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Purification and Characterization of Peptidyl Prolyl *cis-trans* Isomerase (PPIase) from *Bacillus stearothermophilus* SIC1

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The peptidyl prolyl *cis-trans* isomerase(PPIase, EC 5.2.2.8) from *Bacillus stearothermophilus* SIC1 was extracted from the cells treated with by lysozyme. PPIase was purified from the cell extracts by heat treatment, ammonium sulfate precipitation, ion exchange chromatography and finally gel filtration (FPLC). The purity of purified the enzyme after Superose 12 column chromatography was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE). The molecular weight of the purified PPIase was estimated as 18,000 by SDS-PAGE. The 39 amino acid residues from the N-terminus were determined by the protein sequencer. The enzyme showed the optimum pH at 8.0 and was stable at the range of pH 7.0 to 8.0.

The enzyme was considerably stable after heat treatment at 60℃ for 30 minutes, and the enzyme was quite stable up to 65℃. The presence of the PPIase in the refolding solution accelerated the isomerization rate of the assay peptide.

Key words: PPIase, protein folding, *Bacillus stearothermophilus* SIC1

Introduction

Peptidyl prolyl *cis-trans* isomerase (PPIase) catalyzes the *cis-trans* isomerization of Xaa-pro peptide bonds in oligopeptides and accelerates the slow, rate-limiting steps in the refolding of several proteins *in vitro* (Fischer et al., 1984; Lang et al., 1987; Line et al., 1988). It is thought to be essential for protein folding during protein synthesis in the cell (Brandts et al., 1975; Fischer et al., 1984).

PPIase from porcine kidney is a cytosolic protein of 17 kDa molecular weight. Amino acid sequence surprisingly revealed this protein to be identical to cyclophilin from bovine thymocytes(Takahasi et al., 1989; Fischer et al., 1989). Cyclophilin had been identified originally as the major high affinity binding protein for the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984; Harding et al., 1986). PPIase is known to be bound by the immunosuppressive drug(Lang et al., 1987; Takahasi et al., 1989) such as cyclosporin A (CsA). The enzymatic activity is inhibited by cyclosporin A (Handschsumacher et al., 1984). Later studies have led to the hypothesis the the effect of cyclosporin A (CsA) is mediated through inhibition of the peptidyl prolyl *cis-trans* isomerization activity of PPIase in T-helper lymphocyte activation. These facts suggest that PPIase plays an important role in the regulation of T-cell activation (Hander et al., 1987; Bunjes et al., 1981; Elliot et al., 1984; Emmel et al., 1989).

One action of CsA thought to be central to many of its immunosuppressive effects is its ability to suppress the events occurring early in T-cell activation, such as lymphokine gene transcription in response to signals initiated at the antigen receptor. Recently CsA was found to specifically inhibit the appearance of DNA binding activity of nuclear proteins involved in T-cell activation (Crabtree et al., 1989; Elliot et al., 1984; Emmel et al., 1989). Since CsA inhibits the activity of PPIase, the PPIase activity may be required

for DNA binding and transcriptional activation of nuclear proteins (Emmel et al., 1984).

Studies on PPIase in the *B. stearothermophilus* should be useful in obtaining information on the function of PPIase *in vivo* because *B. stearothermophilus* SIC1 is prokaryotic organism that can be handled genetically.

In this paper, We demonstrated that the *B. stearothermophilus* SIC1 contains PPIase activity, so we propose that this enzyme is catalysed the isomerization of proline peptide bonds, a reaction efficiently accelerated by PPIase. Thus we examined whether the inhibition of PPIase activity is related with the immunosuppressive drug.

Materials and Methods

Microorganism, medium, cultivation

The microorganism used in this study was a strain of *Bacillus stearothermophilus* SICI was discribed previously (Zang, et al., 1988).

The medium for enzyme production consisted of the following composition (L-broth) 1% bacto tryptone, 0.5% yeast extract, 1% NaCl. The pH was adjusted to pH 7.3 prior to autoclaving. The preculture was carried out aerobically in 500ml shaken flasks containing 100ml of the medium at 60℃ for 24 hours on a circular shaker at 160 rpm. The cultivation for the production of enzyme was carried out under the same conditions as the preculture at 60℃ for 24 hours.

Materials

Bacto tryptone and yeast extract were purchased from Difco Laboratories (Detroit, Mich, USA). The tetrapeptide substrate N-succinyl-Ala-Ala-Pro-Phe-P-nitroanilide (pNA) and chymotrypsin were purchased from Sigma (St. Louis, MO, USA). Isoproply-β-D-thiogalactoside (IPTG) was purchased from Wako Pure Chemeical Industries (Osaka). RNase TI was purchased from Boehringer Mannheim Yamanouchi (ToKyo). All other reagent-grade chemicals were obtained from Wako Pure Chemical Inc. (Tokyo).

Protein assay.

The protein concentration was measured by a BCA protein assay reagent (Pierce Chemical Company, Rockford, Illinois, USA).

Activity measurement of PPIase.

The PPIase activity was measured in a coupled assay with chymotrypsin using the oligopeptide, *N*-succinyl-Ala-Ala-Pro-Phe-pNA (Fischer et al., 1984). The pNA moiety can be cleaved off rapidly by chymotrypsin only when the preceding Ala-Pro peptide bond is in the *trans* conformation. The synthetic peptide (1.68 mM solution) was preincubated with appropriate concentration of PPIase in 0.035 M HEPES-buffer, pH 7.8, containing 2-mercaptoethanol, and the assay was started by mixing with 0.75 mM chymotrypsin in a spectrophotometer cell at 10℃. At a high protease concentration, hydrolysis of the *trans* peptide occurs in a few seconds. The *cis* peptide, however, cannot be cleaved unless Ala-Pro is isomerized from *cis* to *trans*. The changes was monitored by the increase in the absorbance at 390 nm using a Shimadzu UV 160 spectrophotometer (Shimadzu Corp., Kyoto).

Determination of molecular weight by gel filtration

Estimation of molecular weight of PPIase was carried out by gel filtration (EPLC) with a colunm of Superose 12. The column was equilibrated and proteins were eluted with 50 mM Tris-HCl (pH 7.8) at a flow rate 12 ml/hour. The proteins were detected by absorption at 280 nm and by assaying the enzyme activity. The standard proteins used for calibration were cyanocobalamin (1,350), myoglobulin (17,000), bovine serum albumin (67,000), aldolase (158,000), catalase (232,000), ferritin (440,000), and thyroglobulin (669,000) as marker proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of enzyme was determined by SDS-PAGE according to the method of Laemmli (Laemmli et al., 1988). Samples and marker proteins mixed with an equal volume of sample buffer were boiled for 5 minutes. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 or silver staining. The following moleiular weight standards (Bio-Rad Laboratories, CA, USA) were used: egg white lysozyme, 14,000; soybean trypsin inhibitor, 21,000; bovine carbonic anhydrase, 31,000; egg white ovalbumin, 43, 000; bovine serum albumin, 66,000; rabbit muscle phosphorylase b, 97,000.

Amino acid sequence

The N-terminal amino acid sequence was determined by a protein sequencer (473A Protein Sequencer, Applied Biosystems, USA) connected with an amino acid derivative analyzer (120A PTH Analyzer, Applied Biosystems, USA).

The catalytic effect of PPlase on slow protein folding of RNase TI

The unfolded RNase TI was prepared by incubation of 100 μM recombinant RNase TI in the solution (8.0 M urea, 0.1M Tris-HCl and 1 mM EDTA, pH 8.0) for 2 hours at 25℃. Refolding was initiated by diluting 25 μl unfolded protein with 975 μl of 0.1M Tris-HCl/ 1 mM EDTA (pH 8.0), containing the appropriate concentrations of PPlase in the UV-spectrophotometer cell at 10℃. The refolding reaction was monitored at 320 nm (2 nm band width) using a Hitchi UV-spectrophotometer (Kiefhaber et al., 1990; Kozak et al., 1983).

Results

Purification of PPlase from *B. stearothermophilus* SIC1

Preparation of crude extracts

A 10ℓ culture of *B. stearothermophilus* SIC1 was grown at 55℃ in L-broth medium. The cells were harvested (100 g wet weight), washed with 0.1 M Tris-HCl (pH 7.8) buffer and treated with lysozyme in 50 ml of 0.1 M Tris-HCl (pH 7.8) containing 5 mM 2-mercaptoethanol at 37℃ for 30 minutes, and disrupted by sonicator. Cell extract was centrifuged at 15,000 rpm for 30 minutes to remove cell debris. The supernatant was incubated at 60℃ for 30 minutes and then centrifuged at 15,000 rpm for one hour to remove denatured proteins.

Ammonium sulfate precipitation

Supernatant collected from heat treatment was treated with ammonium sulfate. The fractions showing the enzymatic activitis were collected between a saturation of 30~60% ammonium sulfate. Solid ammonium sulfated was added to the enzyme solution to reach 40% saturation and allowed to stand for at least 1 hour. The supernatant was collected and again

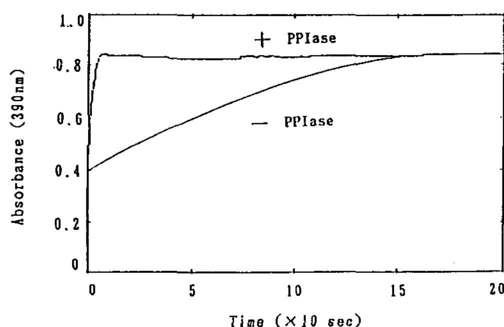

Fig. 1. Assay of PPlase activity *B. stearothermophilus* SIC1.
The enzyme activity was measured using the short synthetic peptide (*N*-succinyl-Ala-Ala-Pro-Pro-Phe-pNA) as the substrate in a two-step reaction coupled to chymotrypsin as previously described under "Meterials and Methods". The reaction was performed in 21 ml HEPES (35 mM) buffer, pH 7.8, at 10℃ in the presence of 50 μl assay peptide (1.8 mM) and 20 μl chymotrypsin (0.76 mM) and monitored at absorbance 390 nm. Symbols; +PPlase, the presence of PPlase; −PPlase, the absence of PPlase.

ammonium sulfate was added to reach 60% saturation. Supernatant was discarded and the precipitate was dissolved with minimal volume of buffer (10 mM Tris-HCl, pH 7.8). The enzyme solution was dialyzed against the same buffer with several times.

DEAE Sepharose CL-6B column chromatography

The dialyzed samples were applied to a column of DEAE Sepharse CL-6B (ϕ2.5×10cm, Pharmacia, Uppsala, Sweden) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol. Elution of the enzyme was performed with a linear gradient of 0.3M NaCl at flow rate 5 ml/min. The PPIase activity was detected in the 0.1 M NaCl. The fractions with PPIase activity were colleacted (Fig. 2).

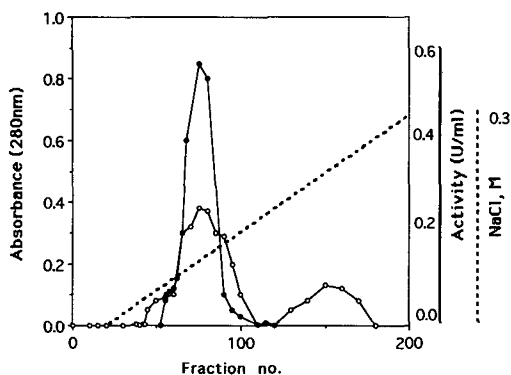

Fig. 2. DEAE Sepharose CL-6B column chromatography of crude PPIase from *B. stearothermophilus* SICI.
The crude enzyme was loaded onto a DEAE Sepharose CL-6B column (ϕ1.5×10 ℃) equilibrated with 10 mM Tris-HCl buffer, pH 7.8. Elution was done with a linear gradient of 0 to 0.3 M NaCl in 20 mM Tris-HCl buffer (pH 7.8), as indicate dotted line. The fractions of eluent were collected at flow rate of 5 m/min. Symbols; ●, PPIase activity; ○, absorbance at 280 nm. Line ···, NaCl concentration.

Sephadex G-75 gel filtraiton

The pooled fractions from DEAE Separose CL-6B were applied on a Sepadex G-75 column (Pharmacia) (ϕ1.5×90cm) equilibrated with 0.15 M NaCl/10 mM Tris-HCl (pH 8.0) buffer. The activity fractions were pooled and concentrated using an ultrafiltration at 4℃.

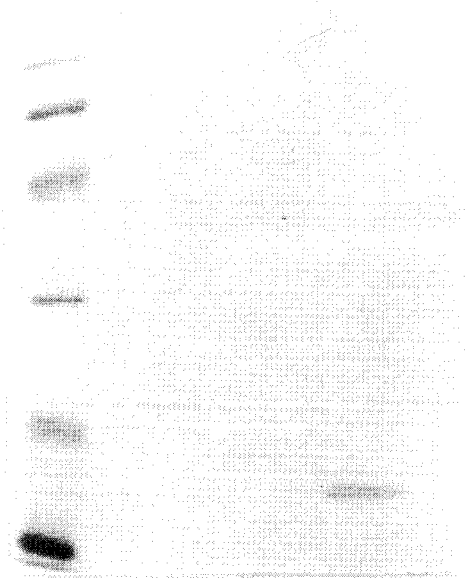

Fig. 3. SDS-PAGE of PPIase from *B. stearothermophilus* SIC1.
Lane M, molecular weight standards; lane 1, Sample (2 μg) filtration (FPLC). Molecular weight markers (from top to bottom: rabbvit muscle phosphorylase (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), soy bean trypsin inhibitor (20 kDa), lysozyme (14.3 kDa). Protein bands were stained with silver stain.

Superose 12 gel filtration

The fractions were applied to Superose 12 column equilibrated with 0.5 M NaCl/10 mM Tris-HCl (pH 8.0) buffer. PPIase was purified as a single band by sodium dodecyl sulfated polyacrylamide gel electrphoresis (SDS-PAGE) at this step (Fig. 3).

A summary of the purification after solubilization was shown in Table 1. The PPIase was purified

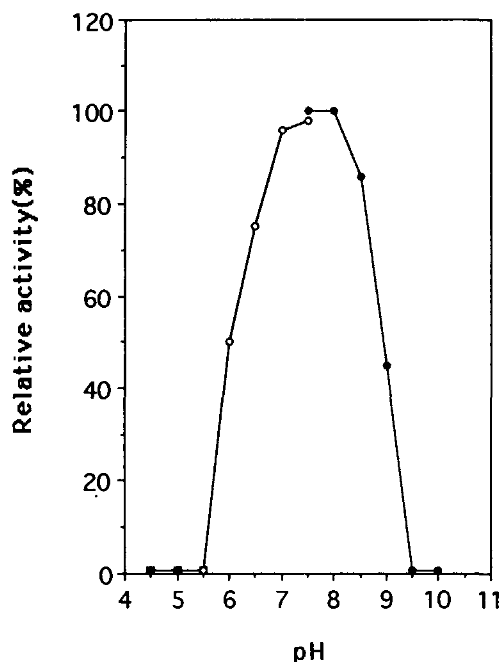

Fig. 4. Effect of pH on the enzyme activity.
The reaction mixture (500 μl), consisting of 200 μl of 60 mM potassium phosphate buffer (pH 5.5~7.5) and 60 mM Tris-HCl buffer (pH 7.0~**9.0**), **300** μl of the enzyme solution (200 nM), was incubated at variour pHs at 37℃ for 30 mim and the residual activity was assayed. The highest activity is denoted at 100%, Symbols: ■, pH 4.0~5.5; ○, pH 5.5~7.5; ●, pH 7.0~9.0

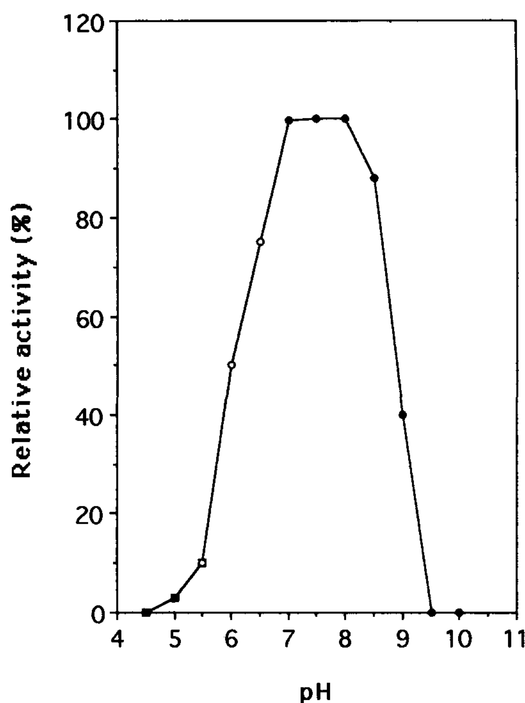

Fig. 5. Effect of pH on the enzyme stability.
The enzyme activity was measured at a different range of pH with 50 mM sodium acetate buffer (pH 4.0~5.5), 50 mM potassium phosphate buffer (pH 5.5~7.5), and 50 mM Tris-HCl buffer (pH 7.0~9.0), 200 μl of the enzyme solution (200 nM), was incubated at 60℃ for 30 mim and the residual activity was assayed. The highest activity is denoted at 100%, Symbols: ■, pH 4.0~5.5; ○, pH 5.5~7.5;p ●, pH 7.0~9.0

about 246 fold, and the activity yield was 25% after solubilization from the cell fraction.

Determination of molecular wight

A single protein band was obtained by SDS-PAGE of the protein fraction from Superose 12, homogenous protein. The molecular weight of the protein was found to be approximately 18,000 daltons with comparison to the standared protein markers in SDS-PAGE (Fig. 3).

N-terminal amino acid sequence

The 39 amino acid residues from the N-terminus of *B stearothermophilus* SIC1 PPIase were determined by protein sequencer. The sequence was Ala-Lys-Lys-Gly-Tyr-Ile-Leu-Met-Glu-Asn-Gly-Gly-Lys-Ile-Glu-Phe-Glu-Leu-Phe-Pro-Asn-Glu-Ala-Pro-Val-Thr-Val-Ala-Asn-Phe-Glu-Lys-Leu-Ala-Asn-Glu-Gly-Phe-Tyr.

Effect of pH

The activity of the purified enzyme was measured at various pHs from 4.5 to 10. The enzyme had an potimum pH at 8.0 in the reaction at 37℃ for 30 minutes. The purified enzyme had almost no activity under pH 5.0 and above pH 9.0 (Fig. 4.) The effect of pH on the enzyme stability was also examined. The

enzyme was incubated in buffers of various pH for 60 minutes at 30℃ and the residual activity was investigated by determining the residual activity (Fig. 5).

Effect of temperature

The enzyme activity was also examined at various temperature; the activity was quite stable up to 62℃ from 30 minutes, and the enzyme retained 50% fo its activity even after heat treatment at 65℃ for 30 minutes. The enzyme activity, however, was completely lost after 30 minutes at 70℃. The optimum temperature for enzyme activity was 60℃ (Fig. 6).

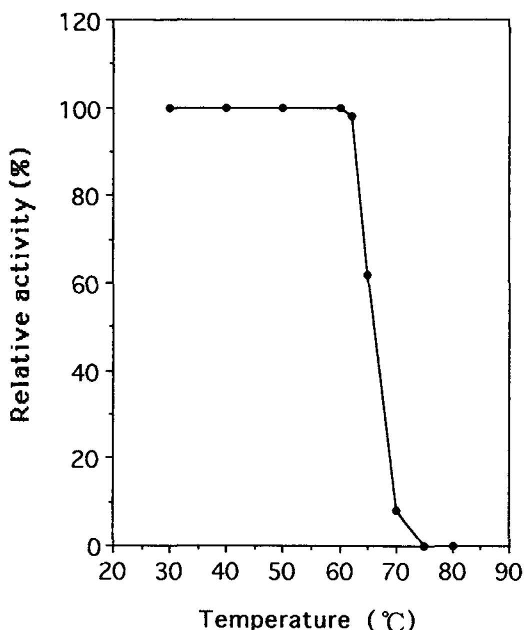

Fig. 6. Effect of temperature on PPIase stability. The reaction mixture (500 μl), consisting of 400 μl 50 mM Tris-HCl buffer (pH 8.0) and 100 μl of the enzyme solution, was incubated at various temperatures for 30 min and the remaining activity were assayed with pNA as a substrate. The highest activity is denoted as 100%.

The catalytic effect of PPIase on slow protein folding of RNase TI

The folding reaction of the PPIase was performed using RNase TI as a catalysis of protein folding. Slow folding kinetics are determined by the isomerization of two prolines, Tyr (38)-Pro (39*) and Ser (54)-Pro (55*) both forms are *cis* in the native protein (Heinemann et al., 1982). The slow reaction of RNase TI in the absence and presence of the PPIase was used. The results in Fig. 7 demonstrate that the presence of the PPIase in the refoding solutions accelerated the isomerization of the unfolded RNase TI.

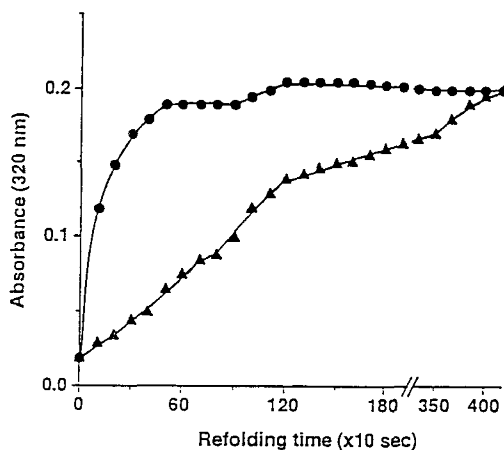

Fig. 7. Thc catalytic effect of PPIase on slow protein folding of RNase TI. The refolding of RNase TI in the absence (▲) and 200 nM presence (●) of PPIase. Refolding conditions were performed by a 40-fold dilution of unfolded protein (RNase TI). The refolding of RNase TI was monitored by the increase in spectrophotometer at 320 nm.

Discussion and Consideration

The purification and characterization of PPIase from *B. stearothermophilus* SIC1 are reported in this paper.

The enzyme was purified from 10 liter culture using the sequencial purification steps such as ammonium sulfate, heat treatment, ion exchang chromatography, gel filtration (Table 1). The enzyme was purified to 264 fold starting from the culture filtrate, and the activity yield was 25%. The purity of the enzyme sample after Superose 12 column chromatography

Table 1. Purification of PPlase from *B. stearothermophilus* SIC1

Step	Total Protein (mg)	Total activitey (units)	Specific activity (units/mg)	Fold	Yield (%)
Cell extact	1985	99.0	0.05	1.0	100
DEAE Sepharose	185	61.5	0.33	6.7	62.4
CL-6B	9				
Sephadex G-75	2	44.0	4.86	97.6	45
Superose 12		24.6	12.3	246	25

was examined by SDS-PAGE. The enzyme appeared to be a single protein band with a molecular weight of 18,000 daltons. The 39 amino acid residues from the N-terminus of the purified PPIase were determined by protein sequedcer.

We examined the dependences of pH and temperature of *B. stearothermophilus* PPIase. The optimal pH for the enzyme activity was at pH 8.0, measured over a pH range from 5.0 to 9.0. The purified PPIase exhibited the maximum activity at 60℃ and stale up to 65 ℃.

The presence of the PPIase present in refolding solution accelerated the isomerization rate of the assay peptide. PPIase from different eukaryotic species could by inhibited either by CsA or FK 506 in 10~20 nM (Schönbrunner et al., 1991). In order to explor the relationship of *B. stearothermophilus* enzyme to other PPIases, we examined the inhibition of PPIase activity by CsA and FK 506 using N-succinyl-Ala-Ala-Pro-Phe-pNA as the substrate. However, neither drug showed any inhibitory effects on the PPIase activity.

Molecular cloning of *B. stearothermophilus* PPIase gene would help in understanding the structure and function relationships for PPIase *in vivo*, providing as the genetic and molecular biological tools.

References

Brandts, J. F., H. R. Halorson and M. Brennan. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residuces. Biochemistry, 14, 4953~4963.

Bunjes, D., C. Hardt, M. Rollinghoff and H. Wagner. 1981. Cyclosporin A-mediates immunosuppression of primary cytotxic T cell responses by impairing the release of interlukin 1 and interleukin 2. Eur. J. Immunol., 11, 657~661.

Crabtree, G. R. 1989. Contingent genetic regulatory events in T-lymphocyte activation. Science, 243, 355-361.

Elliott, J. F., Y. Lin, S. B. Mizel, R. C. Bleuckley, D. E. Harnish and V. Paetkau. 1984. Induction of interleukin 2 messenger RNA inhibited by cyclosorin A. Science, 226, 1439~1441.

Emmel, E. A., C. L. Verweij, D. B. Durand, K. M. Higgins, E. Lacy and G. R. Crabtree. 1989. Cyclosporin A specifically inhibits function of nuclease proteins involved in T cell activation. Science, 246, 1617~1620.

Fischer, G., H. Bang and C. C. Mech. 1984. Nachweis einer enzymkatalyse für die *cis-trans* isomerisierung der peptidebindung in prolinhaltigen peptiden. Biomed. Biochim. Acta., 10, 1101~1111.

Fischer, G., B. Wittmann-Liebold, K. Lang, T. Kiefhaber and F. X. Schmid. 1989. Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins. Nature, 337, 476~478.

Handschumacher, R. E., M. W. Harding, J. Rice, R. J. Drugge and D. W. Speicher. 1984. Cyclo-

philin A specific cytosolic binding protein for cyclosporin A. Science, 226, 544~547.

Haendler, B., R. H. Warbinek and E. Hofer. 1986. Complementary DNA for human T-cell cyclophilin. The EMBO Journal, 6, 947~950.

Heinemann, U. and W. Saenger. 1982. Specific protein-uncleic recognition in ribonuclease TI-2'-guanylic and complex: an X-ray study. Nature, 299, 27~31.

Kiefhaber, T., R. Quaas, U. Hahn and F. X. Schmid. 1990. Folding of ribonuclease TI. 2. kinetic models for the folding and unfolding reactions. Biochemistry, 29, 3062~3070.

Kiefhaber, T., R. Quaas, U. Hahn and F. X. Schmid. 1990. Folding of ribonuclease TI. 1. existence of multiple unfolded states created by proline isomerization. Biochemistry, 29, 3053~3061.

Lang. K. Schmid, F. X. and G. Fischer. 1987. Catalyis of protein folding by prolyl isomearse. Nature, 329, 268~270.

Lang, K. and F. X. Schmid. 1988. Protein-disulphide isomerase and prolyl isomerase act differently and independently as catalysts of protein folding. Nature, 331, 453~455.

Lin L-N., H. Hasumi and Brands, J. F. 1983. Catalysis of proline isomerization during protein-folding reactions. Biochim. Biophys. Acta., 956, 256~266.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277, 680~685.

Schönbunner, E. R., S. Mayer, M. Tropschug and G. Fischer. 1991. Catalysis of protein folding by cyclophilins from different species. J. Biol. Chem., 266, 3630~3635.

Takahashi, M., T. Hakano and M. Suzuki. 1989. Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. Nature, 337, 473~475.

Zang, M., H. Nakai and T. Imanaka. 1988. Useful host-vector systems in *B. stearothermophilus.* Appl. Environ. Microbiol., 54, 3162~3164.

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