[BIO36] Structural conformation of *Bacillus stearothermophilus* F1 protease and effect of modification on its thermostability

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Introduction

The extracellular F1 serine protease, produced by a thermophilic *Bacillus stearothermophilus* F1, has been isolated and characterized as one of a serine protease. F1 protease was stable in the pH range of 8.0 to 10.0, with an optimum activity at pH 9.0. The enzyme was stable for 24h at 70°C (Rahman *et al.*, 1994).

The family of serine proteases has been extensively studied from psychrophilic, mesophilic, thermophilic and hyperthermophilic origin to gain insight in thermostability (Wintrode *et al.*, 2001; Almog *et al.*, 2002; Smith *et al.*, 1998; Voorhorst *et al.*, 1997). Thus an improvement of thermostability is required for the industrial application of the enzyme. In this paper we reported the enhancement of thermostability of F1 protease by replacement of trytophan residue with arginine, creating addition ion pairs.

Materials and Methods

Model Building

F1 protease sequence was aligned with thermitase from *Thermoactinomyces vulgaris* (1THM) using the Homology module of InsightII (MSI, CA). This target-template alignment was logon to a server running Modeller (Sali and Blundell, 1993). An initial model was built using Modeller for automated homology modeling. The generated results were in PDB format. The initial model of F1 protease was loaded into InsightII module (MSI, CA) for visualization.

Molecular dynamics simulation

All calculation and graphical analyses were run on a Silicon Graphics Indigo2 workstation. The InsightII/Discover package (MSI/CA) was used to perfom energy minimization and molecular dynamics simulation using CVFF forcefield. The molecular dynamics simulation mutant (W200R) was carried out in vacuo.

Construction of Mutated F1 Protease gene

The amplification and transformation was performed according to GeneTailor Site-Directed Mutagenesis System, instruction manual (Invitrogen, USA). Trypthopan 200 was replaced with arginine residue, this substitution was introduced in a single mutant on F1 protease gene.

Expression and purification of mutated F1 protease

Overnight recombinant cell culture was inoculated into 100ml LB medium containing ampicillin (40 μg/ml) and incubated at 37°C, 200 rpm until an OD_{660} of 0.5. IPTG (40 ug/ml) was used to induce the expression of the recombinant protein. The cell culture was harvested after 24h of induction time. The crude enzyme medium was put into a hot waterbath at 70°C for 3h and centrifuged at 10,000xg for 10 minutes to remove the denaturant proteins.

Assay of the Protease Activity

Protease activity was determined by a method of Rahman *et al.* (1994). The absorbance was read at 450 nm using a spectrophotometer (Pharmacia).

Thermostability test

For the thermostability study, the F1 protease and mutated F1 protease were pre-incubated at 85°C at different times. The enzymes then were taken out and immediately frozen, prior to being assayed.

Results and Discussion

Model building

F1 protease gene showed a few sequences identities with other serine protease gene with thermitase from *Thermoactinomyces vulgaris*

(PDB code: 1THM) as a best template for F1 protease sequence. The optimal alignment of the F1 protease sequence (target) to the sequence of thermitase (template) showed 61% sequence identity. The initial structure of F1 protease was refined and the final structure comprises 9 β strands and 6 α helices arranged in a singles domain. As in all serine proteases, F1 protease contains a catalytic triad, comprising Asp 39, His 72 and Ser 226.

Choice of mutation

The three dimensional structure of F1 protease was successfully predicted. The structure comparison of serine protease from subtilisin BPN', F1 protease, stetterlysin and pyrolysin had revealed a tremendous increase of the ion pairs content in hyperthermostable stetterlysin and pyrolysin (Voorhorst *et al*., 1997). In stetterlysin, its largest network composed from four amino acid residues (Figure 1A). However there were only three amino acid residues involved the largest network on F1 protease structure (Figure 1B). These amino acid residues forming the ion pair networks of both F1 protease and stetterlysin were at a conserved region. In a previous homology modelling study of the conformational and structural behavior of the *Pyrococcus furious* pyrolysin and *Thermococcus stetter*i stetterlysin using computational technique (Voorhorst *et al*., 1997), certain features concerning the molecular basis of thermostability for subtilisin like-serine

protease were noted. One of the most important factors leading to increased thermostability of pyrolysin and stetterlysin were the formation of networks ion pair (salt bridges) between the oppositely charged residues. In order to know the effect of intersubunit ion pairs on the structural stability, mutant was designed in which Trp200 was replaced by arginine (W200R), as present in stetterlysin. The analysis of molecular dynamic simulations of mutated F1 protease revealed that an additional three new ion pairs between Arg200 and Asp202 (Figure 1C) compared to wild type.

PCR Amplification of the Mutated Protease gene

Site-directed mutagenesis has become one of the most commonly methods used in molecular biology. A mutant was designed in which Trp200 was replaced by arginine. The mutagenic primer and reverse primer were design based on the wild type sequences. Both primers were used to amplify a new mutant with possible additional ion pairs. The method of site-directed mutagenesis by PCR was performed by single step reaction with two overlapping primers, one of which contains the target mutation. The mutated F1 protease was successfully amplified from the recombinant wild type F1 protease gene and designated as W200R. The purified plasmid was sent for automated sequencing to confirm the mutated site (data not shown).

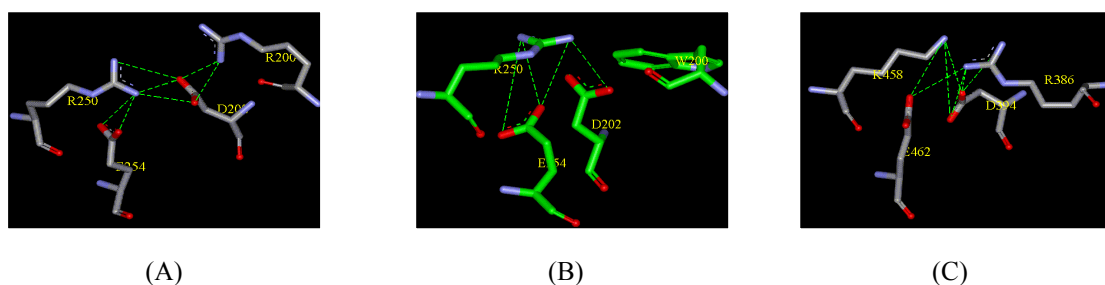| (A) | (B) | (C) |

FIGURE 1 Ion pairs network (A) the largest ion pairs network in stetterlysin (B) structure wild type of F1 protease structure (C) mutated F1 protease structure after dynamics simulation.

Expression of the F1 protease gene in E.coli

Recombinant mutated F1 protease was expressed in *E.coli* DH5α and the proteolytic activity was screened on LB-ampicillin containing 2% skim milk by its ability to form a halo zone (data not shown). Expression of the both recombinant wild type and mutated F1 protease (W200R) was shown in Table 1. Both enzymes were partially purified by heat treatment due to precipitate the mesophilic *E.coli* proteins (Schalk *et al.*, 1992).

TABLE 1 Intracellular F1 protease activity with E.coli DH5α after induction of 24h by IPTG.

Expression vector	Protease activity (U/ml)
pTrcHis/ lac Z (negative control)	0.00
pTrcHis/ wild type (positive control)	18.49
pTrcHis/ W200R	18.89

Thermostability test

The thermostability of the mutated F1 protease was examined by measuring the protease activity at different times of incubation at 85°C.

The half-life of the W200R was 10 minutes longer than that the wild type enzyme at 85°C. The results showed that the recombinant mutated F1 protease (W200R) is considerably more stable than the recombinant wild type F1 protease. We substituted the tryptophan to arginine at position 200, which seem to be involved in stabilizing hydrogen bond and ion pairs.

Acknowledgement

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