Journal of Biochemistry and Molecular Biology, Vol. 33, No. 6, November 2000, pp. 493-498

JOURNAL OF
Biochemistry
and
Molecular Biology

© BSRK & Springer-Verlag 2000

Identification of a Mature form and Characterization of Thermostability of a Serine-type Protease from *Aquifex pyrophilus*

Yun-Kyeong Kim[†,‡], In-Geol Choi[†,¶], Wonwoo Nam[‡] and Yeon Gyu Yu[†,*]
[†]Structural Biology Center, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul, 136-650,
[‡]Department of Chemistry, Ewha Womans University, Seoul, 120-750, Korea

Received 19 October 2000, Accepted 9 November 2000

***Aquifex pyrophilus*, a hyperthermophilic bacterium, has a serine-type protease that is located at the cell wall fraction with a mature size of 43 kDa. Molecular cloning of the protease gene revealed that it has an ORF of 619 amino acids with homologous catalytic site of serine-type proteases [Choi, I.-G., Bang, W.-K., Kim, S.-H., Yu, G. Y., *J. Biol. Chem.* (1999), Vol. 274, pp. 881-888]. Constructs containing different regions of the protease gene, including a alanine-substituted mutant at the active site serine, were constructed, and the factors affecting the expression level of the cloned protease gene in *E. coli* were examined. The presence of the C-terminus hydrophobic region of the protease hindered over-expression in *E. coli*. Also, the proteolytic activity of the expressed protein appeared to toxic to *E. coli*. An inactive form that deleted both of the N-terminal signal sequence and the C-terminal polar residues was over-expressed in a soluble form, purified to homogeneity, and its thermostability examined. The purified protein showed three disulfide bonds and three free sulfhydryl group. The thermal denaturation temperature of the protein was measured around 90°C using a differential scanning calorimeter and circular dichroism spectrometry. The disulfide bonds were hardly reduced in the presence of reducing agents, suggesting that these disulfide bonds were located inside of the protein surface.**

Keywords: *Aquifex pyrophilus*, Hyperthermophile, Protease, Thermostability, Expression

*To whom correspondence should be addressed.
Tel: 82-2-958-5936; Fax: 82-2-958-5939
E-mail: ygy@kistmail.kist.re.kr

[¶]Present Address: Department of Chemistry and Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720, USA.

Introduction

Hyperthermophiles had been isolated from various geothermal places such as deep-see vent, hot spring, and volcanic deposits. Due to the exceptional stability of proteins from hyperthermophiles, these organisms were interesting as a source of industrially important enzymes, such as protease. Most of hyperthermophiles belong to Archaea except two genera of eubacteria, *Aquifex* and *Thermotoga*. *Aquifex pyrophilus* is a Gram-negative bacterium whose optimum growth temperature is 85°C (Huber *et al.*, 1992). Previously, we identified a gene from *A. pyrophilus* homologous to serine-type proteases. It has conserved regions containing three canonical residues (Ser, His, Asp) consisting of a catalytic site of serine-type proteases (Choi *et al.*, 1997). Furthermore, we discovered that the precursor form of the protease is processed from 64 kDa form into a 43 kDa mature form. The mature form of the protein is localized at the cell wall. Compare to subtilisin, or other serine-type protease, the *Aquifex* protease has unique properties; it has a non-homologous region in the middle of the open reading frame. Also, cystein residues were found more frequently in the cloned *Aquifex* protease compared to other serine-type proteases (Choi *et al.*, 1999).

To investigate the characteristic of the *Aquifex* protease, the nature of the mature form and conditions for over-expression should be identified. In addition, the thermostability of the protein was thoroughly examined. In this study, we constructed various forms of the *Aquifex* protease and examined their expression level. Also, we examined thermal unfolding properties of the inactive form whose active site serine is substituted with alanine. In addition, the number of disulfide bond and the minimal region of the mature form were identified.

Materials and Methods

Materials Restriction enzymes and T4 DNA ligase were obtained from Promega (Madison, USA). Sequencing primers were purchased from Bioneer (Seoul, Korea). The *E. coli* strain

used for plasmid DNA amplification was DH5α [*supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]. *E. coli* BL21(DE3) was used for over-expression of the recombinant protein. All other chemicals and reagents used in this study were reagent grade.

Construction of various forms of *A. pyrophilus* protease A full-length open reading frame of the *Aquifex* protease, deletion mutants at the C-terminus and/or N-terminus and mutants with point mutation were prepared. The region representing the full length of ORF (amino acid 1-619) was amplified by PCR with two primers representing the 1st-8th and 611th-619th codons containing extra *Nco*I and *BamHI* restriction sites, respectively. After treatment of *Nco*I and *BamHI*, the amplified DNA fragments were ligated with a linearized pET28a vector with the same restriction enzymes to generate expression vector WT. Three deletion mutants at the N-terminus region, which were representing 51th to 619th residue (ΔN1), 102th to 619th residue (ΔN2) and 121th to 619th residue (ΔN3), were constructed by inserting amplified DNA fragments into *Nde*I and *Not*I site of pET21a vector. The mutant lacking both of the N- and C-terminus region (ΔN2-ΔC) was prepared by inserting the DNA fragment spanning 102th to 553th residue into the *Nde*I and *Hind*III site of pET21a vector. The active site serine of the two deletion mutants (ΔN2 and ΔN2-ΔC) was substituted with alanine to generate inactive mutants [(ΔN2(S502A) and ΔN2-ΔC(S502A)] using Quickchange™ site-directed mutagenesis kit (Stratagene, La Jolla, USA).

Expression and purification of a mutant, ΔN2-ΔC(S502A) An alanine substituted mutant, which deleted both the signal sequence and C-terminus hydrophobic region, ΔN2-ΔC(S502A), was expressed in *E. coli* as previously described (Kim and Yu, 2000). The expression vector was transformed into the *E. coli* strain BL21(DE3) and the cells were grown at 37°C in Luria-Bertani's (LB) broth with ampicillin (100 μg/ml). When the optical density of the culture at 600 nm reached 0.7, the expression of the protein was induced by adding IPTG at a final concentration of 1.0 mM. After 3 h of induction, the cells were harvested by centrifugation at 5,000 × g for 15 min. About 10 g of cell pellet (wet weight) was suspended in 50 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol and phenylmethylsulfonyl fluoride) and passed through a French press twice under 12,000 psi. The cell debris was removed by centrifugation at 15,000 × g for 20 min and the cell lysate was incubated for 45 min at 70°C. Denatured *E. coli* proteins were removed by centrifugation at 15,000 × g for 20 min, and the heat-treated sample was loaded onto a Q-sepharose column (1.5 × 20 cm; Phamacia LKB Biotechnology, Inc., Uppsala, Sweden) equilibrated with 10 mM Tris-HCl, pH 8.0. Proteins were eluted with 400 ml of a linear gradient of 0-0.8 M NaCl in the equilibration buffer. The fractions containing the expressed protein were pooled, concentrated and dialyzed against 10 mM Tris-HCl, 20 mM NaCl. Then, the sample was loaded onto a gel filtration column (Superdex 200, Amersham Pharmacia Biotech. Uppsala, Sweden). The fraction containing the expressed protein was pooled and used for further study.

Measurement of CD and DSC The circular dichroism (CD) spectra of purified protein were measured using Jasco 7000 spectrophotometer (Jasco, Japan) at a protein concentration of 0.5 mg/ml in 10 mM Tris-HCl, pH 8.0. A thermal denaturation curve was obtained by monitoring the ellipticity at 220 nm as temperature was increased at a rate of 1°C/min. A differential scanning calorimetry (DSC) of 0.5 mg/ml protein in 50 mM Na-PO_4, pH 7.0 buffer was performed with a Nano Differential Scanning Calorimetry (Calorimetry Science Corp. USA) as a heating rate of 1°C/min.

Characterization of sufhydryl group The thiolate ion has a higher value of molar extinction coefficient at 240 nm (4,000 $M^{-1}cm^{-1}$) than the un-ionized thiol group (Graminski *et al.*, 1989). The adsorption value at 240 nm of the purified protein was monitored at different pH values, and the number of free sulfhydryl group of the protein was calculated from the difference of absorption values of protonated form (pH 7.0) and deprotonated form (pH 10).

Assays A gel staining method was used to measure protease activity. Proteins were separated on 0.1% gelatin containing gel and the stained as described by Kleiner and Stetler-Stevenson (1994). Proteins were assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

Expression of various forms of the *A. pyrophilus* protease Although the cloned protease gene had an ORF of 619 amino acids, the size of the mature form was observed as 43 kDa. This indicating a post-translational processing from a precursor form. In order to characterize the possible processed regions, various forms of the protease gene were constructed. Three distinct groups of mutants were generated. First, deletion mutants of the N-terminal regions were prepared. The N-terminal region of 50, 101 and 120 amino acids were deleted to prepare ΔN1, ΔN2 and ΔN3 mutants, respectively. Secondly, the C-terminal 67 residue was deleted from ΔN2 to prepare ΔN2-ΔC. Lastly, inactive forms of proteases, ΔN2(S502A) and ΔN2-ΔC(S502A), were constructed by introducing alanine substitution at the 502th active site serine residue in ΔN2 and ΔN2-ΔC, respectively (Fig. 1). The correctness of the designed mutation was confirmed by sequencing of the constructed expression vectors.

Characterization of protease constructs Previously, the sequence analysis of the protease gene showed signal sequence at the N-terminus and a hydrophobic region at the C-terminus. Since the size of mature form, 43 kDa, is much smaller than the expected size of the ORF, both regions may be processed. To examine the role of these regions in activity and localization, the prepared mutants were expressed in the *E. coli* strain BL21(DE3), and the level of expression, localization and activity was examined. When the whole ORF

Name of Construct	Schematic Map	Activity in Gelatin Gel	Over-Expression	Location
WT	1 — S502 — 619	+	−	pellet
ΔN1	51 — S502 — 619	+	−	pellet
ΔN2	102 — S502 — 619	+	−	pellet
ΔN3	121 — S502 — 619	+	−	pellet
ΔN2-ΔC	102 — S502 — 553	+	−	soluble
ΔN2-(S502A)	102 — A502 — 619	−	−	pellet
ΔN2-ΔC(S502A)	102 — A502 — 553	−	+	soluble

Fig. 1. Schematic representation of the constructs of *Aquifex pyrophilus* protease. The pro-sequence at the N-terminus was indicated as gray box, and the hydrophobic region at the C-terminus was represented as black box. The active site serine at 502th residue was indicated as a bar. The activity of the constructs expressed in *E. coli* was measured by gelatin staining assy. The expression level of ΔN2-ΔC(S502A) construct was higher than 5% of total protein, however, all other construct were failed to be expressed as a distinct protein bands in coomasie stained gel. The crude extracts of *E. coli* containing the expression vectors were centrifuged at 100,000 × g for 40 min, and the protease activities of the soluble or pellet fraction were examined using gelatin-gel staining method.

of the protease gene (WT) was expressed, its was recovered at the insoluble fraction. When the proteolytic activity of the insoluble fraction was assayed in gelatin-containing gel, the active protease bands were observed as an intact as well as processed form. Although the expressed protease band was detected in western analysis, it could not be distinguished from background protein bands in coomasie stained gel (data not shown). The three N-terminus region-deleted mutants behaved similarly to WT clone. Their expression level was very low so that they could only be detected in western analysis. All three mutants were active in a gelatine-gel assay, and they were recovered in insoluble fraction. When the C-terminus hydrophobic region was removed (ΔN2-ΔC), the mutant protein was recovered at soluble fraction with a low level of expression. These results indicated that the N-terminal region affected neither the proteolytic activity nor the solubility of the expressed protein; however, the hydrophobic region at the C-terminus of the protein was responsible for the localization at the pellet or membrane fraction.

The proteolytic activity of the transformed protease gene may cause a toxic effect on the expression host. In fact, the *E. coli* cells transformed with the cloned protease gene were grown very poorly, and the cells were lysed in some cases. To examine the effect of proteolysis activity on the expression level, inactive mutants that had alanine substitution at the active site serine, ΔN2(S502A) and ΔN2-ΔC(S502A), were transformed. The two mutants had no protease activity as expected; however, they showed different expression level as well as localization. The mutant with an intact C-terminus located at the insoluble fraction, and its expression level is very low like WT clone. However, the protein was expressed with a high level in the cytosolic fraction when the hydrophobic C-terminus region was removed. These results indicated that the activity of the expressed protein had a toxic effect on the host cell. In addition, the hydrophobic C-terminus region was responsible for the solubility as well as the low level-expression of protease.

Purification of inactive form of *A. pyrophilus* The ΔN2-ΔC(S502A) clone showed high-level expression and covered

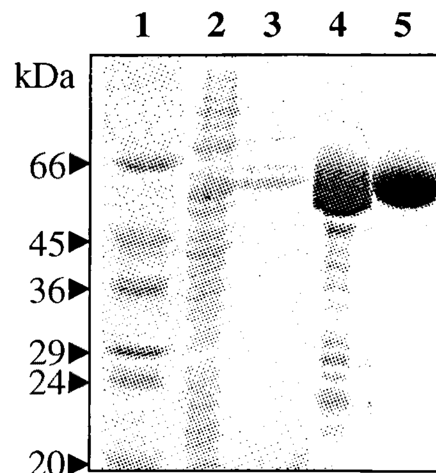

Fig. 2. SDS-PAGE of the expressed protein [ΔN2-ΔC(S502A)] at different stage of purification. Lane 1, Molecular weight maker proteins; lane 2, crude extract of *E. coli* after induction with 1 mM of IPTG for 3 h; lane 3, soluble fraction of crude extract after heat-treatment at 70°C for 45 min; lane 4, purified protein after Q-sepharose anion exchange chromatography; lane 5, purified protein after mono-Q anion exchange chromatography (FPLC).

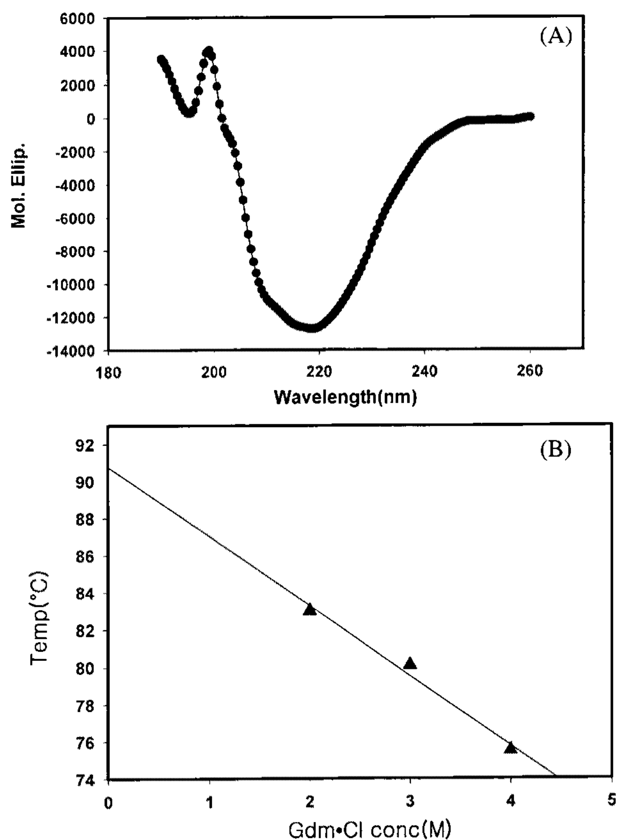

Fig. 3. CD analysis of the thermostability of the purified protein. (A) The CD spectrum of the purified protein, [ΔN2-ΔC(S502A)], was measured using a Jasco 7000 spectrophotometer in 10 mM Tris-HCl, pH 8.0. (B) Linear extrapolation to estimate the thermostability of expressed protease. The temperature dependent denaturation of the protein was measured by monitoring the ellipticity at 220 nm on CD spectrometry of protein sample in different concentration of guanidium hydrochloride.

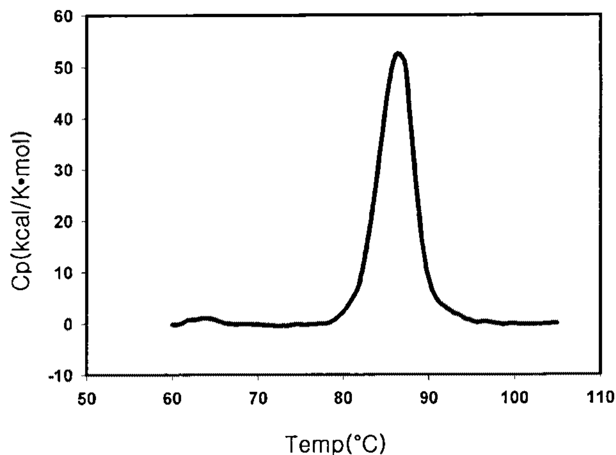

Fig. 4. Calorimetric recording of the purified protein, [ΔN2-ΔC(S502A)]. The purified protein was heated with a rate of 1°C/min in 10 mM Tris-HCl buffer, and the heat capacity of the sample was plotted against temperature.

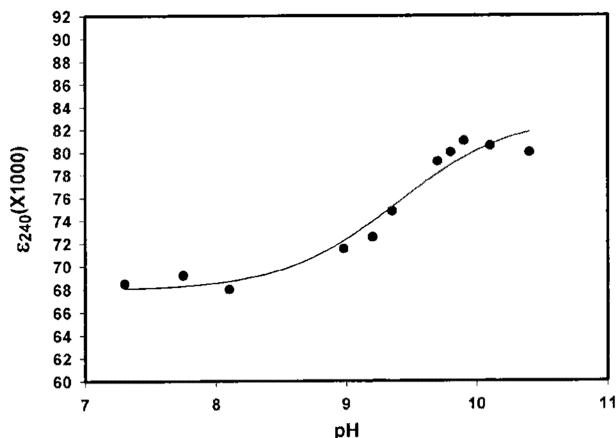

Fig. 5. Titration curve of thiol groups of the purified protein, [ΔN2-ΔC(S502A)]. The absorption value of the purified protein at 240 nm was measured in different pH conditions. The molar extinction coefficients calculated from the absorption values were plotted against corresponding pH values.

the minimal region of the cloned gene for proteolytic activity. Hence, the mutant protein was further purified to characterize the physical stability of the *A. pyrophilus* protease. The ΔN2-ΔC(S502A) clone was expressed in the *E. coli* strain BL21(DE3) using a pET expression system. A 50 kDa protein was expressed in a soluble fraction after 3 h of induction with 1 mM IPTG. The expression level of the mutant protein was about 5-10% of total cellular protein (Fig. 2, lane 2). The expressed protein remained in soluble fraction against heat treatment for 45 min at 70 (Fig. 2, lane 3). The protein was further purified to more than 95% purity by Q-sepharose anion exchange column and gel filtration chromatography (Fig. 2, lane 4, 5).

Thermostability of protease The thermostability of the purified mutant protein of *A. pyrophilus* protease was examined by measuring the melting temperature using CD spectrometer and DSC. The CD spectrum of the protein had a minimum at 220 nm (Fig. 3a). The temperature-dependent denaturation of the protein was measured by monitoring the ellipticity at 220 nm of the protein sample in different concentration of guanidium hydrochloride. The melting temperature was calculated from the convection point of temperature dependent ellipticity at 220 nm and plotted against the concentration of guanidium hydrochloride. The melting temperature of the protein was obtained as 90°C from extrapolating to 0 M guanidium hydrochloride (Fig. 3b). The melting temperature was also obtained by using DSC. The graph of the heat capacity of the protein showed a peak maximum at 87°C (Fig. 4). The values of melting temperature obtained from two different methods were in good agreement indicating that the mutant protein had a stable conformation like other proteins from *A. pyrophilus*.

Characterization of sulfhydryl group Sequence analysis indicated that the cloned protease had 9 cystein residues. The presence of multiple cystein residues was unusual among serine-type protease. Only a few serine-type proteases, such as aqualysin, have cystein residues that could form disulfide bonds (Kwon *et al.*, 1988). To examine whether the cystein residues formed disulfide bonds, the number of free cystein was measured by monitoring the extinction coefficient at 240 nm (ε_{240nm}) during pH titration. The titration of the-SH group took place between pH 9-10, and the number of free cystein was calculated as 3 by the difference in the E_{240nm} value of the protonated and deprotonated sulfhydryl group (Fig. 5). When the protein is incubated with 5 mM of dithiothreitol for 1 h, the number of free -SH group was not changed (data not shown). These results indicated that 6 out of 9 cysteins formed disulfide bonds in the expressed protein, and the disulfide bonds were not exposed to the surface and hardly accessible to reducing agent.

Discussion

Factors affecting high-level expression of the cloned *Aquifex* protease Protease is one of the most important industrial enzymes. For characterization of enzymatic properties and industrial application, mass-production of the protease is required. To set up an efficient expression system, the factors that affect the expression level should be elucidated. In this study we identified several factors that contribute to the low level expression of protease. First, the hydrophobic region at the C-terminus decreased the expression level. Although this region assumed to be processed in *Aquifex pyrophilus*, it served as a membrane-anchoring signal when the protein is expressed in foreign host such as *E. coli*. Since the high level expression of the hydrophobic sequence may perturb the integrity of the membrane structure or electrochemical potential, it decreased the viability of host cells. As a result, the protease containing the C-terminus hydrophobic region could not be expressed in a high level. The other factor preventing high-level expression was the intrinsic proteolytic activity. Hence, for mass-production of a catalytically active form, a deletion of the C-terminus region with a proper secretion signal would be required.

Stability of the protease The stability of the cloned protease was examined in a crude extract of *A. pyrophilus*. The mature form of protease was very stable that the half-life at 105°C was measured about 6 h (Choi *et al.*, 1999). The expressed protein representing 102th-553th residue of the cloned gene showed resistance against the heat treatment like other protein from hyperthermophiles (Choi *et al.*, 1998). However, the melting temperature of the expressed protein was measured as 90, which was lower than expected. There are several possible reasons. First, the expressed protein had differences in several positions compare to wild-type. It had an alanine substitution at 502th residue that may perturb the overall stability of the protein. In addition, the expressed protein had a few extra residues at both N- and C-terminus, since it was about 7 kDa larger than that of the mature protein. These extra sequences may contribute to the lowered thermostability of the protein. Another possible factor was disulfide bond. The disulfide bond(s) that contributed to the stability of the mature protease may lack in the expressed protein. Previously, the thermostability of the mature form was significantly reduced in the presence of a reducing agent, such as dithiothreitol. This suggests that reducible disulfide bond(s) may contribute to its thermostability (Choi *et al.*, 1999). In contrast, the melting temperature of the over-expressed mutant, ΔN2-ΔC(S502A), was only marginally changed in the presence of a reducing agent (data not shown). We assumed that the reducible disulfide bond that contributed thermostability of the protease was not formed in the expressed protein, and the observed melting temperature represented of the stability of the reduced form. The cystein residues would form disulfide bond in the mature form and affect the stability of protein. In many case, the disulfide bonds stabilize the conformation of proteins (Arai *et al.*, 2000; Sreekrishna *et al.*, 2000). A detailed 3-dimensional structure of the protein would be required for elucidation of the molecular nature of the extreme thermostability of the protease.

Acknowledgments We thank Dr. Duck-Yeon Lee for his advise in operation of differential scanning calorimetry. This work was supported by the grants from the Ministry of Science and Technology, Korea.

References

Arai, M., Hamel, P., Kanaya, E., Inaka, K., Miki, K., Kikuchi, M. and Kuwajima, K. (2000) Effect of an alternative disulfide bond on the structure, stability, and folding of human lysozyme. *Biochemistry* **39**, 3472-3479.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Choi, I. G., Bang, W. G., Kim, S.-H. and Yu, Y. G. (1999) Extremly thermostable serine-type protease from *Aquifex pyrophilus*. *J. Biol. Chem.* **274**, 881-888

Choi, I. G., Cho, C. S., Cho, Y. and Yu, Y. G. (1998) Overexpression, purification, and characterization of heat stable aldolase from *Methanococcus jannaschii*, a hyperthermophilic archaea. *J. Biochem. Mol. Biochem.* **31**, 130-134.

Choi, I. G., Kim, S. S., Ryu, J. R., Han, Y. S., Bang, W. G., Kim, S. H. and Yu, Y. G. (1997) Random sequence analysis of genomic DNA of hyperthermophile: *Aquifex pyrophilus*. *Extremophiles* **1**, 125-134.

Dyson, H. J., Jeng, M. F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D. S., Kuprin, S. and Holmgren, A. (1997) Effects of buried charged groups on cysteine thiol ionization and reactivity in *Escherichia coli* thioredoxin: structural and

functional characterization of mutants of Asp 26 and Lys 57. *Biochemistry* **36**, 2622-2636.

Graminski, G. F., Kubo, Y. and Armstrong, R. N. (1989) Spectroscophic and kinetic evidence for the thiolate anion of glutathion at the active site of glutathion S-transferase. *Biochemistry* **28**, 3562-3568.

Haney, P. J., Stees, M. and Konisky, J. (1999) Analysis of thermal stabilizing interactions in mesophilic and thermophilic adenylate kinases from the genus *Methanococcus*. *J. Biol. Chem.* **274**, 28453-28458.

Huber, R., William, T., Huber, D., Trincore, A., Burggraf, S., Konig, H., Rachel, R., Rockinger, I., Fricke, H. and Stetter, K. O. (1992) *Aquifex pyrophilus*, gen. Nov. sp. Nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria, *Syst. Appl. Microbiol.* **15**, 340-351.

Kelly, S. M. and Price, N. C. (1997) The application of circular dichroism to studies of protein folding and unfolding. *Biochim. Biophys. Acta.* **1338**, 161-185.

Kim, S. S. and Yu, Y. G. (2000) Molecular cloning of an extremely thermostable alanine racemase from *Aquifex pyrophilus* and enzymatic characterization of the expressed protein. *J. Biochem. Mol. Biol.* **33**, 82-88.

Kleiner, D. E. and Stetler-Stevenson, W. G. (1994) Quantitative zymography: detection picogram quantities of gelatinases. *Anal. Biochem.* **218**, 325-329.

Lee, D. Y., Ahn, B. Y. and Kim, K. S. (2000) A thioredoxin from the hyperthermophilic archaeon Methanococcus jannaschii has a glutaredoxin-like fold but thioredoxin-like activities. *Biochemistry* **39**, 6652-6659.

Nayak, S., Rathore, D., and Batra, J. K.(1999) Role of individual cysteine residues and disulfide bonds in the structure and function of *Aspergillus ribonucleolytic* toxin restrictocin. *Biochemistry* **38**, 10052-10058.

Kwon, S.-T., Matsuzawa, H. and Ohta, T. (1988) Determination of the positions of the disulfide bonds in aqualysin I (a thermophilic alkaline serine protease) of *Thermus aquaticus* YT-1. *J. Biochem.* **104**, 557-559.

Sreekrishna, K., Nells, L., Potenz, R., Cruze, J., Mazzaferro, P., Fish, W., Fuke, M., Wedemeyer, W., Welker, E., Narayan, M. and Scheraga, H. A. (2000) Disulfide bonds and protein folding. *Biochemistry* **39**, 4207-4216.