Enzymatic Activity, Thermal Stability and Solubility of SIB1 FKBP22

Ohnmar Ye Win[1]

Abstract

SIB1 FKBP22 is the protein from *Shewanella* sp., a psychrotrophic bacterium which is adaptable to cold environments (– 5˚C to 20˚C). This strain most rapidly grows at 20˚C, but the highest cell density is obtained at 4˚C. This strain grows well at 4˚C and can slowly grow even at – 4˚C. This strain cannot grow at 30˚C. It is expected to produce cold – adaptedenzymes and some of them are superior to mesophilic and thermophilic ones in the application field. SIB1 FKBP22 is a homodimer and each subunit contains the C – terminal catalytic domain and N – terminal dimerization domain. FKBP represents FK506 binding protein. This protein exhibits peptidylprolyl*cis* – *trans*isomerase activity for peptide. In a denatured state of proteins,all peptide bonds assume energetically stable *trans* conformation. Exception is the peptide bond N – terminal of the proline residue. This bond exists in an equilibrium state, in which 80% of this bond assumes *trans*conformation and 20% assumes *cis* conformation. PPIase does not change this equilibrium state, but equally accelerates both rates from *trans* to *cis* and from *cis* to *trans*. For proteins containing *cis*proline residues in a native state, *cis* – *trans*isomerization of the proline residue has been reported to be a rate –limitingstep of protein folding. By using RNaseT_1 refolding assay, SIB1 FKBP22 exhibits peptidylprolyl*cis* – *trans*isomerase activity like other MIP – like FKBP subfamily proteins. It is proposed that PPIase activity facilitates the efficient folding of proteins containing *cis*proline not only *in vivo* but also *in vitro*. In order to analyze the thermal denaturation and renaturation activity of SIB1 FKBP22, its mutants such as C – domain$^+$ and V37R/L41R were constructed. By comparing the thermal stability of the wild type protein and its mutants, V37R/L41R exhibits the fully reversible activity; C – domain$^+$ has the nearly fully reversible activity and SIB1 FKBP22 has the reversible activity but not fully comparable with its mutants. It is proposed that SIB1 FKBP22 and its mutants have thermal stability. Plasmids are important elements in modern DNA technology. Cold – shock expression vectors, pCold^{TM} DNA, are used to perform efficient protein expression utilizing promoter derived from cspA gene, which is one of the cold – shock genes. In the present study, the expression of dsRED and EGFP is observed by applying cold – shock induction.It can be noted that the insoluble amount of dsRED and EGFP is eliminated after the coexpression of SIB1 FKBP22.

Keywords: SIB1 FKBP22, its mutants, *PPIase*, refolding assay, thermal stability, solubility.

Introduction

The psychrotrophic bacterium *Shewanella* sp. strain was isolated in Kanaya laboratory from water deposits in a Japanese oil reservoir (Kato, *et al*., 2001). A variety of microorganisms grow on this planet. They greatly differ in growth temperatures. Microorganisms that grow at 0˚C and below are classified as psychrophiles and psychrotrophs. The terminology of psychrophile gives to microorganisms that can grow only at 20˚C and below. The terminology of psychrotroph gives to microorganisms with the upper limit of growth temperature of 25 ˚C and above (Kanaya, 2006).

Psychrophiles and psychrotrophs are the bacteria that can grow at low temperatures. In these bacteria, a variety of the systems, which facilitate protein

[1] Associate Professor, Botany Department, Loikaw University

folding processes, must be developed, because protein folding reactions are generally slow at low temperatures. Acceleration of the peptidyl-prolyl isomerization reaction by PPIases may be the function of one such system. This reaction is normally slow, especially at low temperatures, if it is not assisted by PPIases (Suzuki, *et. al.*, 2004).

Proteins are synthesized as a single polypeptide chain, folded into a correct structure, and exhibit various functions. It remains to be fully understood what kind of structure and function of proteins correlate with one another. This is one of the major problems that remain to be solved in the biology field. One strategy to solve this problem is to analyze adaptation mechanisms of proteins from extremophiles to unusual conditions, such as high temperature, low temperature, high pH, low pH, high pressure, high salt concentration, non-aqueous condition, and so on (Kanaya, 2006).

When polypeptides are synthesized at ribosomes, peptide bonds are connected in *trans*form. In the case of peptide bonds N-terminal of the proline residues, however, some of them form *cis* peptide bonds incorrectly folded proteins. Consequently, *trans*-to-*cis* conversions of these peptide bonds (prolylisomerizations) should occur during protein folding reactions. The *cis-trans*isomerizations of peptide bonds N-terminal of the proline residues are catalyzed by peptidylprolyl*cis-trans*isomerases (PPIases) (Suzuki *et al*., 2004).

Plasmids are important elements in modern DNA technology for two reasons: they can be easily isolated and transferred to other host cells merely by mixing purified plasmid DNA with the desired host cells inappropriate solutions and by use of restriction enzymes any gene can be inserted into the plasmid DNA (Saul, F.A., 2003).

In the present study, SIB1 FKBP22 is the protein from *Shewanella* sp. SIB1. The refolding assay of RNase T_1 on SIB1 FKBP 22 was carried out. Moreover, the thermal stability of SIB1 FKBP22 & its mutants: C-domain$^+$ and V37R/L41R was determined. In addition, SIB1 FKBP22 was coexpressed with recombinant proteins: dsRED and EGFPin *E. coli* cells using cold shock induction; and analyzed in 15% SDS-PAGE for expression levels. Depending on their results, PPIase and chaperone activities of SIB1 FKBP22 were discussed.

Materials and Methods

In this research work, RNase T_1 refolding assay for enzymatic activity of SIB1 FKBP22, the thermal stability of the wild type and its mutants, and the expression level of dsRED and EGFP harboring pColdII vector with SIB1 FKBP22 were conducted at the Laboratory of Molecular Biotechnology, Department of Material and Life Science, Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University, Japan in 2006.

Enzymatic Activity

For the RNase T_1 refolding assay, RNase T_1 was first unfolded by incubating the solution containing 20mM Tris-HCl (pH 8.0), 1mM EDTA, 5.6M guanidine hydrochloride, and 16μM RNase T_1 (Funakoshi) at 10°C for overnight. Refolding was then initiated by diluting this solution 80-fold with 20mM Tris-HCl (pH 8.0) containing SIB1 FKBP22. The final concentrations of RNase T_1 and SIB1 FKBP22 were 0.2μM and 21nM, respectively. The refolding reaction was monitored by measuring the increase in tryptophan fluorescence with F-2000 spectrofluorometer (Hitachi Instruments). The excitation and emission wavelengths were 295 and

323nm, respectively, and the band width was 10nm. The refolding curve was analyzed with double exponential fit.

Circular Dichroism (CD)

The CD spectra were recorded on a J-725 automatic spectropolarimeter from Japan Spectroscopic Co., Ltd. The proteins were dissolved in 20mM sodium phosphate (pH 8.0) and incubated for 30 minutes at the temperatures indicated prior to the CD measurement. For measurement of the far-ultraviolet (far-UV) CD spectra (220nm), the protein concentration was approximately 0.2mg/ml and a cell with an optical path length of 2 mm was used. The mean residue ellipticity, θ, which has units of deg cm^2dmol^{-1}, was calculated by using an average amino acid molecular weight of 110. The reversibility of thermal denaturation was verified by reheating the samples (Suzuki *et al*., 2005).

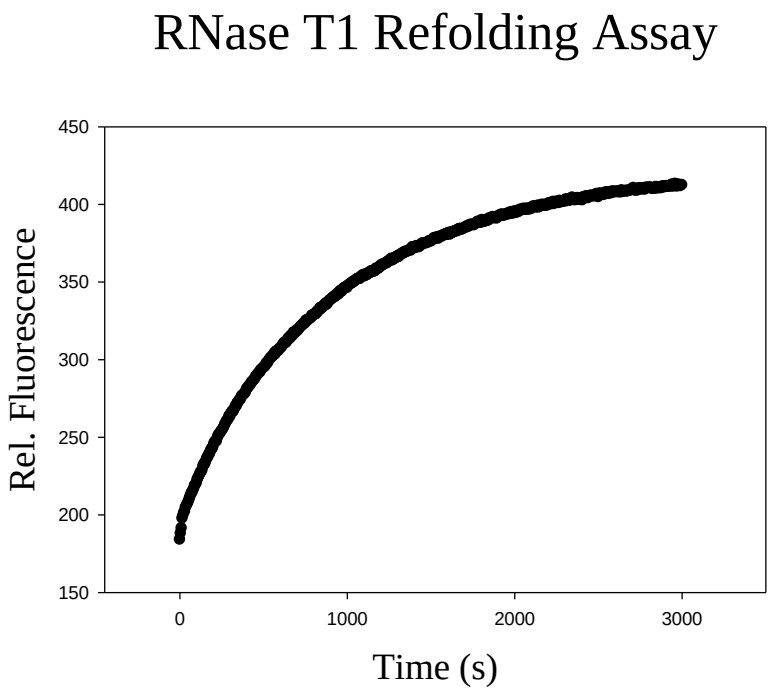
Coexpression of SIB1 FKBP22 & recombinant proteins: dsRED& EGFP in the *E. coli* cells with cold-shock induction

Cold-shock expression vectors, pCold™ DNA, are designed to perform efficient protein expression utilizing promoter derived from cspA gene, which is one of the cold-shock genes. A 200 ml of LB media containing 0.1% chloramphenicol (30µg/ml) and ampicillin (50µg/ml) was inoculated with 2% preculture of SIB 1 FKBP 22 and dsRED; and EGFP respectively. The cultures were incubated at 37 °C until OD_{660} is 0.5. After adding 0.1% of 1mM IPTG, the cultures were transferred to 30°C for 1 hour and transferred to 15 °C for 20 hours. Cells were centrifuged at 15,000 rpm at 4°C for 1 minute, suspended in TE buffer, disrupted by sonication. And then, cell debris and supernatant were harvested by centrifugation at 15,000 rpm at 4°C for 30 minutes to assay in 15% SDS-PAGE to get the soluble and insoluble amounts of recombinant proteins.

Results

PPIase Activity

When the PPIase activity was determined by RNase T_1 refolding assay, SIB1 FKBP22 exhibited much high activity. The acceleration effect of SIB1 FKBP22 on RNase T_1 refolding reaction was detected at 21nM (Figure 1.).



Standard Error of Estimate = 0.6398

	Coefficient	Std. Error	t	P
y0	190.5686	0.2482	767.9367	<0.0001
a	20.2187	0.5067	39.9008	<0.0001
b	0.0066	0.0003	25.0894	<0.0001
c	211.5674	0.4328	488.7889	<0.0001
d	0.0010	0.0000	273.9472	<0.0001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	4	1937991.8948	484497.9737	1183638.0715	<0.0001
Residual	596	243.9604	0.4093		
Total	600	1938235.8552	3230.3931		

Figure 1.PPIase activity of SIB1 FKBP22.The increase in tryptophan fluorescence at 323nm during refolding of RNaseT_1 (0.2μM) is shown as a function of the refolding time. Refolding reaction was carried out at 10°C in the presence of 21nM of SIB1 FKBP22 (thick solid line).

Comparison of thermal stabilities of SIB1 FKBP22 and its mutants:- V37R/L41R and C-domain+

Thermal stability of SIB1 FKBP22

To examine the thermal stability of SIB 1 FKBP 22, the far-UV CD spectra of SIB1 FKBP22 was measured at temperature ranges from 4 to 70°C and vice versa. The thermal denaturation curve of SIB 1 FKBP 22 was measured by monitoring a change in the CD values at 220nm, 1°C/min. The denaturation curve (trace 1) and the renaturation curve (trace 2) are shown in (Figure 2).

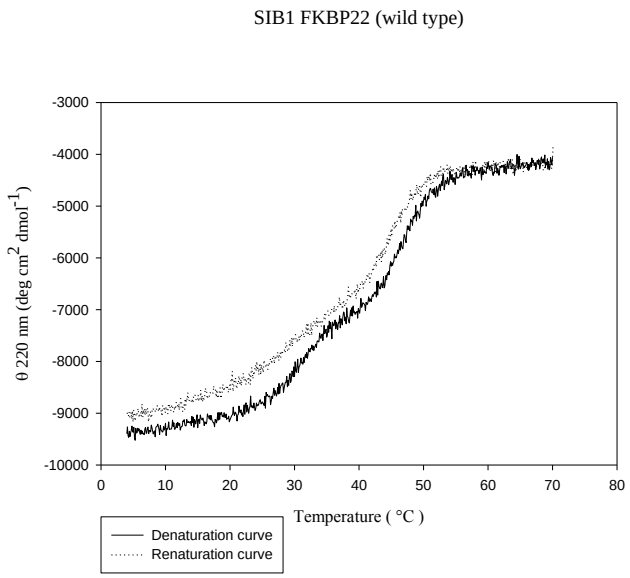


Figure 2. Thermal denaturation and renaturation curves of SIB1 FKBP22. The [θ] values of denaturation curve (trace 1) and renaturationcurve (trace 2) of SIB1 FKBP22 at 220nm are shown as a function of temperature. The protein was dissolved in 20mM sodium phosphate (pH 8.0) at 0.20mg/ml for SIB1 FKBP22. A cell with an optical path length of 2mm was used. Temperature was linearly raised and reduced at 1°C/min.

Thermal stability of V37R/L41R

To examine the thermal stability of V37R/L41R, the far-UV CD spectra of V37R/L41R was measured at temperature ranges from 4 to 70°C and vice versa. The thermal denaturation curve of V37R/L41R was measured by monitoring a change in the CD values at 220nm, 1°C/min. The denaturation curve (trace 1) and the renaturation curve (trace 2) are shown in (Figure3).

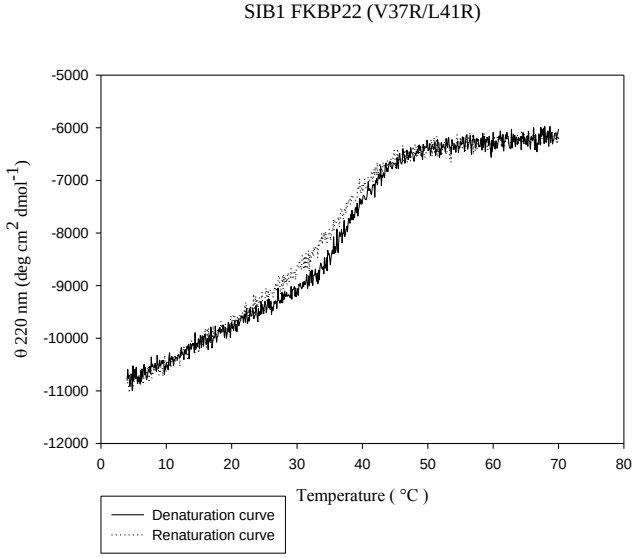


Figure 3. Thermal denaturation and renaturation curves of V37R/L41R.The [θ] values of denaturation curve (trace 1) and renaturation curve (trace 2) of V37R/L41R at 220nm are shown as a function of temperature. The protein was dissolved in 20mM sodium phosphate (pH 8.0) at 0.20mg/ml for V37R/L41R. A cell with an optical path length of2mm was used. Temperature was linearly raised and reduced at 1°C/min.

Thermal stability of C-domain$^+$

To examine the thermal stability of C-domain$^+$, the far-UV CD spectra of C-domain$^+$ was measured at temperature ranges from 4 to 70°C and vice versa. The thermal denaturation curve of C-domain$^+$ was measured by monitoring a change in the CD values at 220nm, 1°C/min. The denaturation curve (trace 1) and the renaturation curve (trace 2) are shown in (Figure 4).

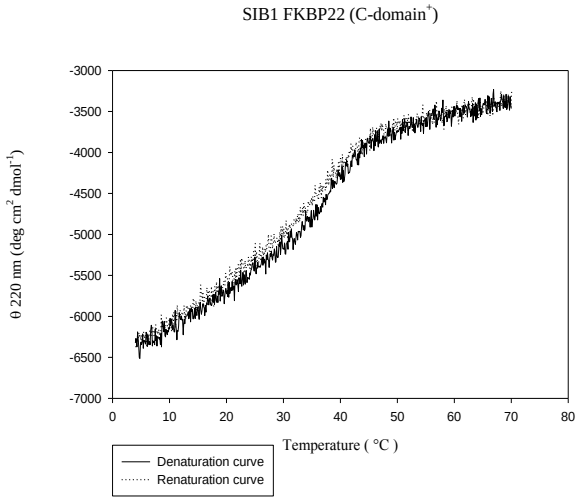


Figure 4. Thermal denaturation and renaturation curves of C-domain$^+$. The [θ] values of denaturation curve (trace 1) and renaturation curve (trace 2) of C-domain$^+$ at 220nm are shown as a function of temperature. The protein was dissolved in 20mM sodium phosphate (pH 8.0) at 0.20mg/ml for C-domain$^+$. A cell with an optical path length of 2mm was used. Temperature was linearly raised and reduced at 1°C/min.

Coexpression of SIB1 FKBP22 &Recombinanat Proteins in the *E. coli* cells with cold-shock induction

Coexpression of SIB1 FKBP22 &dsREDin the *E. coli* cells with cold-shock induction

By using pColdII vector, the insolubility of dsRED in the coexpression with SIB1 FKBP22 (Lane 8) was greatly reduced. On the contrary, some insoluble form of dsRED was found in the expression of dsRED only (Lane 5) (Figure 5).

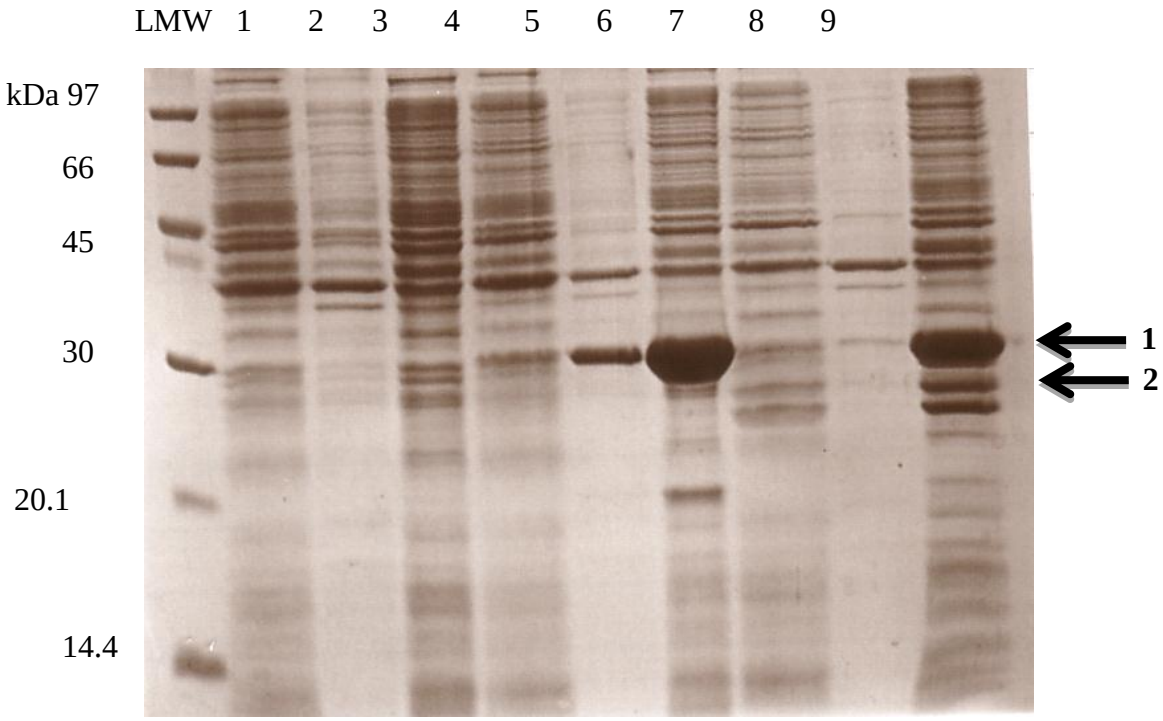


Figure 5. Estimation of the soluble and insoluble amounts of dsRED by coexpressionin the presence and absence of SIB1 FKBP22 (15% SDS-PAGE). dsRED and SIB1 FKBP22 were overproduced in the *E. coli* cells as described under experimental procedure. Lanes 4 & 7 (dsRED; dsRED and SIB1 FKBP22 harboring pColdII vectors) before cold-shock induction, and Lanes 2 & 3 (pColdII vector) after cold-shock induction were used as the positive and negative controls. Lanes 5 (insoluble)& 6 (soluble) (dsRED) and Lanes 8 (insoluble)& 9 (soluble) (dsRED and SIB1 FKBP22) after cold-shock induction were analyzed to show the insoluble and soluble amounts of dsRED on 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Arrows 1 and 2 indicate dsRED and SIB1 FKBP22, respectively after expression. The positions of the standard proteins contained in a low-molecular-weight marker kit (Pharmacia Biotech) are shown along with the gel together with their molecular masses.

Coexpression of SIB1 FKBP22 & EGFP in the *E. coli* cells with cold-shock induction

There was no insoluble form of EGFP in the coexpression with SIB1 FKBP22 (Lane 7).

On the contrary, a considerable amount of insoluble form of EGFP was found in the expression of EGFP only (Lane 4) (Figure6).

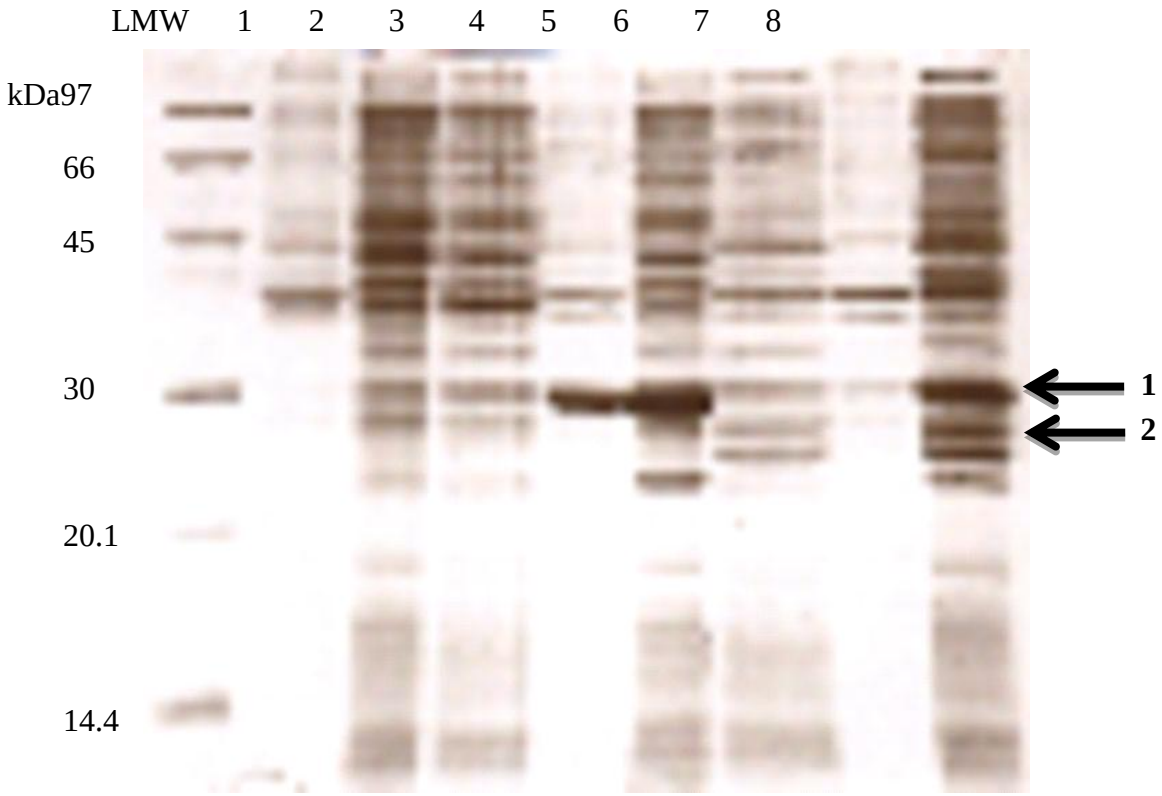


Figure 6. Estimation of the soluble and insoluble amounts of EGFP by coexpressionin the presence and absence of SIB1 FKBP22 (15% SDS-PAGE). EGFP and SIB1 FKBP22 were overproduced in the *E. coli* cells as described under experimental procedure. Lanes 3 & 6 (EGFP; EGFP and SIB1 FKBP22 harboring pColdII vectors) before cold-shock induction, and Lanes 1 & 2 (pColdII vector) after cold-shock induction were used as the positive and negative controls. Lanes 4 (insoluble)& 5 (soluble) (EGFP) and Lanes 7 (insoluble)& 8 (soluble) (EGFP and SIB1 FKBP22) after cold-shock induction were analyzed to show the insoluble and soluble amounts of EGFP on 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Arrows 1 and 2 indicate EGFP and SIB1 FKBP22, respectively after expression. The positions of the standard proteins contained in a low-molecular-weight marker kit (Pharmacia Biotech)are shown along with the gel together with their molecular masses.

Discussion and Conclusion

Suzuki,*et al*., (2005) stated that SIB1 FKBP22 and C-domain^{+} both exhibit the maximal PPIase activity at 10°C and their activities are greatly reduced at 20°C. These results suggest that subtle conformational change around the active-site causes great reduction of the enzymatic activity. Stoller G. *et al*., (1995) isolated a peptidyl-prolyl*cis-trans*isomerase (PPIase) which is specifically associated with the 50S subunit of the *E. coli* ribosome. PPIases are enzymes that catalyse protein folding both *in vitro* and *in vivo*. Hartl F.U., (1996);and Bukau*et al.*, (1998) reported that this property is similar to that of a molecular chaperone which interacts with partially or

fully unfolded proteins and increases the yield of fully folded proteins by preventing the formation of misfolded aggregates.

In the present study, the acceleration effect of SIB1 FKBP22 on RNase T_1refolding reaction was observed. It has been noted that SIB1 FKBP22 has had high PPIase activity at 10°C (Figure 1). This finding is agreed with those mentioned by Suzuki, *et al*., (2005); Stoller G. *et al*., (1995); Hartl F.U., (1996); and Bukau*et al.*, (1998).

Suzuki, *et al*., (2005) reported that all DSC curves reproduced by repeating thermal scans of N-domain$^+$, C-domain$^+$, and SIB1 FKBP22 are highly reversible. The denaturation curve of SIB1 FKBP22 clearly showed two well separated transitions. Deconvolution of the thermogram according to a non-two-state denaturation model gives melting temperature (T_m) values of 32.5°C and 46.6°C for these transitions. These T_m values are nearly equal to those of C-domain$^+$ (35.6°C) and N-domain$^+$ (44.7°C), suggesting that the thermal unfolding transitions of SIB1 FKBP22 at lower and higher temperatures represent those of its C-domain and N-domain, respectively.

In this study, analyses of the thermal stability of SIB1 FKBP22 and its variants: C-domain$^+$ and V37R/L41R by CD (Figure 2, 3 & 4.) indicate that thermal unfolding of SIB1 FKBP22 and its variants: C-domain$^+$ and V37R/L41R is initiated at >25°C. In thermal denaturation, V37R/L41R exhibits the fully reversible activity (Figure 4); C – domain$^+$ has the nearly fully reversible activity (Figure 3) and SIB1 FKBP22 has the reversible activity but not fully comparable with its mutants (Figure 2). SIB1 FKBP22 unfolded through an intermediate state. The T_m values for the first and second transitions are roughly estimated to be 25 and 43°C for wild type; 39.45˚C for V37R/L41R; and 38.14˚C for C – domain$^+$, respectively. It can be concluded that SIB1 FKBP22 has weak chaperone activity which is in agreement with those discussed by Suzuki, *et al.*, 2004 (unpublished data).

pColdII vector has cspA gene, one of the cold-shock genes. Cold-shock expression vectors, pCold™ DNA, are designed to perform efficient protein expression utilizing promoter derived from cspA gene, which is one of the cold-shock genes.

In the present investigation, pColdII vector was used to coexpress SIB1 FKBP22 and dsRED; and SIB1 FKBP22 and EGFP respectively. Before cold shock induction, there was no target protein in the coexpression of SIB1 FKBP22 and the recombinant proteins. After cold shock induction, there was a considerable soluble amount of target protein in the coexpression of SIB1 FKBP22 and the recombinant ones. It can be noted that using this vector helps the expression of recombinant proteins.However, there is still insoluble amount of dsRED and EGFP by using pCold II vector. After the coexpression ofSIB1 FKBP22, the insoluble amount of dsRED and EGFP can be efficiently decreased(Figure 5 & 6). It can be concluded that SIB1 FKBP22 is able to diminish the insoluble amount of recombinant proteins: - dsRED and EGFP. This is indicated that SIB1 FKBP22 is a cold – adapted enzyme.

Acknowledgements

I am greatly indebted to Dr. Myat Nyunt, Pro – rector, and Dr. Soe Myint Thein, Pro – rector, Loikaw University for permitting me to present this paper. I would like to express my indebtedness to Dr. Sann Sann Oo, Professor (Head), Botany Department, Loikaw University, for helpful suggestions regarding this work.My sincere thanks are due to my Japan supervisor, Dr. Shigenori Kanaya, Professor, Kanaya Laboratory, Department of Biotechnology, Osaka University, Japan, for his valuable advice, guidance and encouragement, and for kindly providing me with all the facilities of the laboratory necessary in this work.I would like to acknowledge my gratitude to all the members in Kanaya Laboratory (2005-2007), Department of Biotechnology, Osaka University, Japan, for their invaluable guidance, advice, suggestion and comment.I am grateful to Dr. MyatMyat Moe, Professor

(Head); and Dr. KhinLattLatt Maw, Professor, Botany Department, Dagon University for permitting me to participate in 2nd. Myanmar Korea Conference on Useful Plants.

References

Bukau, B., and Horwich, A.L. (1998) The Hsp70 and Hsp60 Chaperone Machines. *Cell*,Vol. 92, pp. 351-366.

Hart, F.U. (1996) Molecular Chaperones in Cellular Protein Folding. *Nature*,Vol. 381, pp. 571-579.

Kanaya, S. (2006) Lectures by Power Point Presentation.

Kato, T., Haruki, M., Imanaka, T., Morikawa, M. &Kanaya, S. (2001) Isolation and Characterization of PsychotrophicBacteria from Oil-reservoir Water and Oil Sands. *Appl. Microbiol. Biotechnol.*,Vol. 55, pp. 794-800.

Saul, F.A., Mourez, M.L., Normand, B.V., Sassoon, N., Bentley, G.A., Betton, J., (2003) Crystal Structure of a Defective Folding Protein.*J.Protein Science*, Vol. 12, pp. 577-585.

Suzuki, Y., Haruki, M., Takano, K., Morikawa, M., and Kanaya, S. (2004) Possible involvement of an FKBP family Member Protein from a PsychrotrophicBacterium *Shewanella* sp. SIB1 in Cold-adaptation.*Eur. J. Biochem.*,Vol. 271, pp. 1372-1381.

Suzuki, Y., Takano, K., and Kanaya, S. (2005) Stabilities and Activities of the N- and C-domains of FKBP22 from a PsychrotrophicBacterium overproduced in *E. coli*. *FEBS J.*,Vol. 272, pp. 632-642.

Stoller, G.,Rucknagel, K.P., Nierhaus1, K.H., Schmid, F.X., Fischer, G., Rahfeld, J.U.(1995) A Ribosome-associated Peptidyl-Prolyl*cis-trans* Isomerase identified as the Trigger Factor. *EMBO J.*, Vol.14, pp. 4939-4948.

ABBREVIATIONS

CD	Circular Dichroism
C-domain	Domain of Carboxyl (COO^-) group
conc.	Concentration
DNA	Deoxyribonucleic Acid
dsRED	a variant of the *Discosomastriata* marine anemone fluorescent protein
EDTA	ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
FKBP	FK506-Binding Protein
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kiloDalton
LB	Luria-Bertani Media
LMW	Low Molecular Weight Marker
M	molar solution
ml	mililitre
mM	milimolar
NaCl	Sodium Chloride
N-domain	Domain of Amino(NH_2) group
nm	nanometre
ng	nanogram
OD	Optical Density
PPIase	peptidylprolyl*cis-trans*isomerase
rpm	revolution per minute
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Eletrophoresis
SIB1 FKBP22	FKBP22 protein from *Shewanella*sp. SIB-1
T_m	melting temperature
Tris	tris(hydroxymethyl)aminomethane
UV	Ultraviolet
WT	Wild Type
µl	microlitre
µg	microgram