 100 μl. The reaction was initiated by the addition of 10 μl of α-chymotrypsin (3.5 mg/ml). The PPIase activity was measured by a UV/VIS spectrophotometer at 390 nm. The coupling enzyme assay [7, 13] consisted of the reversible PPIase reaction coupled with the irreversible α-chymotrypsin, which catalyzes hydrolysis of the C-terminal amide bond of peptide. In this reaction, α-chymotrypsin has the isomer selectivity and cleaves the Phe-p-nitroanilide amide bond of only *trans* isomer of substrate. One unit was defined as the amount of enzyme to isomerize the test peptide from *cis* to *trans* form in 1 min.

Purification of PPIase

All operations were carried out at 4°C. The *E. gracilis* cells harvested were suspended in 250 ml of Tris-HCl buffer (pH 8.0), containing 4 mM EDTA and 10 mM DTT. After cells were disrupted by French Press at 10,000 psi, cell-free extract was obtained by centrifugation at 10,000 rpm for 25 min. Solid ammonium sulfate was added to the supernatant to give a final concentration of 80%. After standing on ice for 20 min, the precipitate was collected by centrifugation as described above, and the pellet was solubilized in 10 mM Tris-HCl buffer (pH 8.0). The solution was applied onto a Superdex G-75 gel permeation column (50×1.5 cm) on FPLC that had previously been equilibrated with 10 mM Tris-HCl (pH 8.0) buffer. The column was eluted at a flow rate of 1 ml/min. Fractions were collected every 2 min, and protein level was monitored at 280 nm. Enzyme fractions were pooled and applied onto a Mono-Q anion-exchange column on FPLC (5×50 mm) with 10 mM Tris-HCl (pH 8.0) buffer. After measuring PPIase activity, the enzyme fractions were pooled and dialyzed for 3 h in 10 mM potassium phosphate (pH 6.0) buffer for the next step, Mono-S cation-exchange (5×50 mm) chromatography. Pooled enzyme suspension concentrated using ultrafiltration cell (Amicon) was loaded onto a Superdex S-200 gel permeation column on FPLC (10×40 mm), and the enzyme was eluted with 10 mM Tris-HCl (pH 8.0) buffer at a flow rate of 1 ml/min. Enzyme activity in every fraction was measured as described above.

SDS-PAGE and Molecular Mass Determination

The purity of PPIase was examined by SDS-PAGE with the following standard proteins as molecular mass markers: ovalbumin (43 kDa), anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa), insulin (β-chain; 3.4 kDa). After electrophoresis, gels were stained with Coomassie-brilliant blue R250. The molecular mass of the native enzyme was determined by size exclusion gel chromatography on FPLC using a Superdex S-200 column (50×1.5 cm), which was previously calibrated with standard proteins: bovine serum albumin (66 kDa),

carbonic anhydrase (29 kDa), cytochrome (12.9 kDa), aprotinin (6.5 kDa).

Substrate Specificity of PPIase

The k_{cat}/K_m values of substrates for the PPIase were calculated with seven tetrapeptides, varying in amino acid Xaa in succinyl-Ala-Xaa-Pro-Phe-nitroanilide, by the first-order rate constant of enzyme catalyzed reactions and uncatalyzed reactions of the same peptides. Reactions were carried out in assay mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 2 μl of test peptide (2.5 mg/ml DMSO), and PPIase (0.015 mM) in a final volume of 100 μl. The reaction was initiated by the addition of 10 μl of α-chymotrypsin (3.5 mg/ml), and the activity was measured by a UV/VIS spectrophotometer at 390 nm.

Inhibition of PPIase by Immunosuppressive Drug

The inhibition studies were performed by adding immunosuppressive ligands such as cyclosporin A, FK506, or rapamycin in DMSO at concentrations between 1 and 10 μM. The activity of PPIase was determined as described by Fischer *et al.* [5].

RESULTS AND DISCUSSION

Purification of PPIase from *E. gracilis*

Proteins in cell-free extract of cultured cells were pooled by ammonium sulfate precipitation, and the solubilized cell protein was applied onto Superdex G-75 gel filtration chromatography. After measuring PPIase activity as described in Materials and Methods, fractions with enzyme activity were collected and applied to a Mono-Q ion-exchange column following a Mono-S ion-exchange column. PPIase was found in fractions passed thorough the column of both ion-exchange chromatography steps, and it did not bind to the columns. The enzyme fractions were concentrated by ultrafiltration, and the enzyme solution was further applied to a Superdex S-200 gel permeation column on FPLC as a final step. After these steps, the enzyme was found as a single purified band on SDS-PAGE, as shown in Fig. 1. The overall procedure of purification is illustrated in Table 1. The specific activity at the last purification step increased about 27-fold with 6.7% recovery.

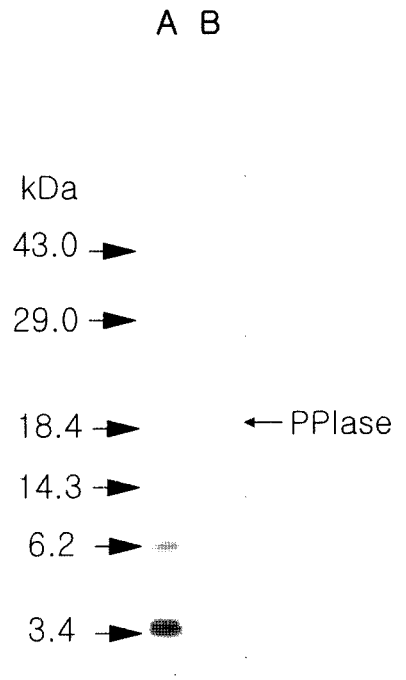

Fig. 1. 15% SDS-PAGE of PPIase purified from *E. gracilis*. Lane A: Standard marker proteins (ovalbumin, 43 kDa; anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa; bovine trypsin inhibitor, 6.2 kDa; insulin (β-chain), 3.4 kDa; insulin (α-chain). Lane B: Purified PPIase from *E. gracilis* (1 mg) after Superdex S-200 gel filtration chromatography on FPLC.

PPIase is a ubiquitous protein found in cells and various organisms. Numerous studies attempted to purify and characterize three families of PPIase [9, 11, 21, 24, 27, 28, 32]. Furutani *et al.* [11] reported purification of 16 kDa PPIase from *Methanococcus thermolithotrophicus* as a bacterial source with 2.6% of enzyme recovery, and Rainer *et al.* [27] also purified 17 kDa PPIase from *Tolypocladium inflatum*, an imperfect fungi, with 17% of enzyme recovery. PPIases from maize were in both cytosolic and microsomal fractions [24] and in the endoplasmic-reticulum lumen [32] and were purified with different purification folds.

Table 1. Purification steps of PPIase from *E. gracilis*

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
30–80% $(NH_4)_2SO_4$	10	148,000	482	307	1	100
Superdex G-75	30	121,600	132	921	3	82
Mono-Q	5	16,330	3	5,433	18	11
Mono-S	3	14,400	2.4	6,000	20	9.7
Superdex S-200	2	9,900	1.2	8,250	27	6.7

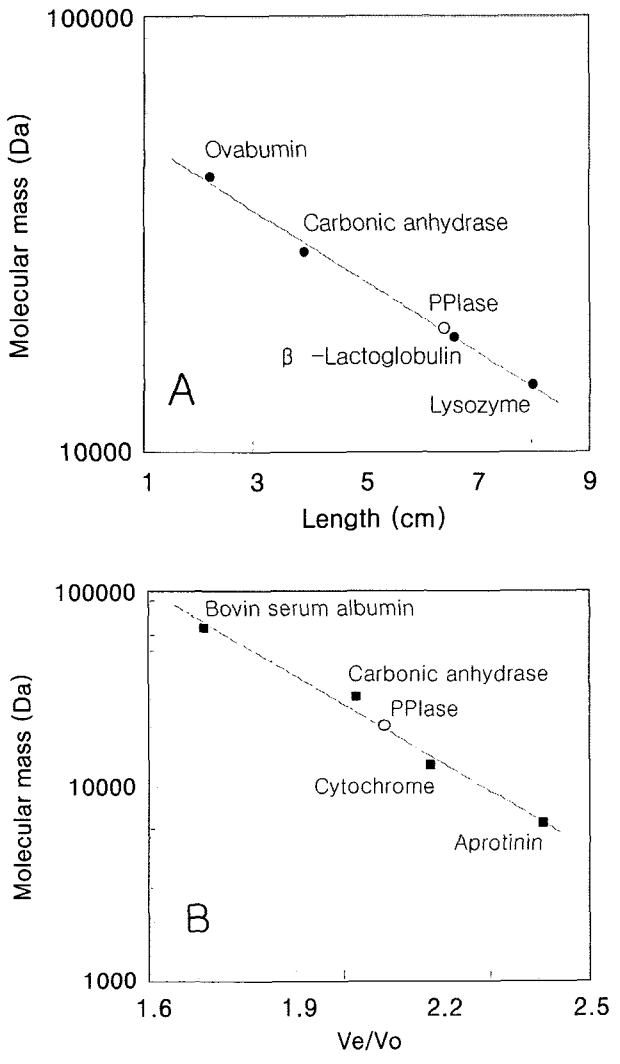

Fig. 2. Determination of molecular mass of PPIase from *E. gracilis*.

A: SDS-PAGE revealed that D-HIV dehydrogenase migrates as a single protein band with a molecular mass of about 19 kDa, compared to standard proteins (ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa). B: Molecular mass determinations of the native enzyme were carried out by size exclusion chromatography, using a Sephadex G-150 column, which was previously calibrated with standard proteins (bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome 12.9 kDa; aprotinin, 6.5 kDa).

Determination of Molecular Mass of Purified PPIase

The molecular mass of the denatured form of the purified PPIase was determined, as shown in Fig. 2(A). SDS-PAGE showed that PPIase migrated as a single protein band with a molecular mass of about 19 kDa, based on comparison with standard proteins [ovalbumin, 43,000 Da; anhydrase, 29,000 Da; β-lactoglobulin, 18,400 Da; lysozyme, 14,300 Da; bovine trypsin inhibitor, 6,200 Da; insulin (β-chain), 3,400 Da]. The molecular mass of the native enzyme was determined by size exclusion gel chromatography on FPLC using a Superdex S-200 column, which was previously calibrated with standard proteins [bovin serum albumin, 66,000 Da; carbonic anhydrase, 29,000 Da; cytochrome, 12,900 Da; aprotinin, 6,500 Da]. The elution volume of PPIase indicated a molecular mass of 19 kDa, assuming that the protein has a globular structure (Fig. 2B). Both methods gave consistent molecular masses for PPIase, and these results suggest that the native form of the enzyme is composed of a single peptide subunit.

PPIase homologue has been isolated from a variety of animal sources as well as from yeast, bacteria, and plants [32]. In many organisms, as in mammals, multiple isoforms are expressed, and they are localized to different subcellular compartments [10]. The first purified PPIase, a novel cyclophilin, exhibited a molecular mass of 18 kDa [5]. Price *et al.* [25] reported a 21 kDa PPIase containing an NH_2-terminal signal sequence, which was suggested to mediate translocation into the endoplasmic reticulum. The 23 kDa PPIase isoform contains an NH_2-terminal signal sequence, like 21 kDa PPIase [19]. Several isoforms of PPIase are known to contain the rotamase (PPIase) domain with at least 50% homology and with certain residues completely conserved, particularly in the rotamase active site [34].

Assay of Peptidyl-Prolyl *Cis/Trans* Isomerization of Prolyl Peptides

The typical reaction activity curves for the *cis/trans* isomerization of succinyl-Ala-Ala-Pro-Phe-p-nitroanilide

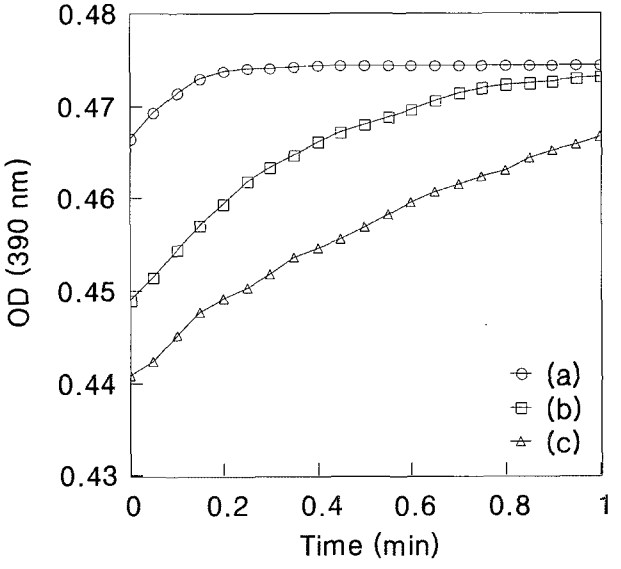

Fig. 3. *In vitro* peptidyl-prolyl *cis/trans* isomerization of Suc-Ala-Ala-Pro-Phe-p-nitroanilide by PPIase from *E. gracilis*. The assay mixture contained 100 mM Tris-HCl (pH 8.0) buffer, 2 μl of test peptide (1.25 mg/ml DMSO), and PPIase in a final volume of 100 μl. The reaction was initiated by the addition of 10 μl of α-chymotrypsin (3.5 mg/ml).

(a) Control assay with *E. gracilis* PPIase only, (b) control assay with addition of both PPIase and cyclosporin A, (c) assay without both PPIase and cyclosporin A.

are shown in Fig. 3; (a) in the presence of *E. gracilis* PPIase only, (b) in the presence of both PPIase and cyclosporin A, and (c) in the absence of both PPIase and cyclosporin A. There were abrupt changes of absorbance at the early phase of reaction, probably during the mixing time. This abrupt change in absorbance reflects a rapid reaction of the *trans*-substrate with α-chymotrypsin. The remaining *cis*-substrate does not react with α-chymotrypsin. Non-enzymatic conversion of *cis* to *trans* peptide is the rate-limiting step for the whole coupled reaction; therefore, it appears as a slow exponential increase of absorbance in this curve. The addition of PPIase accelerated reaction velocity of this rate-limiting step and the substrate isomerized from *cis* to *trans* by PPIase can further be hydrolyzed by α-chymotrypsin. The data in Fig. 3 revealed a much larger change of absorption and faster reaction by *E. gracilis* PPIase compared to the experiments of Harrison and Stein [13] where the change of absorbance due to remaining *cis*-peptide was only 0.13 and the initial burst phase lasted for about 10 seconds after mixing with substrate.

Substrate Specificity of PPIase from *E. gracilis*

Synthetic prolyl peptide substrates in solution exist in equilibrium of *cis/trans* conformer. Since PPIase catalysis depends on the population of *cis* isomer in peptide substrates, the *cis/trans* equilibrium constant or the percentage of *cis* isomers in an equilibrium mixture of peptide substrate is very important for the catalytic activity of PPIase. Harrison and Stein [13] studied the equilibrium constant of several different peptide substrates and they calculated K*eq*=[*cis*]/[*trans*] of the substrate, succinyl-Ala-Xaa-Pro-Phe-ρ-nitroanilide, at 10°C. Kofron *et al.* [18] reported that the *cis/trans* equilibrium constant varied depending on the medium in which the peptide was dissolved, and that the presence of metal salt and water content also affected the equilibrium. The isomer distributions at equilibrium of seven different peptide substrates, varying Xaa in Suc-Ala-Xaa-Pro-Phe-ρ-nitroanilide, in DMSO are shown in Table 2. The *cis/trans* equilibrium constants were estimated to be from 0.14 (Ile) to 0.63 (Gly), corresponding to 12.00% to 38.52% of the *cis* population, respectively, under experimental condition.

Table 3. Substrate specificity of *E. gracilis* PPIase toward succinyl-Ala-Xaa-Pro-Phe-ρ-nitroanilide.

Peptide No.	Xaa	k_{ne} (10^2 s^{-1})	k_{cat}/K_m (10^5 s^{-1})	Relative activity
1	Ala	2.89	61.19	100
2	Gly	2.15	3.53	5.77
3	Glu	2.38	3.33	5.44
4	His	3.52	20.17	32.96
5	Ile	3.88	12.45	20.35
6	Leu	3.42	20.57	33.62
7	Lys	2.54	38.36	62.69

The k_{cat}/K_m values of substrates for the PPIase for isomerization were calculated with seven different tetrapeptides, varying in amino acid Xaa in succinyl-Ala-Xaa-Pro-Phe-nitroanilide, by the observed first-order rate constant of enzyme catalyzed reactions and uncatalyzed reactions of the same peptides. Reactions were carried out in assay mixture containing 0.1 M Tris-HCl buffer (pH 8.0), 2 μl of test peptide (2.5 mg/ml DMSO), and PPIase (0.015 mM) in a final volume of 100 μl.

The k_{cat}/K_m values were compared with the values of substrate specificity of PPIase. The k_{cat}/K_m value for a PPIase-catalyzed reaction can be calculated from the first-order rate constant observed and the rate constant for non-enzymatic isomerization according to Eq. (1) [16]

$$k_{cat}/K_m=(k_{obs}-k_{ne})/[E] \quad (1)$$

where k_{obs} is the observed first-order rate constant for the enzyme reaction of PPIase and k_{ne} represents the rate constant for non-enzymatic isomerization. [E] is the total concentration of PPIase. The values of k_{cat}/K_m for *E. gracilis* PPIase catalyzed *cis/trans* isomerization of 7 different synthetic peptides are listed in Table 3, along with their first-order rate constant for uncatalyzed reactions: Non-enzymatic isomerization rate constants ranged from 3.88×10^2/s (Ile) to 2.15×10^2/s (Gly). The least reactive substrate for PPIase isomerizes spontaneously most slowly, and the most reactive substrate non-enzymatically reacts with the highest rate. On the basis of the specificity data, all substrates can be classified into 3 groups; the group with high specificity (Ala), the groups with very low specificity (Glu and Gly), and the group with intermediate specificity (Leu, Ile, Lys, and His). The k_{cat}/K_m value for

Table 2. *Cis/trans* equilibrium constants of prolyl peptide in 0.1 M Tris-HCl buffer (pH 8.0). Synthetic prolyl peptide substrates exist in an equilibrium of *cis/trans* conformer in solution. The equilibrium constants (K*eq*=[*cis*]/[*trans*]) of several different peptide substrates were calculated with succinyl-Ala-Xaa-Pro-Phe-ρ-nitroanilide at 10°C.

Peptide No.	Structure of substates	K*eq*=*tran*/*cis*	Percent *cis* peptide
1	N-Suc-Ala-Ala-Pro-Phe-ρNA	0.16	13.38
2	N-Suc-Ala-Gly-Pro-Phe-ρNA	0.63	38.52
3	N-Suc-Ala-Glu-Pro-Phe-ρNA	0.43	29.94
4	N-Suc-Ala-His-Pro-Phe-ρNA	0.16	14.20
5	N-Suc-Ala-Ile-Pro-Phe-ρNA	0.14	12.00
6	N-Suc-Ala-Leu-Pro-Phe-ρNA	0.15	12.78
7	N-Suc-Ala-Lys-Pro-Phe-ρNA	0.22	17.93

Ala-peptide was 61.19×10^5/s. The values of Glu- and Gly-peptides were estimated to be 3.33×10^5/s and 3.53×10^5/s, respectively. k_{cat}/K_m values of the rest of the peptides ranged from 12.45×10^5/s to 38.36×10^5/s.

Inhibition of PPIase Activity by Immunosuppressive Drug

PPIase binds selectively to immunosuppressive ligands, such as cyclosporin A, FK506, and rapamycin. Immunophilin-immunosuppressive ligands complexes are able to inhibit the clonal expansion of T cells [29], and they exert toxic effects on other cellular components. In contrast to other immunosuppressant binding proteins [22], immunophilins exhibit at a PPIase activity that catalyzes slow conformational interconversion at around the angle ω of prolyl bonds in oligopeptides and proteins *in vitro* [9, 11, 12, 34] and *in vivo* [2, 20, 31]. The families have been named as cyclophilins (Cyps), FK506-binding proteins (FKBPs), and parvulins [26]. Cyps are able to bind the cyclosporin A (CyA), whereas FKBPs bind the FK506 and rapamycin. After binding immunosuppressive ligands to immunophilins, the immunosuppressant inhibits the enzymatic activity of the immunophilins, and the complex is presented to cellular components [10, 15].

The PPIase from *E. gracilis* bound cyclosporin A; however, it had no affinity to FK506 and rapamycin (Fig. 4), such as cyclophilin from *Tolypocladium inflatum* [27]. The PPIase purified from *E. gracilis* was highly sensitive to cyclosporin A in the nanomolar range, and the rotamase activity of PPIase was fully inhibited with 10 μmol of cyclosporin A. FK506 and rapamycin had no effect on the ratamase activity of the PPIase. These results provide direct evidence that PPIase from *E. gracilis* can be regarded as a family of the Cyps. In the case of FK506-binding protein from *Methanococcus thermolithotrophicus,* 50% of rotamase activity was inhibited by 250 nM FK506 [19]. A similar inhibitory ratio (50%) was found for the microsomal cyclophilin from maize at 5 nM cyclosporin A concentration [32]. In this study, no FKBP was found in *Euglena,* and it would be interesting to elucidate the functions of cyclosporin A binding protein-type PPIase in *E. gracilis* in protein folding.

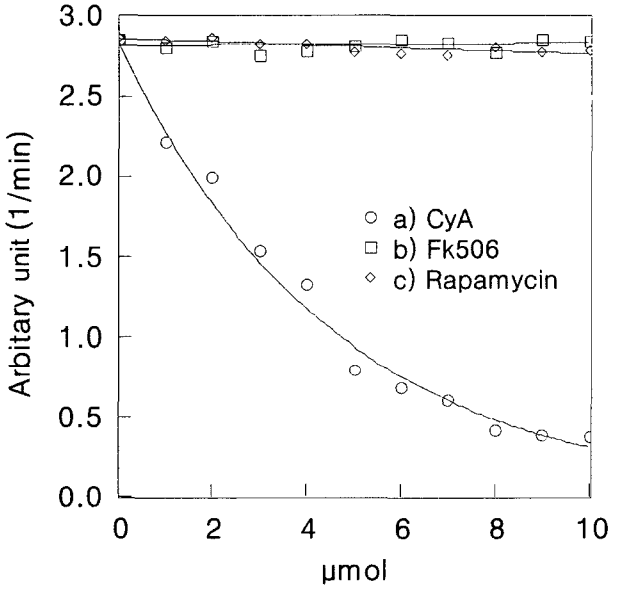

Fig. 4. Inhibition of PPIase activity by immunosuppressive ligands such as a) cyclosporin A, b) FK506, and c) rapamycin at different concentrations.

The assay mixture contained 100 mM Tris-HCl (pH 8.0) buffer, 2 μl test peptide (1.25 mg/ml DMSO), and PPIase in a final volume of 100 μl. The reaction was initiated by the addition of 10 μl of α-chymotrypsin (3.5 mg/ml).

Acknowledgments

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