Two Flexible Loops in Subtilisin-like Thermophilic Protease, Thermicin, from *Thermoanaerobacter yonseiensis*

Hyeung Jin Jang, Chang-Hun Lee, Weontae Lee and Yu Sam Kim*

Department of Biochemistry, College of Science, Protein Network Research Center, Yonsei University, Seoul 120-749, Korea

Received 28 May 2002, Accepted 13 August 2002

A gene that encodes a thermostable protease, coined thermicin, has been isolated from *Thermoanaerobacter yonseiensis* that is expressed and characterized in *E. coli*. In order to elucidate the molecular characteristics on thermostability of the enzyme, molecular modeling and mutagenesis technology were applied. In the modeling structure, the structural core, including the active site, was well conserved; whereas, the two loop regions were unique when compared to thermolysin. The mutant enzyme with the small loop deleted (D190-I196), based on modeling structural information, showed identical enzyme activity. However, when the large loop was deleted (P233-P244), a little lower K_m and even a lower k_{cat} was found. This indicates that the large loop could influence catalytic activity. However, the unfolding temperature (T_m), which was determined by a differential-scanning calorimetry for the mutant enzyme deleted the small loop, was 96°C. This is 14°C lower than that for the parent thermicin. These results suggest that the small loop may play a role in maintaining the proper folding of the enzyme at high temperatures, whereas the large loop might be related to catalysis.

Keywords: Thermicin, *Thermoanaerobacter yonseiensis*, Thermophilic protease

Introduction

Some organisms are able to grow at temperatures above the boiling point of water, where the proteins from the organism are extremely stable at high temperatures. Elucidation of the molecular-basis thermostability of the proteins is important, not only to the comprehensive understanding of how a protein keeps its native conformation, but also to the development of

enzyme applications in industry. To identify the key factors that are responsible for the increased thermostability, an initial attempt was made to conduct the multiple amino acid sequence comparisons of homologous enzymes that are isolated from various organisms with different growth temperatures (Argos *et al.*, 1979). Extensive structural comparisons led to the conclusion that the overall conformation of each structure is quite similar to that of its counterpart proteins from mesophilic organisms; only slight differences in structure (including small deletions and/or insertions) make thermophilic proteins more stable than their mesophilic counterparts (Imada *et al.*, 1991; Day *et al.*, 1992; Yip *et al.* 1995; Wallon *et al.*, 1997). However, the proposed factors that are responsible for thermostability include the following: number of ion pairs (Perutz *et al.*, 1975; Yip *et al.*, 1995; Vogt *et al.*, 1997), number and type of hydrogen bonds per residue (Shortle *et al.*, 1992), amount and type of solvent accessible surface area (Chan *et al.*, 1995), amino acid composition (Fleming *et al.*, 1997), hydrophobic core size (Eriksson *et al.*, 1992), cavity size (Eriksson *et al.*, 1992), and multimer association regions of the protein (Wallon *et al.*, 1997). These suggestions on thermostability have sometimes been controversial (Kim *et al.*). An increased number of ion pairs appeared to be the only identifiable difference that could lead to thermostability in a thermophilic citrate synthase (Russel *et al.*, 1997); whereas, in psychrophilic (cold-active) citrate synthase, an increased number of ion pairs was known to cause an increase in the resistance to low temperature denaturation (Russel *et al.*, 1998).

In order to understand the roles of particular side chains in proteins, specific sites of some proteins were substituted for other amino acids (Yutani *et al.*, 1987; Matsumura *et al.*, 1988; Lim *et al.*, 1989; Sandberg *et al.*, 1991). This raises the possibility that even a single amino acid substitution may considerably increase the thermostability of the protein, although the chance to significantly increase the protein stability depends on the overall structure of a protein, as well as the local environment of the side chain residues. Therefore, a systematic mutational analysis of the proteins with the three-

*To whom correspondence should be addressed.
Tel: 82-2-2123-3448; Fax: 82-2-392-3488
Email. yskim@yonsei.ac.kr

dimensional (3-D) structural information is essential for discovering the correlation between the structure and the stability of proteins.

In a previous work (Jang *et al.*, submitted), we presented the cloning and expression for a gene that encodes a novel thermostable protease, thermicin, from the anaerobic bacteria. Interestingly thermicin contains neither cysteine residues in the mature enzyme nor the calcium-binding motif, which indicates that there might be other factors beside those that are related to thermostability that were previously mentioned.

In this paper, we report that thermicin has two unique loops that are responsible for thermostability and catalytic activity.

Materials and Methods

Molecular modeling The homology modeling of thermicin was based on the sequence alignment of subtilisin of both the firefly thermite (Teplyakov *et al.*, 1989) and subtilisin of *Bacillus lentus*. using the FASTA (<http://www2.ebi.ac.uk/fasta3/>) program. The sequence alignment for the structurally-conserved region was performed by structural data that was extracted from the X-ray coordinates of firefly thermite (PDB code: 1THM) and subtilisin of *Bacillus lentus* (PDB code: 1ST3). The pair-wise sequence homologies of these two templates against Thermicin were 37.7% and 39.9%, respectively. The PredictProtein server at EMBL (<http://www.embl-heidelberg.de/predictprotein/predictprotein.html>) predicted the secondary structures of thermicin. The initial template structures of thermicin were generated only for heavy atoms. Combining the use of conjugate-gradient energy minimization and molecular dynamics calculations in the MODELLER (< bibio >) program further optimized the modeling structures. Structurally-variable regions were engineered by combining the use of the PDB coordinate-searching and fragment-fitting routine, and was further relaxed by 1000 steps of conjugate-gradient energy minimization until its energy derivation reached 0.001 kcal/mol. Molecular dynamic simulation of 10ps at 298 K was also performed in the MODELLER program. After all of the coordinates were assigned, a conformational search for side chains were performed using the side chain rotamer library. In addition, local structure refinement procedures were also incorporated for each structural segment by successive use of energy minimization and molecular dynamics simulations. Regulating the distortions of bonds and angles finished all of the local refinements, including energy minimizations. Distance-dependent dielectric terms for mimicking the solvent effects were applied for the electrostatic energy calculations. The final thermicin structure was evaluated by the PROCHECK (Laskowski *et al.*, 1993), PROSA (Sippl, 1993) and WHATIF (Vriend and Sander, 1993) programs.

Construction of expression plasmids The deletion of the two loops was performed by the following: polymerase chain reaction (PCR) with oligonucleotide primers, the primer for small loop deletion (L2-2) D190~I196 (DKRT PDI) 5'-G GTA GGA GCA GTG GAC GAA GAC GAC GAA GTA GC -3' & complementary anti-sense primer, the primer for long loop deletion (L1-3) P233~P244 (PFGAD EIMIN KP) 5'-CA GCT TCT GGA AAT GTG * TAC AGG AGC GCC ACA GG 3 & complementary anti-

sense primer and primer for long and small loop deletions (LL12) 5'-G GTA GGA GCA GTG GAC * GAA GAC GAC GAA GTA GC-3', complementary anti-sense primer, and 5'-CA GCT TCT GGA AAT GTG * TAC AGG AGC GCC ACA GG-3', complementary anti-sense primer, respectively, using a Gene Amp PCR system 2400 (Perkin-Elmer Applied Biosystems, Foster City, USA). The asterisks indicate the locations of the deletion sites. The pQEX1 (Jang *et al.*, submitted) that contained the wild-type thermicin gene was used as a template DNA for deletion mutagenesis. The mutations were confirmed with a Model 310 DNA sequencer (Perkin-Elmer Applied Biosystems) using dideoxy chain termination sequencing. The resultant plasmids that carried the mutant thermicin genes (L1-3 and L2-2) were constructed in *E. coli* DH5a, and designated as pL1-3 and pL2-2, respectively. These plasmids were then introduced into *E. coli* M15 (pREP4) by heat shock.

Preparation of mutant enzymes The resulting plasmids were designated pQEXI, pL1-3, and pL2-2. The thermicin and mutant enzymes were expressed in *E. coli* M15 (pREP4) as previously described (Jang *et al.*, submitted). Thermicin and mutant enzymes from *E. coli* M15 (pREP4/pQEXI, pL1-3, and pL2-2) were purified by Ni-NTA affinity chromatography in a column of Ni-NTA Kits (Novagen). This was followed by anion exchange chromatography and SP Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden) (Jang *et al.*, submitted) that yielded purified thermicin as assayed by SDS-PAGE. The enzyme concentration was determined using a BCA assay (Bio-Rad, Richmond, USA). The enzyme concentration in the reaction assays was between 0.5 and 0.75 µg/mL (7-10 nM). In all of the experiments where the rates were compared, the same enzyme preparation with the same enzyme concentration was used.

Enzyme assay The hydrolysis of benzyloxycarbonyl-glycyl-prolyl-p-nitroanilide (Z-Gly-Pro-pNA) and several other substrates was monitored with a spectrophotometer (Shimadzu UV160A and Beckman DU640). The initial hydrolysis rate of all of the substrates was obtained by monitoring the release of the chromophore pNA (p-nitroaniline) at 405 nm ($\epsilon = 9650 \text{ M}^{-1} \text{ cm}^{-1}$ was found constant under all conditions in this study). All of the buffers contained 0.1 M NaCl, unless otherwise specified. The methanol concentration in all of the assays was adjusted to 0.228 mM to enhance the solubility of the Z-Gly-Pro-pNA stock solutions. Methanol was found to inhibit thermicin hydrolysis of ZGP noncompetitively; therefore, the Z-Gly-Pro-pNA hydrolysis rate by thermicin in 0% methanol was extrapolated from the linear plot of rate vs. methanol concentration (data not shown). The pH of each buffer was adjusted to the desired value at 85°C. All of the assays were carried out in 20 mM buffers, acetate/acetic acid at pH 4.2 to 4.7, MES (2-[N-morpholino] ethanesulfonic acid) at pH 4.8 to 6.2, HEPES (N-[2-hydroxyethyl] piperazine-N-2-ethanesulfonic acid) at pH 6.3 to 7.6, TAPS (N-Tris[hydroxymethyl]methyl-3-aminopropane sulfonic acid) at pH 7.7 to 8.6, and CAPS (cyclohexylaminopropane sulfonic acid) at pH 8.7 to 9.3) in the presence of 0.1 or 0.5 M NaCl. All of the aqueous solutions were prepared from deionized water of >18 MΩ from a MilliQ system (Millipore, Bedford, MA). The kinetic parameters, k_{cat} and K_m , were obtained by non-linear fitting of the data to the hyperbolic Michaelis-Menten equation.

Thermal stability of thermicin To determine the effect of loops on the thermal stability of thermicin, differential scanning calorimetry (DSC) was applied to the native enzyme, short-loop deleted mutant, and long-loop deleted mutant enzyme. In the DSC experiments, the purified proteins at a concentration of 2-3 mg/ml in 20 mM potassium phosphate with a pH of 6.8 were heated at a constant rate of 10°C/min until a maximum temperature of 120-125°C was reached.

Results

Molecular modeling of thermicin Figure 1 summarizes

the amino acid sequence alignment, folding threading pattern, and secondary structural elements of thermicin, together with firefly thermitase and subtilisin of *Bacillus lentus*. Table 1 lists the structural statistics that are calculated by different protein analysis programs to evaluate the modeling structure of thermicin. The Ramachandran plot demonstrates that 32% of the residues of the thermicin protein are in the structurally-allowed region. It was also observed that the general fold of thermicin is shared with both the firefly thermitase and subtilisin of *Bacillus lentus*. They had 6 α -helices (helix A-F) and 9 β -pleated sheets (strand 1-9), as shown in the topology diagram (Fig. 2). The structure shows that the distance

Thermicin :	-----VNIIPLFASFSAKEIYTLQKIDKRLNKKAS-----YLRSECVPAIILYSN :	45
Pyrococcus :	--MNKKGLTVLFTAIMLLSVVPVHFVSAGTPPVSSSENSTTSILPNQQ-----VVTKEVSAALNAIMKGG :	63
stetterlys :	--MNRVLSLLIVAVMLLSAIPAAVSVPAVTATPVQPTDIPQQVAVEKPSNFIPGEVLQKEIQKVLISG :	68
Aquifex_py :	-----MPFFFLFLLIILPYGLLFSLDEKFSALKLALLN-----EQKLTYSLSKQ- :	47
aerolysin :	-----MSQYLKRRKKLAQCQRFQ-----GTSGISS-REIVVGYVDSF :	37
AK1 :	--MKFKAIIVSLSLAVSMLFPFLVEA--ASNDGVESPKTVSEINVSHEK-----GAYVQGEVIVQFKEQV :	61
B.Stearo_J :	MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	47
aqalysin_1 :	--MRKTYWLMALFAVLVLGGCQMASRSDPTPTLAEAFWPKEAPVYGLDD-----PEAIPGRYIVVFKKKG :	63
thermitase :	-----MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	37
D13158 :	--MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	37
P41362 :	--MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	43
P27693 :	--MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	43
Q99405 :	-----MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	-
P29599 :	-----MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	-
AB005792 :	--MRRQSLKVMVLSTVALLFMAN-----PAAASEE-----KKEYLIVVEP-- :	43
P29142 :	MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	47
P00783 :	MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	47
P04189 :	MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	47
P35835 :	MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	47
P07518 :	-----MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	-
P00781 :	-----MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	-
P11018 :	-----MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	-
P29139 :	-----MRSKKLWISLLFALTTLIFTMAFSNM---SVQAAGKSS-----TEKKYIVGFKQTM :	-
Thermicin :	-----LPYETLKSKEIKFGGSIKFEIPIIKGWSVNLPCDKLKHFAISIKG-IHFIAED----- :	96
Pyrococcus :	PNMVLIIKTEGKLEAAKTELEKLGAEILDENRVLMNLLVKIKPEKVKELNYISS-LEKAWLNREVKLSP :	132
stetterlys :	KTVRLIISPEKDRAMEVYETLKRGLKIDPISKPDQLQFIVVEMPVSNVEKLAEIPGILHVWKDEMVKLQEP :	138
Aquifex_py :	-----LRLMVTIRNLKHSIGNTERIKLSSEETLLIGEYTLQDIKTFKTLN-VIYIALP-----MKY :	104
aerolysin :	P-----TEALKELNKTGDIKIKHLEKIKAIIVLNIPDNKTEKLEKLGVRVIEEDGVAVAFG :	95
AK1 :	NAAEEAKAKLKEVGATAVPDNDRVKSKFNVLVKGVNVEAVKALNNPLVEY-AEPN-YLFNAW-----TPN :	125
B.Stearo_J :	S-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	103
aqalysin_1 :	QGS-----LLQGGITTLQARLAPQGVVVTQAYTGAQGFQAEMAPQALEAFRQSPD-VEFIEAD--KVVRA :	126
thermitase :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	90
D13158 :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	90
P41362 :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	108
P27693 :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	108
Q99405 :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	-
P29599 :	-----DEVSAQSVEESYDVDVIEHFEEIPIVIAHAELETELKELQNDPN-VKAEEN-----AEV :	-
AB005792 :	VMSQFVDQIDGDEYSISSSQVEDVEIDLHFEFDFIPVLSVELDPQDVEALELDPA-ISYIEED-----AEV :	108
P29142 :	S-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	103
P00783 :	S-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	103
P04189 :	S-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	103
P35835 :	S-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	103
P07518 :	-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	-
P00781 :	-----AMSSAKKDVISEKGGKVKQKQFYVNAAAATLDEKAVKELKKDPS-VAYVEED-----HIA :	-
P11018 :	-----MNGEIRLIPYVTNEQ-----MNGEIRLIPYVTNEQ-----MNGEIRLIPYVTNEQ :	15
P29139 :	-----MERKVHIIPYQVIKQ-----MERKVHIIPYQVIKQ-----MERKVHIIPYQVIKQ :	15
Thermicin :	SLVKLQHHIATQETASRKAN-----DLGYTCKGKTIFAEEDTGIV-PHPDETQPKN----	145
Pyrococcus :	PIVEKDVKTKEPSPEPKMYNSTWVIN---ALQFIQEFQYDGGGVVAVLDTGVDPNHPELSITPDGRRK :	198
stetterlys :	VSPEAGMDGVTPAQQDPKLPDMFMSVYKIHAFTWNIYGVLDGDNVTVAVLDTGIDVGHFELQVTLGDRPK :	208
Aquifex_py :	SPLLDKVALYTRLTDLMDQTN-----LSEYKGRGVIEGADSGIDYTHFAFRG----- :	152
aerolysin :	FSNYTDTQWNVKMNAPRLGLRFS-----HIWRRAFSGYGVKVAVLDTGIDYKHEFTSG----- :	148
AK1 :	DTYYQGYQYSPQNTYTDYAW-----DVTGSSSGQETAVDTGVDYTHPDLDG----- :	172
B.Stearo_J :	HEYAQSVPYGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	149
aqalysin_1 :	WATQSPAPWGLDRIDQRDPLPSN-----SYTYTATGRGVNVVIDTGIRTTREFFG----- :	178
thermitase :	DPYFSSRQYSPQKIQAPQAW-----DIAEGS-GAKTAVDTGVQSNHDPDLG----- :	50
D13158 :	TI-SQTVPWGISFISTQQA-----NRGIFGNGARVAVLDTGIA-SHPDLR----- :	134
P41362 :	TTMAQSVPPGISRVQAPAAH-----NRGLTGSQVKKVAVLDTGIS-THPDLN----- :	153
P27693 :	TTMAQSVPPGISRVQAPAAH-----NRGLTGSQVKKVAVLDTGIS-THPDLN----- :	153
Q99405 :	---AQSVPPGISRVQAPAAH-----NRGLTGSQVKKVAVLDTGIS-THPDLN----- :	42
P29599 :	---AQSVPPGISRVQAPAAH-----NRGLTGSQVKKVAVLDTGIS-THPDLN----- :	42
AB005792 :	TT-MQTVPPGISINRVQAPIAQ-----SRGFTGTGVVAVLDTGIS-NHAPDLR----- :	152
P29142 :	HEYAQSVPYGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	149
P00783 :	HEYAQSVPYGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	149
P04189 :	HEYAQSVPYGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	149
P35835 :	HEYAQSVPYGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	149
P07518 :	---AQSVPPGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	43
P00781 :	---AQSVPPGISQHKAPALH-----SQGYTGSNVKVAVIDSGIDSSHDPDLN----- :	43
P11018 :	IMDVNELPEIKVKAPEMW-----AKGVKGNKIKVAVLDTGCDTSHDPDLN----- :	62
P29139 :	EQQVNELPRGVEMQAPAVW-----NQTR-GRGVKVAVLDTGCDADHDPDLKA----- :	61

Fig. 1. Comparison of amino acid sequences of thermicin with other proteinases.

Thermicin :	-----RITAFYD-----	: 152
Pyrococcus:	-----IIEWKDFDTEGFVDTSFSFSKVNGTLINTTFQVAGCLTLNESTGL	: 245
stetterlys:	IVDIYDASDEGIAQIYYATNVNTENGITVNMTVPVYWGFIYMYGHEQITDYMGTYYVGGINGSEYYLG	: 278
Aquifex_py:	-----RITLTHD-----	: 159
aerolysin:	-----KVVCIN-----	: 155
AK1 :	-----KVIKGYD-----	: 179
B.Stearo_J:	-----VRGGAS-----	: 155
aqalysin_l:	-----RARVGYD-----	: 185
thermitase:	-----KVVGWGD-----	: 57
D13158 :	-----TAGGAS-----	: 140
P41362 :	-----TRGGAS-----	: 159
P27693 :	-----TRGGAS-----	: 159
Q99405 :	-----TRGGAS-----	: 48
P29599 :	-----TRGGAS-----	: 48
AB005792 :	-----TRGGAS-----	: 158
P29142 :	-----VRGGAS-----	: 155
P00783 :	-----VRGGAS-----	: 155
P04189 :	-----VRGGAS-----	: 155
P35835 :	-----VRGGAS-----	: 155
P07518 :	-----VRGGAS-----	: 49
P00781 :	-----VVGGAS-----	: 49
P11018 :	-----QIIIGGKN-----	: 69
P29139 :	-----RITIGGRN-----	: 68

Thermicin :	-----VMNGKK--	: 158
Pyrococcus:	MEYVVKTVVYVSNVTIGNITSANGIYHFGQLPERYFDLNFDDQEDFYVLLVNSTGNGYDIAYVDTDLDY	: 315
stetterlys:	LLPERYFDLNNLTSPDDLMGDLNDVYPVLIVNQSGNFVA-----YIDFNLNNDTDDQPIG	: 335
Aquifex_py:	-----LTTGEVCY	: 167
aerolysin:	-----TIGNTLYK	: 163
AK1 :	-----FVNDNDY--	: 185
B.Stearo_J:	-----FVPSSETN--	: 162
aqalysin_l:	-----ALGGNG--	: 191
thermitase:	-----FVNDNS--	: 63
D13158 :	-----FISSEP--	: 146
P41362 :	-----FVPGEP--	: 165
P27693 :	-----FVPGEP--	: 165
Q99405 :	-----FVPGEP--	: 54
P29599 :	-----FVPGEP--	: 54
AB005792 :	-----FVPGEP--	: 164
P29142 :	-----FVPSSETN--	: 162
P00783 :	-----FVPSSETN--	: 162
P04189 :	-----FVPSSETN--	: 162
P35835 :	-----FVPSSETN--	: 162
P07518 :	-----FVPSSETN--	: 56
P00781 :	-----FVSGES--	: 55
P11018 :	-----ETDDDGKK	: 77
P29139 :	-----ETDDDEGD	: 76

Thermicin :	-----QPYDNGHGTHVAGDAAGNGYA	: 180
Pyrococcus:	DFTDEVPLGQYNVTYDVAVFSSYYGPLNYVLAID-----PNGEYAVFGWGHGTHVAGTVAGYDSN	: 379
stetterlys:	LFTETGDYFQTPDTLVLDIALAKVHIGDMSNPNDYLYTVPTEDGIGYAMFMWDAQGHGTHVAGTVAGYGLP	: 405
Aquifex_py:	KEEIEKG-----NCNQRTDGHGTHVAGTVAGDHEV	: 198
aerolysin:	GTNLR-----KCAARKCHGTHVAGTIAASLN	: 189
AK1 :	-----DPMNLNNHGTHVAGTIAAETNN	: 207
B.Stearo_J:	-----PYDGSSHGTHVAGTIAALNN	: 183
aqalysin_l:	-----QDCNGHGTHVAGTIAAGVITY	: 210
thermitase:	-----TPONGNGHGTHVAGTIAAVTNN	: 85
D13158 :	-----SYHNNNGHGTHVAGTIAALNN	: 167
P41362 :	-----STODGNGHGTHVAGTIAALNN	: 186
P27693 :	-----STODGNGHGTHVAGTIAALNN	: 186
Q99405 :	-----STODGNGHGTHVAGTIAALNN	: 75
P29599 :	-----STODGNGHGTHVAGTIAALNN	: 75
AB005792 :	-----NISDNGHGTHVAGTIAALNN	: 185
P29142 :	-----PYDGSSHGTHVAGTIAALNN	: 183
P00783 :	-----PYDGSSHGTHVAGTIAALNN	: 183
P04189 :	-----PYDGSSHGTHVAGTIAALNN	: 183
P35835 :	-----PYDGSSHGTHVAGTIAALNN	: 183
P07518 :	-----PYDGSSHGTHVAGTIAALNN	: 77
P00781 :	-----YNTDNGHGTHVAGTVAAALDN	: 76
P11018 :	---ED-----AISDYNHGTHVAGTIAANDS	: 100
P29139 :	---PE-----IFKYNHGTHVAGTIAAATEN	: 99

Fig. 1. Continued.

between the N-terminal and C-terminal domains of thermicin is shorter than that of the X-ray structure of thermitase. Interestingly, the structural domains are involved with both a short flexible loop and long loop, which is three α -helices that are wrapped by a single two-stranded β -sheet (Fig. 3). The core active site of thermicin is located on a small cavity that is surrounded by two anti-parallel β -strands and a single α -helix between the N- and C-terminal domains (Fig. 4).

Preparation of deletion mutant thermicin Expression plasmids of the loop deletion mutants, pQE-2-2, pQE-1-3, and pQE-LL12, were subjected to DNA sequencing in order to

confirm that the amino acid deletions had been introduced into these clones. It was confirmed that 7 and 12 amino acid residue deletions had been made during each mutagenesis.

The native and loop-deleted mutant thermicin genes were expressed in *E. coli* M15 (pREP4). This was followed by purification, partially from the culture fluid.

Catalytic properties of mutant thermicin L1-3 and L2-2

As shown in Table 2, the specific activities toward ZGP as an authentic substrate for subtilisin of the mutant L2-2, thermicin deleted small loop, was essentially equivalent to that of the wild-type enzyme, regardless of whether or not they were

Thermicin :	-----SN---SKYKGAPEANLVAVKVLDSYGRSSDLAGMOMVLDN :	221
Pyrococcus :	NDAWDWLSMYSGEWEVFSRLYGWDYTNVTDTFQGVAPGAQIMAIRVLRSDGRGSMWDTIEGMYAATH :	448
stetterlys :	-----TDPVFNGTYGVAPNAQLMBVKKVPGEGFSSATSWINGMTYAASN :	450
Aquifex_py :	-----YRGTAPEAEELIVVKIGNEEFREDKIEALDILAKRIKA :	236
aerolysin :	-----NV---S-AAGVVPKVLIAVKKVYDSGGYSDTAEGTIEAVKA :	229
AK1 :	-----AT---G-IAGMAPNTRILAVRALD-RNMSGTSLSDADATYAADS :	247
B.Stearo_J :	-----SI---G-VLGVSPASLYAVKVLDSSTGGQYSWINGIEWAISN :	223
aqalysin_1 :	-----GVAKK--VNLVAVRVLD-CNMSGSTSGVIAGVDWVTRN :	245
thermitase :	-----ST---G-IAGTAPKASILAVRVLD-NSGGTWTAVANGITYAADQ :	125
D13158 :	-----SI---G-VLGVAPSADLYAVKVLDRNMSGSLASVAQGIETWAINN :	207
P41362 :	-----SI---G-VLGVAPSADLYAVKVLG-ASGGSVSSTAQGLEWAGNN :	226
P27693 :	-----SI---G-VLGVAPNABLYAVKVLG-ASGGSVSSTAQGLEWAGNN :	226
Q99405 :	-----SI---G-VLGVAPSADLYAVKVLG-ASGGSVSSTAQGLEWAGNN :	115
P29599 :	-----SI---G-VLGVAPSADLYAVKVLG-ADGRGAISSTAQGLEWAGNN :	115
AB005792 :	-----SI---G-VLGVAPNVDLYGVKVLG-ASGGSTISGTAQGLEWAGNN :	225
P29142 :	-----SI---G-VLGVSPASLYAVKVLDSSTGGQYSWINGIEWAISN :	223
P00783 :	-----SI---G-VLGVSPASLYAVKVLDSSTGGQYSWINGIEWAISN :	223
P04189 :	-----SI---G-VLGVSPASLYAVKVLDSSTGGQYSWINGIEWAISN :	223
P35835 :	-----SI---G-VLGVAPSADLYAVKVLDSSTGGQYSWINGIEWAISN :	223
P07518 :	-----SI---G-VLGVAPSADLYAVKVLDSSTGGQYSWINGIEWAISN :	117
P00781 :	-----TT---G-VLGVAPNVSLLYALKVLD-SSGGTYSALVSGIEWATQN :	116
P11018 :	-----NG---G-IAGVAPEASLLTVKVLGGENGSGQYEWINGINYAVEQ :	141
P29139 :	-----EN---G-VVGVAPAEADLLTIKVEN-KOSSGQYDWITQGIYVAIEQ :	139

Thermicin :	KEKYNIRVLSIGETPALPTFLDPLVRGVDTLWKN--GIVVVVAAAGNSGPNYN---SITSPGTSRNAIT :	286
Pyrococcus :	G----ADVISMSLGGNAPYLDGTDPESEVAVDELTEK-YGVVFVIAAGNEGPGIN---IVGSPGVATKAIT :	510
stetterlys :	G----ADVISMSLGGGEINDGIESPENFYVLLTDWFGVTFATAAGNEGPTTN---TVHAPGSDDLVIT :	513
Aquifex_py :	YG---KPGVINLSLGLTPLGPHDGTDFISRFIESIVNT-YKIVVVVAAAGNMGNEP---IHDKLNLFQENTSS :	300
aerolysin :	G----ALILSMSLGGP---TDASVLRDASYWYQQ--GAVQIAAAGNSGDGDLPTNNVGYPAKVPSTHA :	289
AK1 :	G----AEVINLSLGGCD---CHTTTLENANVYAWNKGIVVAAAAGNNGSSTT---FEPASVENVIA :	302
B.Stearo_J :	N----MDVINMSLGGP---SGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--SSTVGYPAKVPSTHA :	282
aqalysin_1 :	HR---RPAVANMSLGG---GVSTALDNVAKNSIAA--GVVYVAAAGNDNANACN---YSPARVAEAIT :	302
thermitase :	G----AKVISLSLGGT---VNSGSLQQAVNYAWNKGIVVAAAAGNAGNTAP---NYPAYISNAIA :	180
D13158 :	N----MHVINMSLGGT---SGSSTLELAVNRNNA--GILLVGAAGNTRGRQG---VNYPARMSGVMA :	262
P41362 :	G----MHVANLSLGGP---SPSATLEQAVNSATSR--GVLVVAASGNSGAGS---ISYPARANAMA :	281
P27693 :	G----MHVANLSLGGP---SPSATLEQAVNSATSR--GVLVVAASGNSGAGS---ISYPARANAMA :	281
Q99405 :	G----MHVANLSLGGP---SPSATLEQAVNSATSR--GVLVVAASGNSGAGS---ISYPARANAMA :	170
P29599 :	G----MHVANLSLGGP---SPSATLEQAVNSATSR--GVLVVAASGNSGAGS---ISYPARANAMA :	170
AB005792 :	G----MHVANMSLGGSS---AGSATMEQAVNQATAS--GVLVVAASGNSGAGN---VGFPARANAMA :	280
P29142 :	N----MDVINMSLGGP---SGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--SSTVGYPAKVPSTHA :	282
P00783 :	N----MDVINMSLGGP---SGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--SSTVGYPAKVPSTHA :	282
P04189 :	N----MDVINMSLGGP---TGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--TSTVGYPAKVPSTHA :	282
P35835 :	N----MDVINMSLGGP---TGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--TSTVGYPAKVPSTHA :	282
P07518 :	N----MDVINMSLGGP---TGSTALKTVVDKRVSS--GIVVAAAAGNEGSSGS--TSTVGYPAKVPSTHA :	176
P00781 :	G----LDVINMSLGGP---SGSTALKQAVDKRYAS--GIVVAAAAGNEGSSGS--QNTIGYPAKVDVIA :	175
P11018 :	K----VDLISMSLGGP---SDVPELKEAVKNKVN--GVLVVAAGNEGSDGDERTEELGYPAKVAEVA :	201
P29139 :	K----VDLISMSLGGP---EDVPELHRAVKKKVAS--QLLVMAAGNEGSDGDDRTDELGYEGGKNEVVS :	199

Thermicin :	VGAIVDKR-----TPDIEDDEVAKFSGRGPYL--YKPDVVAPGVKIVSTASGNV :	334
Pyrococcus :	VGAAPVPIVGVVVSQALGYDYYGFYFPAYTNVRIAFFSSRGPRIDGEIKPNVAPGVGIYSSLPFTWI :	580
stetterlys :	VGAFRSSLRW-----QIFYGVGDVADTVASFSSRGPRMDGLLDVPIAPGEMIFSSLPFLWY :	569
Aquifex_py :	FDVIG-----SWAYVDIWMITDS---VEITINSFCQAVSIREGDTK :	339
aerolysin :	AAAVV-----QNGSVPTWSSDGP-----EVDLAPGVNLSITYEGGR :	326
AK1 :	VGAIV-----QYDLRASFSNYET-----WVDVAPGVDIVSTITGNR :	339
B.Stearo_J :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	319
aqalysin_1 :	VGAIT-----SSDARASFSSNYGS-----CVDLAPGASIPSAWYTS :	339
thermitase :	VASTD-----QNDNKSFSSTYES-----WVDVAPGVSIQSTLPGGT :	217
D13158 :	VGAIV-----QNGQRAFSSSTYGP-----EDELISAPGVNVSTYTGNR :	299
P41362 :	VGAIV-----QNNNRASFSSQYGA-----GLDIVAPGVNVSTYTPGST :	318
P27693 :	VGAIV-----QNNNRASFSSQYGA-----GLDIVAPGVNVSTYTPGST :	318
Q99405 :	VGAIV-----QNNNRASFSSQYGA-----GLDIVAPGVNVSTYTPGST :	207
P29599 :	VGAIV-----QNNNRASFSSQYGA-----GLDIVAPGVNVSTYTPGST :	207
AB005792 :	VGAIV-----QNNNRASFSSQYGA-----GLDIVAPGVNVSTYTPGST :	317
P29142 :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	319
P00783 :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	319
P04189 :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	319
P35835 :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	319
P07518 :	VGAIV-----SSNQRAFSSSAGS-----ELDVMAPGVSIQSTLPGGT :	213
P00781 :	VGAIV-----SNKNRAFSSVGA-----ELDVMAPGVSVSTYTPSNT :	212
P11018 :	VGSVS-----VARELSEFSNANK-----ELDLVAPGVNLSITLPPNK :	238
P29139 :	VGAIV-----FDRHASEFSNSNN-----EVDLVAPGEDILSTVPGGK :	236

Fig. 1. Continued.

pretreated with 5 mM dithiothreitol for reduction. However, the L1-3 mutant enzyme-deleted long loop showed lower K_m and k_{cat} than that of others, indicating that the long loop may be related to catalytic activity. However, the possibility cannot be ruled out that the alteration with artificial substrates may not be equivalent to protein substrates. It has been shown that for some serine proteases, including subtilisin, reduced autolysis leads to an apparent increase in instability (Daniel *et al.*, 1982; Takagi *et al.*, 1990; Takagi *et al.*, 2000). Subtilisin is irreversibly inactivated through autolysis at elevated temperatures (Wells *et al.* 1986; Mitchinson *et al.*, 1989; Takagi *et al.* 1990).

Unfolding temperature of the L1-3 and L2-2 mutant thermicin Using differential-scanning calorimetry, an unfolding temperature (T_m) of 96°C was measured for the small loop-deletion mutant, which is 14°C lower than the T_m that was measured for the parent thermicin and the long loop-deleted mutant enzyme (Fig. 5). This result clearly suggests that the small loop in thermicin may play a role, at least partially, in thermostability.

Discussion

One of the common strategies to enhance the thermostability

Thermicin :	PFGADEIMINKP	RSAT	GTSMATPMV	ACAVALL	LEKNS	----	RL	INVEIKNI	LKKT	TATK	INEAG	----	394	
Pyrococcus:	GG	-----	ADFMS	GTSMATPHVS	GVVALL	ISGAKAEG	IYXNPDI	IKKVLES	SGATW	LEGDP	-----	634		
stetterlys:	TVTNN	--SYNY	GIWD	GTSMATPHVS	CAVAL	MSYAKQHN	LTYPDIM	IKRAEL	SAKPV	NGT	-----	630		
Aquifex_py:	-----	-----	LIDL	GNCGLTAVSFS	GRNPL	NGDKN	VFIKID	NVNENF	NRNGWSV	SFTPI	DVKD	-----	391	
aerolysin :	-----	-----	YAYMS	GTSMATPHVT	VAAAL	QALRLASG	KRLT	TPDEV	YQVIT	TAKDI	GPPG	-----	379	
AK1 :	-----	-----	YAYMS	GTSMASPHVAG	LAALL	ASQ	-----	GRNNIE	IRQAIE	QTADK	ISGT	-----	384	
B.Stearo_J:	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGNS	-----	366	
aqalysin_1:	TA	-----	TQTLN	GTSMATPHVAG	VAAAL	YLEQNPSAT	PASVASA	ILNGATT	GRLSG	IGSGSP	NRLL	-----	399	
thermitase:	-----	-----	VASLS	GTSMATPHVAG	VAGL	ASQ	-----	GRSASNI	RAATENT	ADKISGT	-----	262		
D13158 :	-----	-----	VVSL	GTSMATPHVAG	VAAAL	VKSRYP	-----	SYTNNQ	IRQRINQ	ATYLGSS	-----	346		
P41362 :	-----	-----	VASLN	GTSMATPHVAG	AAAL	VKQKNP	-----	SWSNVQ	IRNHLKNT	ATSLGST	-----	365		
P27693 :	-----	-----	VASLN	GTSMATPHVAG	AAAL	VKQKNP	-----	SWSNVQ	IRNHLKNT	ATSLGST	-----	365		
Q99405 :	-----	-----	VASLN	GTSMATPHVAG	VAAAL	VKQKNP	-----	SWSNVQ	IRNHLKNT	ATSLGNT	-----	254		
P29599 :	-----	-----	VASLN	GTSMATPHVAG	AAAL	VKQKNP	-----	SWSNVQ	IRNHLKNT	ATSLGST	-----	254		
AB005792 :	-----	-----	VSSFN	GTSMATPHVAG	VAAAL	VKQKNP	-----	SWSNVQ	IRNHLKNT	ATNLGNT	-----	364		
P29142 :	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGNS	-----	366	
P00783 :	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGNS	-----	366	
P04189 :	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGNS	-----	366	
P35835 :	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGNS	-----	366	
P07518 :	-----	-----	YGAYN	GTSMATPHVAG	AAAL	LILSKHP	-----	TWTNA	QVRDRLES	STATY	LGSS	-----	260	
P00781 :	-----	-----	VTSLN	GTSMASPHVAG	AAAL	LILSKYP	-----	TLSAS	QVRNR	LSS	PATNLGD	-----	259	
P11018 :	-----	-----	YGKLT	GTSMAPHV	S	GALAL	LKSYEEES	FQRKL	SESEV	FAQL	LIRRTL	PLDIA	-----	290
P29139 :	-----	-----	VATFS	GTSMATPHVAG	ALAL	LKQLANAS	FERDL	TEPE	LYAQL	LKRTI	PLGNS	-----	288	

Thermicin :	-----	-----	LWTQ	CS	GMIN	IEEL	LKKV	-----	-----	-----	-----	412				
Pyrococcus:	-----	-----	YTQK	QYTEL	DQ	HGLV	NVTKS	WEIL	KAING	-----	-----	684				
stetterlys:	-----	-----	LIDQ	GFLI	QVDK	ATEE	EKLSQ	EP	TTYIF	AGT	TYTS	FKNP	IEE	PLIPI	679	
Aquifex_py:	-----	-----	GEVHL	WGEN	VAFAS	PDYS	YTIS	SLAS	-----	-----	SSSV	ISV	SGS	SFT	428	
aerolysin :	-----	-----	FD	VFS	GYGL	VDYA	AVVA	ALSR	-----	-----	-----	-----	-----	-----	401	
AK1 :	-----	-----	GT	YFKY	GR	INSY	NAV	TY	-----	-----	-----	-----	-----	-----	401	
B.Stearo_J:	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	381	
aqalysin_1:	YSL	LS	SGSG	STAP	CTCS	SYIT	GSLS	PE	DYN	FQ	PNGT	YY	YSPAG	-----	THRAWLR	450
thermitase:	-----	-----	GT	YWAK	RV	NAYK	EVQY	-----	-----	-----	-----	-----	-----	-----	279	
D13158 :	-----	-----	NLY	CS	GLV	HAG	RA	Q	-----	-----	-----	-----	-----	-----	361	
P41362 :	-----	-----	NLY	CS	GLV	NEA	EA	TR	-----	-----	-----	-----	-----	-----	380	
P27693 :	-----	-----	NLY	CS	GLV	NEA	EA	TR	-----	-----	-----	-----	-----	-----	380	
Q99405 :	-----	-----	NLY	CS	GLV	NEA	EA	TR	-----	-----	-----	-----	-----	-----	269	
P29599 :	-----	-----	NLY	CS	GLV	NEA	EA	TR	-----	-----	-----	-----	-----	-----	269	
AB005792 :	-----	-----	NQ	CS	GLV	NEA	EA	TR	-----	-----	-----	-----	-----	-----	379	
P29142 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	381	
P00783 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	381	
P04189 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	381	
P35835 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	381	
P07518 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	275	
P00781 :	-----	-----	FY	YKGL	IN	VQAA	QAQ	-----	-----	-----	-----	-----	-----	-----	274	
P11018 :	-----	-----	KT	LACN	GL	YLTAP	DELA	EAKAEQ	-----	-----	SHLL	TL	-----	-----	319	
P29139 :	-----	-----	PKME	CN	GL	LYLT	AVEEL	SRIFT	-----	-----	Q	R	VAGIL	-----	318	

Fig. 1. Continued.

of the enzyme is to introduce disulfide bonds into the protease by SDM. Introduction of a disulfide bond into the subtilisin E from *Bacillus subtilis* resulted in an increase of 4.5°C in T_m of the mutant enzyme, but there was no change in its catalytic efficiency (Takagi *et al.*, 1990). However, the properties of the mutant enzyme were found to revert to those of the wild-type enzyme. Enhanced stability of subtilisin was observed as a result of the mutations of Asn109 and Asn218 to Ser. The analog that contained both mutations showed an additive effect on thermal stability. Thermostability of the alkaline protease from *Aspergillus oryzae* is important because of its extensive use in manufacturing soy sauce. The optimal temperature of the wild-type enzyme was enhanced from 51 to 56°C by the introduction of a disulfide (Cys 169-Cys 200) bond. Another strategy for improving the stability of the protease was by replacing the polar amino acid groups with the hydrophobic groups. The presence of positively-charged amino acids in the N-terminal turn of a helix is undesirable because of the possibility of repulsive interactions with the helix dipole. Replacement of Lys by Ser or Asp resulted in an increase in the thermostability of the neutral protease from *B. subtilis* in the range of 0.3 to 1.2°C (Eijsink *et al.*, 1992).

Our comparison shows the distribution of the amino acid composition of thermophilic and mesophilic proteases. A higher occurrence of charged-amino acids was also observed in the proteins from *T. yonseiensis*. The percentage of

aspartate, glutamate, arginine, and lysine of the cloned protease was about 21.6%. This is similar to the value that was found in pyrolysin from *Pyrococcus furiosus*, and is much higher than the proteases from mesophiles or thermophiles (Table 6). These charged-residues may be a participant in the formation of salt bridges, since the range in surface area is much narrower. The distribution of these structural features does not differ significantly between the mesophilic and thermophilic proteins. Therefore, for proteases, it is not possible to find a structural feature that differentiates thermophiles from mesophiles. This suggests that pairwise comparisons of thermophilic structures to mesophilic structures contain no useful information in uncovering the structural basis of thermostability.

From our comparison of a large group of proteins from the same family, we observed two potential factors in the difference between thermophilic and mesophilic protein stability—the loops at the amino side of the protease from thermophilic bacteria and thermal factors. Both of these observations can be related to molecular flexibility. The deletion of the small loop in the thermicin may allow access to conformations that are destabilizing for thermophilic proteins at high temperatures. Fewer glycyl residues in the thermophilic structures may serve to decrease the flexibility of this region, thereby providing greater stability at higher temperatures (Panasik *et al.*, 2000). Localization of

Table 1. Comparison of amino acids on the substrate binding site

Binding Pocket site	Target residue	Substituted residues
P2'	F 204	F or Y
P1'	R 246	variable
P2'-P1 lining	A 162	Conserved A or few S
	N 165	Highly conserved
	T 249	T or S, N and V, A, K, Q
	S 252	Conserved S and few G, V, K
P1'-P2 lining	H 64	Highly conserved
	D 29	Highly conserved
	T 30	T or D
P1	T 135	T or E, P, D, G, N, A etc (variable)
	G 164	Highly conserved
	S 166	S or G, D
	T 174	T or N, S, G, K etc. (variable)
P2	N 62	N or I, L, V, S, H (variable)
P1-P3 lining	S 131	S or N (Highly conserved)
P2-P3 lining	L 98	Conserved L or few C, F
P3	F 101	Variable
	G 102	Variable
	G 133	Highly conserved
P4	R 103	Variable
	I 109	I or A, V and other hydrophobic residues
	I 132	I or L, W and other hydrophobic residues
	E 134	E or many P, G and few S
	P 136	P or many D, S and variable

temperature effects in 'hinge regions' between elements of secondary structure are also suggested in the work of Fields and Somero (Fields *et al.*, 1998). They concluded that some lactate dehydrogenases adapted to cold temperatures by increasing the flexibility of small areas in the molecule that affect the mobility of adjacent active-site secondary structural elements. They also observed that the thermostable lactate dehydrogenases have a smaller number of glycines in the loop regions between helices. For eight-stranded α/β barrels, it was shown earlier that the loops at the amino-terminal face of the barrel are more important for thermostability than those at the other face (Urfer *et al.*, 1992). While other researchers (< biblio >) have explored the concept of rigidity as a means of adaptation to high temperatures, its role as a general mechanism has not been proven experimentally.

A result from this study also suggests that the mobility of inserted amino acid sequences as loops is important in understanding the difference between mesophilic and

Fig. 2. Ribbon Drawing from the Molecular modeling structure of thermicin (red ribbon). Blue ribbon indicates the structure of thermitase. Two protein structures are superimposed for comparison. Long loop for catalytic activity and short loop for thermostability were enclosed by yellow circles. This structural diagram was produced by the Insight II version 98.0 program (Molecular Simulations Inc.). **Fig. 3.** Schematic Drawing of Simplified modeling structure of thermicin. Red, blue, and green colors indicate α -helix, β -sheet, and loops, respectively. The drawing was produced by the MOLSCRIPT version 2.1 (Kraulis, 1991) and Raster3D version 2.6 (Bacon and Anderson, 1988).

thermophilic structures. The average main chain and side chain thermal factors for the thermophilic structures were found to be consistently higher than for mesophiles. This

Fig. 4. Location of active site of thermicin, based on modeling structure. Catalytic triads and binding motif are also drawn.

Table 2. Comparison of kinetic parameters for the hydrolysis of synthetic peptide by native and mutant thermicins

Thermicin and loop deletion mutants	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($10^5 \text{s}^{-1} \text{M}^{-1}$)
thermicin	11.481 (± 1.1)	32299.58 (± 30)	2813.3
Mutant L1-3 (deleted long loop)	8.906 (± 0.2)	29976 (± 20)	3365.82
Mutant L2-2 (deleted small loop)	11.3045 (± 1)	32552.05 (± 40)	2879.56

^aEnzyme activity was assayed using a acetone-precipitated sample by the method of Wells *et al.* L-Gly-Pro-pNA (Sigma Chemical Co., St. Louis, Mo.) was the substrate

change in thermal factors correlates with the two published structures of psychrophilic proteins - citrate synthase (Russell *et al.*, 1998) and malate dehydrogenase (Kim *et al.*, 1999). For both citrate synthase and malate dehydrogenase, the thermophilic counterpart had significantly larger thermal factors than the psychrophile. It should be noted that in the case of citrate synthase, both of the structures were solved in the same laboratory using the same programs (Russell *et al.*, 1998). Higher thermal factors for thermostable enzymes, as compared to mesophilic enzymes, have been seen in other cases as well (Yip *et al.*, 1995). One interpretation of these observations is that the thermal factors are dominated by the dynamic component. Also, where structures are determined, thermophilic enzymes are more flexible than mesophilic enzymes at room temperature. This explanation, however, is contradicted by experimental work, which demonstrates that protein flexibility decreases as the temperature decreases (Cao *et al.*, 1998).

The dynamic component of thermal factors represents a degree of conformational flexibility, while the static component would reflect different protein conformations in each unit cell of one crystal. A recent publication by Zavodszky *et al.* suggests that thermophilic proteins are indeed more conformationally-rigid than mesophilic proteins at the same temperature.

Therefore, our observations could be explained by multiple conformations for thermophilic proteins at mesophilic temperatures. If this is true, then the high thermal factors that were observed for thermophiles are due to static rather than dynamic disorder. At room temperatures, thermophilic enzymes may be frozen into a number of slightly different conformations. The existence of multiple conformations of the enzyme could also explain why thermophilic enzymes are not functionally-active at mesophilic temperatures. Thermostability is a prerequisite for the successful application of enzymes in industrial processing. The industrial use of proteases in detergents or for leather processing requires that the enzyme be stable at higher temperatures.

In conclusion, the small loop in thermicin plays a critical role in thermostability; whereas, the large loop may be involved in catalysis. However, how the loops are mechanistically involved in the enzyme characteristics, and how well they are correlated to the various explanations in different thermostable enzymes, remains to be elucidated.

Acknowledgments This work was supported by grants from the Korea Science and Engineering Foundation through Bioproducts Research Center and Protein Network Research Center (2000-2-0607).

Fig. 5. Differential-calorimetric scanning of thermicin. The purified proteins, at a concentration of 1~2 mg/ml in 10mM sodium acetate, pH 5.0, were heated at a constant rate of 2°C/min up to 125°C. Top, middle, and bottom plates indicate those for native thermicin, mutant thermicin L1-3 (deleted long loop), and mutant thermicin L2-2 (deleted small loop), respectively. X- and Y-axis indicate temperature (°C) and heat flow (W/g), respectively. The inverted peaks indicate that at this temperature, heat was consumed to denature the proteins.

References

- Argos, P., Rosseman, M. G., Gsau, U. M., Zuber, H., Frank, G. and Tratschin, J. D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5698-5703.
- Bacon, D. J. and Anderson, W. F. (1988) A Fast Algorithm for Rendering Space-Filling Molecule Pictures. *J. Mol. Graph.* **6**, 219-220.
- Cao, W., Lu, J., Welch, S. G., Williams, R. A. and Barany, F. (1998) Cloning and thermostability of TaqI endonuclease isoschizomers from thermus species SM32 and Thermus filiformis TOKGA1. *Biochem. J.* **333**, 425-431.
- Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W. and Rees, D. C. (1995) Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science* **267**, 1463-1469.
- Daniel, R. M., Cowan, D. A., Morgan, H. W. and Curran, M. P. (1982) A correlation between protein thermostability and resistance to proteolysis. *Biochem. J.* **207**, 641-644.
- Day, M. W., Hsu, B. T., Joshu-Tor, L., Park, J., Zhou, Z. H., Adams, M. W. W. and Rees, D. C. (1992) X-Ray crystal structure of the oxidized and reduced forms of the rubredoxin from the marine hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Protein Sci.* **1**, 1494-1507.
- Eijssink, V. G. H., Vriend, vanden Burg, G. B., vander Zee, J. R. and Venema, G. (1992) Increasing the thermostability of a neutral protease by replacing positively charged amino acids in the N-terminal turn of α -helices. *Protein Eng.* **5**, 165-170.
- Eriksson, A. E., Baase, W. A., Zhang, X. J., Heinze, D. W., Blaber, M., Baldwin, E. P. and Matthews, B. W. (1992) Response of a protein structure to cavity creating mutations and its relation to the hydrophobic effect. *Science* **255**, 178-183.
- Fields, P. A. and Somero, G. N. (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenoid fishes. *Proc. Natl. Acad. Sci. USA* **95**, 11476-11481.
- Fleming, T. and Littlechild, J. (1997) Sequence and structural comparison of thermophilic phosphoglycerate kinases with a mesophilic equivalent. *Comp. Biochem. Physiol.* **118A**, 439-451.
- Imada, K., Sato, M., Tanaka, N., Katsube, Y., Matsuura, Y. and Oshima, T. (1991) Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus* at 2.2 Å resolution. *J. Mol. Biol.* **222**, 725-738.
- Kim, Y. K., Choi, I. G., Nam, W. and Yu, Y. G. (2000) Identification of a mature form and Characterization of Thermostability of a Serine-type Protease from Aquifex pyrophilus. *J. Biochem. Mol. Biol.* **33**, 493-498.
- Kim, S. Y., Hwang, K. Y., Kim, S. H., Sung, H. C., Han, Y. S. and Cho, Y. (1999) Structural basis for cold adaptation. *J. Biol. Chem.* **274**, 11761-11767.
- Kraulis, P. J. (1991) MOLSCRIPT: A Program to Produce Both Detailed and Schematic Plots of Protein Structures. *J. Appl. Crystal.* (1991) **24**, 946-950.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 47-60.
- Lim, W. A. and Sauer, R. T. (1989) Alternative packing arrangements in the hydrophobic core of λ repressor. *Nature* **339**, 31-36.
- Matsumura, M., Becktel, W. J. and Matthews, B. W. (1988) Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature* **334**, 406-410.
- Mitchinson, C. and Wells, J. A. (1989) Protein engineering of disulfide bonds in subtilisin BPN'. *Biochemistry* **28**, 4807-4815.
- Nakamura, S., Tanaka, T., Yada, R. Y. and Nakai, S. (1997) Improving the thermostability of Bacillus stearothermophilus neutral protease by introducing proline into the active site

- helix. *Protein Eng.* **10**, 1263-1269.
- Panasik, N., Brenchley, J. E. and Farber, G. K. (2000) Distributions of structural features contributing to thermostability in mesophilic and thermophilic α/β barrel glycosyl hydrolases. *Biochim. Biophys. Acta* **1543**, 189-201.
- Perutz, M. F. and Raidt, H. (1975) Stereochemical basis of heat stability in bacterial ferredoxins and in hemoglobin A2. *Nature* **255**, 256-259.
- Russell, R. J. M., Ferguson, J. M. C., Hough, D. W., Danson, M. J. and Taylor, G. L. (1997) The crystal structure of citrate synthase from the hyperthermophilic archaeon *Pyrococcus furiosus* at 1.9 Å resolution. *Biochemistry* **36**, 9983-9994.
- Russell, R. J. M., Gerike, U., Danson, M. J., Hough, D. W. and Taylor, G. L. (1998) Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. *Structure* **6**, 351-361.
- Sali, A. and Blundell, T. L. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779-815.
- Sandberg, W. S. and Terwillinger, T. C. (1991) Influence of interior packing and hydrophobicity on the stability of a protein. *Proc. Natl. Acad. Sci. USA* **88**, 1706-1710.
- Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L. and Engel P. C. (1998) Protein thermostability in extremophiles. *Biochimie* **80**, 933-941.
- Shortle, D. (1992) Mutational studies of protein structures and their stabilities. *Q. Rev. Biophys.* **25**, 205-250.
- Sippl, M. J. (1993) Boltzmann's principle, knowledge-based mean fields and protein folding. An approach to the computational determination of protein structures. *J. Comput.-Aided. Mol. Des.* **7**, 473-501.
- Takagi, H., Hirai, K., Maeda, Y., Matsuzawa, H. and Nakamori, S. J. (2000) Engineering subtilisin E for enhanced stability and activity in polar organic solvents. *J. Biochem.* **127**, 617-625.
- Takagi, H., Takahashi, T., Momose, H., Inouye, M., Maeda, Y., Matsuzawa, H. and Ohta, T. (1990) Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. *J. Biol. Chem.* **265**, 6874-6878.
- Teplyakov, A. V., Kuranova, I. P., Harutyunyan, E. H., Frommel, C. and Hohne, W. E. (1989) Crystal structure of thermitase from *Thermoactinomyces vulgaris* at 2.2 Å resolution. *FEBS Lett.* **244**, 208-212.
- Urfer, R. and Kirschner, K. (1992) The importance of surface loops for stabilizing an eightfold α/β barrel protein. *Protein Sci.* **1**, 31-45.
- Vogt, G., Woell, S. and Argos, P. (1997) Protein thermal stability, hydrogen bonds, and ion pairs. *J. Mol. Biol.* **269**, 631-643.
- Vriend, G. and Sander, C. (1993) Quality-Control of Protein Models-Directional Atomic Contact Analysis. *J. Appl. Crystallogr.* **26**, 47-60.
- Wallon, G., Kryger, G., Lovett, S. T., Oshima, T., Ringe, D. and Petsko, G. A. (1997) Crystal structure of *Escherichia coli* and *Salmonella typhimurium* 3-isopropylmalate dehydrogenase and comparison with their thermophilic counterpart from *Thermus thermophilus*. *J. Mol. Biol.* **266**, 1016-1031.
- Wells, J. A. and Powers, D. B. (1986) *In vivo* formation and stability of engineered disulfide bonds in subtilisin. *J. Biol. Chem.* **261**, 6564-6570.
- Yip, K. S., Stillman, T. J., Britton, K. L., Artymiuk, P. J., Baker, P. J., Sedelnikova, S. E., Engel, P. C., Pasquo, A., Chiaraluce, R. and Consalvi, V. (1995) The structure of *Pyrococcus furiosus* glutamate dehydrogenase reveals a key role for ion-pair networks in maintaining enzyme stability at extreme temperatures. *Structure* **3**, 1147-1158.
- Yutani, K., Ogasahara, K., Tsujita, T. and Sugino, Y. (1987) Dependence of conformational stability on hydrophobicity of the amino acid residues in a series of variant proteins substituted at a unique position of tryptophan synthase a subunit. *Proc. Natl. Acad. Sci. USA* **84**, 4441-4444.
- Zavodszky, P., Kardos, J., Svingor, A. and Petsko, G. A. (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. *Proc. Natl. Acad. Sci. USA* **95**, 7406-7411.