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Peptidyl-prolyl *cis-trans* isomerase from *Bacillus subtilis*

A prokaryotic enzyme that is highly sensitive to cyclosporin A

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Cyclophylins are members of a class of proteins with peptidyl-prolyl *cis-trans* isomerase activity. These enzymes bind the immunosuppressive agent, cyclosporin A (CsA), which acts as a competitive inhibitor. The peptidyl-prolyl *cis-trans* isomerase from *Bacillus subtilis* (PPIase) was purified to homogeneity in a 4-step purification procedure, which resulted in a 100-fold protein purification with a yield of 5%. Coomassie blue-stained SDS-PAGE revealed a single band of about 18 kDa. PPIase activity was determined using synthetic peptides as substrates in a 2-step reaction coupled to chymotrypsin. Treatment of *Bacillus subtilis* PPIase by CsA revealed an inhibition constant of K_i=175 nM, which differs from cyclophilin of enterobacteria such as *E. coli* or *Salmonella typhimurium* and is in the range of human enzymes.

Bacillus subtilis; Peptidyl-prolyl *cis-trans* isomerase; Cyclosporin A

1. INTRODUCTION

Cyclophilins are conserved, ubiquitous proteins of as yet unknown function. Originally they were purified from bovine spleen [1,2] as the major binding proteins for the immunosupressant, cyclosporin A (CsA). These proteins are widely distributed and found in high concentrations in prokaryotes, eukaryotes and in different cell compartments [1,3–12]. They are known to possess peptidyl-prolyl *cis-trans* isomerase activity (PPIase), a function accepted to be necessary for rate-determining steps in protein folding [13–17]. This only known enzymatic activity in vitro was first detected in bovine PPIase by *cis-trans* isomerization of synthetic Xaa–Pro oligopeptide bonds [18]. Later studies have shown that the PPIase was identical to cyclophilin [19,20] and that CsA blocks the enzymatic activity. The best studied action of CsA is its suppression of T-helper lymphocyte activation, leading to immunosuppression. In lymphocytes, CsA blocks the transcriptional activation of a subset of genes essential for the activation process. These facts suggest that cyclophilins represent a new class of proteins that probably play an important role in the regulation of T-lymphocyte activation and proliferation [21–23]. Interestingly, the cellular target of the structurally unrelated immunosuppressant, FK-506, is a cytosolic protein that does not resemble cyclophilin in structure, but that possesses similiar peptidyl-prolyl *cis-trans* isomerase activity [24,25]. FK-506 blocks the enzymatic activity of the FK-506 binding protein (FKBP), however, it does not bind CsA.

In contrast to eukaryotic PPIase or cyclophilins that have been studied in more details, little information is available on prokaryotic cyclophilins [6]. Two different cyclophilins were found in *E. coli*, one of cytosolic origin, and the other located in the periplasm [3,8,26]. A third one has been described recently in *Salmonella typhimurium* [27]. The comparison of the amino acid sequences of the prokaryotic enzymes with those of eukaryotic origin revealed a low degree of conservation. Also, the enzymes from the Gram-negative bacteria are less sensitive to cyclosporin A than the eukaryotic cyclophilins [28].

In this work we show the purification of PPIase isolated from *Bacillus subtilis*, a Gram-positive prokaryote that is sensitive to cyclosporin A in a range comparable to PPIase of eukaryotic sources. Recently, a 17 kDa PPIase was also isolated from *Streptomyces chrysomallus*, a Gram-positive bacterium, which, like the *Bacillus* enzyme, is sensitive to cyclosporin A and shows inhibition and binding characteristics comparable to PPIase of eukaryotic sources such as mammals, plants, fungi and yeast [29].

2. EXPERIMENTAL

2.1. *Materials*

DEAE-Sepharose, phenyl-Superose (FPLC) and MONO Q (FPLC)

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sizing columns were obtained from Pharmacia LKB-Biotechnology; TSK-HW55(S) was purchased from Merck; succ-Ala-Ala-Pro-Phe-4-nitroanilide, chymotrypsin and 3[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS) were obtained from Sigma; all other substrates for the PPIase-activity assay were obtained from Bachem: succ-Ala-Phe-Pro-Phe-4-nitroanilide, succ-Ala-Ala-Glu-Phe-4-nitroanilide, succ-Ala-Lys-Pro-Phe-4-nitroanilide; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Serva Feinbiochemica Heidelberg and 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS) was from Roth.

2.2. *Purification*

Large scale purification of *B. subtilis* PPIase was accomplished from 10 l of *B. subtilis* JH642 cells. The cells were grown in 2 × YT-medium, containing Bactotryptone (Oxoid) (16 g/l), yeast extract (10 g/l) and sodium chloride (5 g/l), and harvested at early mid-log phase by centrifugation at 4°C for 5 min at 7,500 rpm in a Beckmann GSA-rotor. Typically 40 g of cell-paste was recovered from 10 l of culture and frozen at −80°C. 50 ml of buffer A, containing 50 mM Tris-Cl, pH 8.5, 4 mM dithiothreitol (DTT), 15% glycerine, 0.1% CHAPS and 5 mM EDTA was added to the cell paste. After 1 h incubation with 100 mg of lysozyme cells were disrupted by Ultraturax for 5 × 1 min and subsequently the cell slurry was sonicated three times each for 1 min. The cell debris were removed by centrifugation at 4°C for 30 min at 18,000 rpm. The supernatant, about 125 ml, was applied to a DEAE-Sepharose column (1.8 × 12 cm) equilibrated with buffer A. Using a linear gradient of 0–200 mM KCl (buffer B), active fractions, measured by PPIase activity, were pooled at KCl concentrations of 110–170 mM KCl (about 50 ml, Fig. 1a) and concentrated at 4°C using an Amicon ultrafiltration device equipped with YM3 and UM2 filters. The concentrated extract, about 2 ml, was applied to a TSK-HW55(S) gel filtration column (1.6 × 180 cm), equilibrated with buffer C containing 35 mM HEPES, pH 7.8, and 0.1 M KCl. Fractions containing PPIase, as indicated with horizontal bars (Fig. 1b), were pooled and brought to a 20% ammonium sulfate concentration. Subsequent hydrophobic interaction chromatography on phenyl-Superose HR 5/5 FPLC-column (Pharmacia), equilibrated with buffer D, containing 0.1 M phosphate, pH 8.5, and 10% ammonium sulfate lead to nearly purified enzyme. Using a step gradient from 0.1 M phosphate, pH 8.5, and 10% ammonium sulfate to 50 mM phosphate, pH 8.5, and 10% glycerine (buffer E), active PPIase fractions were pooled as shown in Fig. 1c. After dialysis against 3.5 mM HEPES, pH 7.8, final purification to homogenity was achieved by strong anion-exchange chromatography on MONO Q HR 5/5 FPLC-column (Pharmacia), equilibrated with buffer F, containing 6 mM Tris-Cl, pH 6.8, 2 mM DTT and 2 mM EDTA. A linear gradient of 0–200 mM NaCl (buffer G) resolved three active enzyme pools of PPIase-activity. Purification without hydrophobic interaction chromatography (phenyl-Superose HR 5/5) resulted in co-purification of PPIase and the cold-shock protein, CspB [30].

2.3. *PPIase activity assay and inhibition studies with immunosuppressants*

PPIase activity was determined using synthetic peptides as substrates in a 2-step reaction coupled to chymotrypsin as described [18]. To measure the inhibition of enzyme activity by CsA (cyclosporin A), FK506 and other modified agents, like CsH (dihydro-cyclosporin), or linear CsA, the enzyme was pre-incubated with the immunosuppressive agent before the addition of chymotrypsin and the substrate. The final concentration of EtOH in the reaction did not exceed 1% of the volume, a concentration shown separately not to affect PPIase catalysis.

3. RESULTS AND DISCUSSION

3.1. *Purification of* Bacillus subtilis *peptidyl-prolyl* cis-trans *isomerase*

The isomerase was purified, as described in section 2, from 40 g of *B. subtilis* cells. As shown in Table I the crude homogenate revealed a total activity of 72.197 U with an initial specific activity of 188 U/mg protein. A yield of 5% and a 100-fold purification of PPIase was achieved. Fig. 1 shows protein elution profiles after anion-exchange (A), gel filtration (B), hydrophobic interaction (C) and a final anion-exchange chromatography. First anion-exchange on DEAE-Sepharose and subsequent gel filtration on TSK-HW55(S) lead to essentially homogenous protein, as shown in Fig. 2, lane 3. Final purification was achieved by strong anion-exchange chromatography on MONO Q HR 5/5 FPLC-column (Fig. 2, lane 4). The hydrophobic interaction chromatography on phenyl-Superose HR5/5 FPLC column was only used when high molecular weight proteins were detected after gel filtration. Protein fractions at different stages of the PPIase purification were analyzed on SDS-PAGE (Fig. 2). As shown in Fig. 2, the last step of purification on MONO Q gave a pure enzyme (lane 4) and separated the *B. subtilis* PPIase from the co-purified major cold-shock protein CspB (lane 6).

3.2. *Inhibition by immunosuppressive drugs*

PPIase from different eukaryotic species can be inhibited either by CsA or FK506 in the nM range [17]. In order to explore the relationship of the *Bacillus* enzyme to known types of PPIase we examined the inhibition of PPIase activity by CsA and FK506 using the substrate, succinyl-Ala-Ala-Pro-Phe-4-nitroanilide. A significant inhibition of the bacterial PPIase by CsA was detected (Fig. 3) and an apparent inhibition constant $K_{i,app}$=175 nM was determined. The CsA-binding for two partially characterized isoforms of *B. subtilis* PPI-

Table I

Purification of cyclophilin from 40 g *Bacillus subtilis*

	Protein (mg)	Activity (100 × U)	Specific activity (U/mg protein)	Yield (%)
Supernatant (100,000 × *g*)	609.00	721.97	118.43	100
DEAE-Sepharose	212.00	340.12	160.43	47
TSK-HW55(S)	87.36	256.12	293.20	35
Phenyl-Superose HR 5/5	4.30	129.88	3,020.47	18
MONO Q HR 5/5	0.55	40.07	12,064.00	5

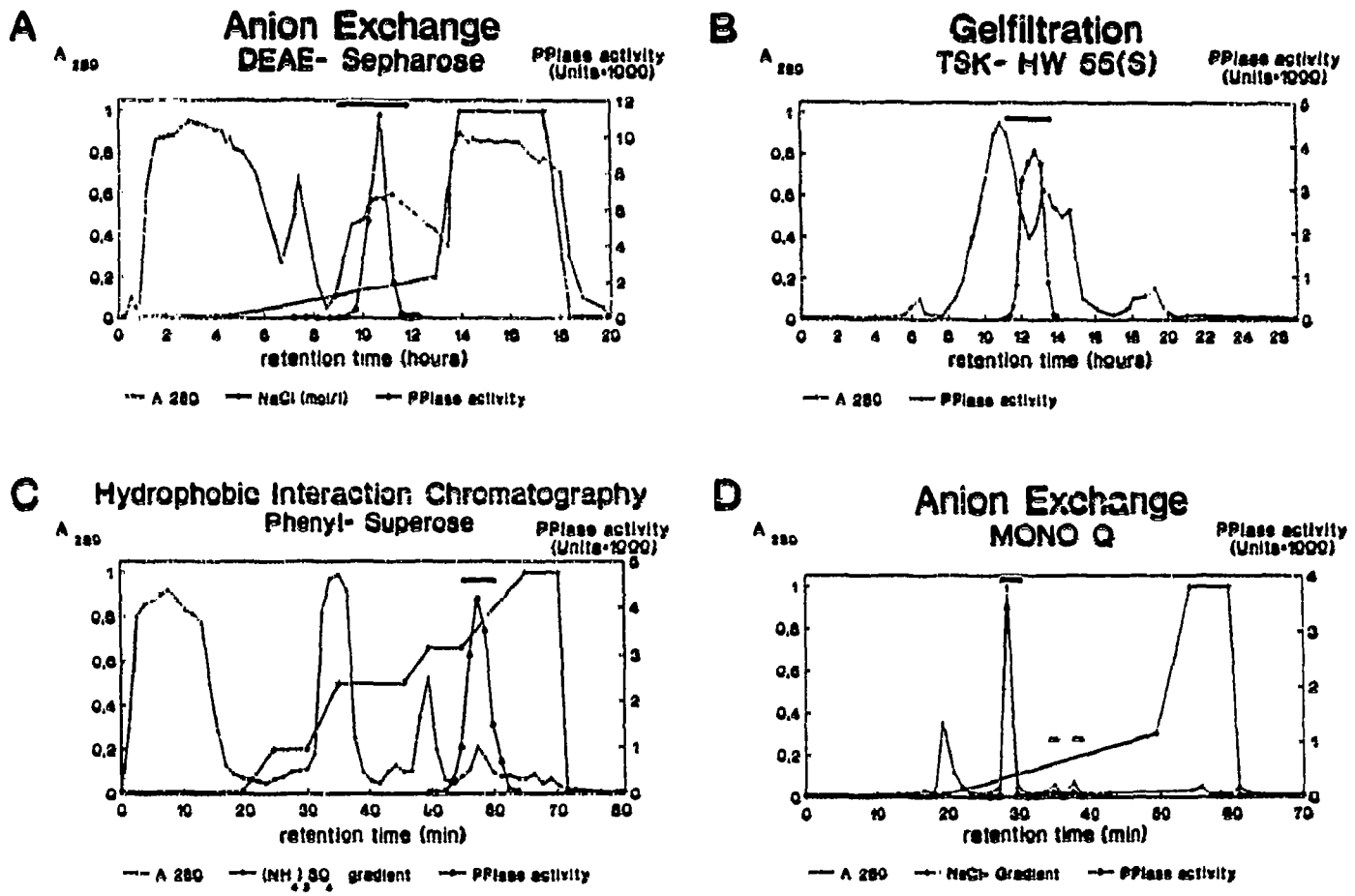

Fig. 1. Elution profiles from (A) DEAE-Sepharose anion exchange, (B) TSK-HW55(S) gel filtration, (C) phenyl-Superose HR 5/5 hydrophobic interaction chromatography and (D) MONO Q HR5/5 strong anion-exchange column. (•) Absorbtion at 280 nm; (+) NaCl or $(NH_4)_2SO_4$ gradient; (▪) PPIase activity; pooled fractions are indicated with horizontal bars.

ase was found to be in the same range (data not shown). In contrast, the PPIase isolated from *E. coli*, which shows some homology to that of human cyclophilin, has been shown to interact very weakly with CsA, as char-

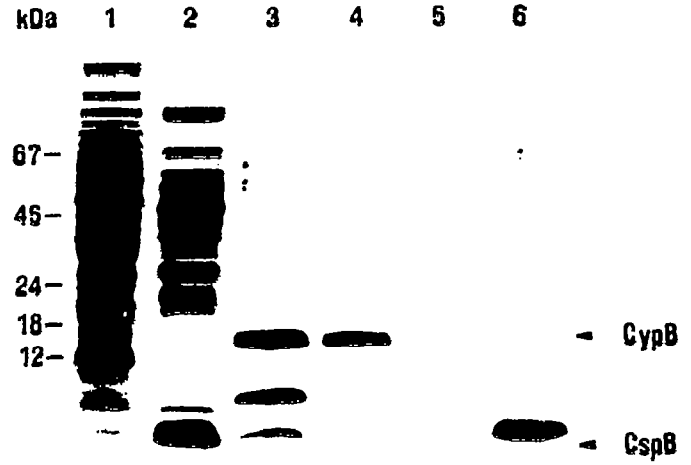

Fig. 2. SDS-PAGE of different fractions obtained during the purification of *Bacillus subtilis* PPIase (CypB). (Lane 1) Crude extract; (lane 2) DEAE-Sepharose fraction (anion-exchange); (lane 3) TSK-HW55(S) fraction (gel filtration); (lanes 4–6) MONO Q fractions (anion-exchange), showing purified CypB (lane 4) and co-purified cold-shock protein, CspB (lane 6).

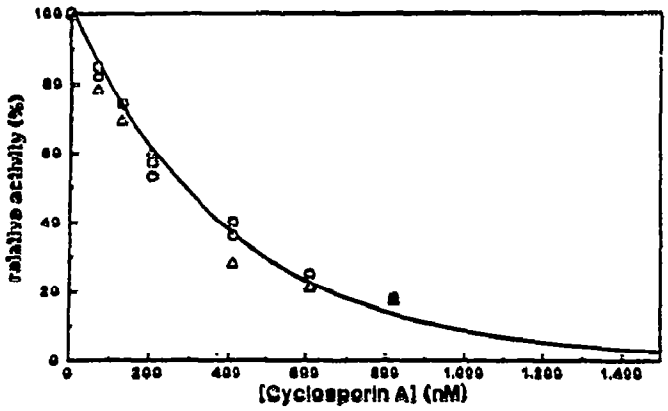

Fig. 3. Inhibition of PPIase activities (12 nM enzyme) at different concentrations of CsA using succinyl-Ala-Ala-Pro-Phe-4-nitroanilide as substrate; the data were analyzed according to an equation for tight-binding inhibition. PPIase activity assay was carried out in three independent experiments, as indicated by different symbols, ○, △, □.

acterized by a K_i greater than 3,000 nM [3]. Sequence alignments of PPIases indicate that the single tryptophan conserved in eukaryotic cyclophilins, which was also implicated as an important residue for CsA binding [31], is absent in the *E. coli* PPIase [3]. Liu et al. [28] have shown that mutation of the natural F112 wild-type cyclophilin, which contains phenylalanine in position 112, to the W112 mutant, which possess tryptophan in the same position, enhances *E. coli* PPIase susceptibility to CsA inhibition by 23-fold, displaying an IC_{50} for CsA in the range of the *B. subtilis* enzyme (130 nM). Recently, an IC_{50} in the range of 30 nM was reported for the PPIase from *Streptomyces* [29], which is still higher than the 6 nM reported for the human cyclophilin [28]. Furthermore the activity of the *B. subtilis* PPIase was not inhibited by FK506 or by other immunosuppressive agents, like CsH and linear CsA (data not shown). In conclusion, the results indicate that the purified PPIase of the Gram-positive bacterium, *B. subtilis*, in contrast to the PPIase of Gram-negative bacteria, is highly sensitive to CsA and therefore defines a new class of bacterial isomerases.

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