Full Paper

# EFFECTS OF HEAT SHOCK PROTEIN CLPC'S α4-β2 LOOP DELETION FROM AN ALKALIPHILIC *BACILLUS LEHENSIS* G1 ON ITS STABILITY AND ACTIVITY

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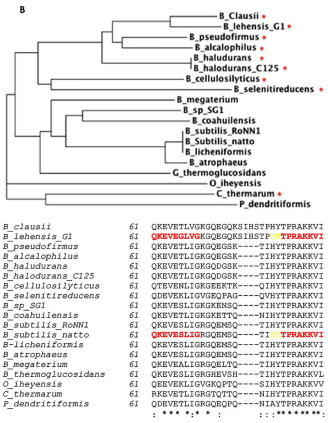
## Graphical abstract




## Abstract

Protein loops are frequently considered as critical determinants that influence not only the function but also the structure of a protein. *Bacillus lehensis* G1 ClpC (WT) has a four-residue insertion at the α4-β2 loop, which is absent in *Bacillus subtillis* ClpC. To foster a deep understanding of the significance of additional residues in the structure and function of ClpC, a deletion mutation involving residues 76-79 (Δ76-79) was constructed. Circular dichroism spectroscopy was used to evaluate the structural perturbations associated with the deletion. The results demonstrated that, the precise configuration of the α4-β2 loop is important for maintaining the structure and function of WT. Δ76-79 leads to severe global destabilisation and unfolding of the secondary structure of the protein, which decreases ATPase activity. The optimum temperature for Δ76-79 is 25 °C, down from 45 °C for WT. The results suggest that the additional four residues at the α4-β2 loop are critical for WT's structure and function.

*Keywords*: Alkaliphilic ClpC, N-terminal loop, deletion, secondary structure, ATPase activity

## Abstrak

Gelung protein sering dianggap sebagai penentu kritikal yang mempengaruhi bukan sahaja fungsi, tetapi juga struktur protein. *Bacillus lehensis* ClpC (WT) mempunyai 4 sisipan residu dalam gelung α4-β2, yang tidak ada dalam *Bacillus subtilis* ClpC. Untuk memupuk kefahaman mendalam berkenaan kepentingan residu tambahan dalam struktur dan fungsi ClpC, mutasi delesi yang melibatkan residu 76-79 (Δ76-79) dijalankan. Spektroskopi edaran dikroisme digunakan untuk menilai gangguan struktur yang berkaitan dengan penghapusan residu. Hasil kajian menunjukkan bahawa konfigurasi gelung α4-β2 yang tepat adalah penting untuk struktur dan fungsi WT. Δ76-79 menyebabkan ketidakstabilan global yang kritikal dan pembukaan lipatan struktur sekunder protein, yang mengurangkan aktiviti ATPase. Walau bagaimanapun, suhu optimum Δ76-79 dikurangkan kepada 25 °C, berbanding dengan ClpC jenis liar, iaitu pada 45 °C. Keputusan mencadangkan bahawa empat residu tambahan dalam gelung α4-β2 adalah berperanan penting didalam menentukan struktur dan fungsi WT.

*kata kunci*: Alkaliphilik ClpC, gegelung terminal-N, delesi, lipatan struktur sekunder, aktiviti ATPase

## 1.0 INTRODUCTION

Being evolutionarily ancient, heat shock proteins (Hsps) are highly conserved in the species from which they originate [1]. Classified according to molecular weight, the examples of Hsps are Hsp10, Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, Hsp110, as well as other small hsp families [1]. Although Hsp groups are highly conserved, they are also associated with varying amino acid sequences, even in proteins which have conserved architecture and are homologues [2]. For instance, the ClpC of Hsp100 requires an adaptor protein which plays a crucial role in general chaperone and degradation activities as well as substrate binding of the ClpCP protease, which includes ClpC hexamerisation, a distinctive and remarkable feature among Hsp100 family proteins [3].

Investigations which are aimed to identify the possible roles of protein loops have provided evidence that such loops are important structural elements for protein function and stability [4–6]. For long, it has been recognised that loops in proteins serve as sites at which active-folding is initiated. Hence, they dominate the folding process of the overall structure by dictating folding reversibility [7]. In terms of functional role, surfaced-exposed loops can potentially interact with ligands, solvent and other biomolecules [4]. The ClpC molecule is a 91 kDa protein consisting of five separate domains: a small N-terminal protein interaction domain, an $AAA^+$ ATPase domain, a coiled-coil M-domain, a second $AAA^+$ ATPase domain, and a C-terminal domain that couples with ClpP. Differing loop regions in the N-terminal domains may result in differences in the binding of substrates and adaptor proteins among Hsp100 proteins [8]. For example, the N-terminal domain of ClpC in *Bacillus subtilis* (*Bs*-ClpCN) binds to the MecA adaptor protein by utilising conserved interface, at the crevice on the surface of the flexible loop region [8]. With regards to the domain structure and sequence of the *Bs*-ClpCN, the N-ClpC hexameric model suggests that the positively charged regions and flexible loop/hydrophobic, as well as disordered residues can be attributed to the binding of adaptor proteins [8]. However, loop regions at the surface of the protein are sometimes regarded as less crucial for protein stability relative to the hydrophobic core and secondary structure. Nevertheless, the hydrophobic patches and irregular hydrogen bonds in these regions have an essential role in protein stability and folding [9–11]. Therefore, the exploration of the evolutionary and functional roles of these loops helps in the understanding of the roles of protein diversity and variability in the process of acquiring new or different functions to support diversification, in different families of the similar superfamily.

So far, most of the best-characterised alkaliphilic species are belong to the genus *Bacillus*. Due to an abundance of well-studied neutralophilic *Bacillus* species such as *Bacillus megaterium*, *B. subtilis* and *Bacillus cereus*, these *bacteria* are a practical tool in researches on alkali adaption. In fact, genomic sequence data is available for all alkaliphilic [12, 13] and neutralophilic species [14], so comparative genomics can be done, to scrutinise the way by which some bacteria in the branches of the *Bacillus* evolutionary tree adapt with alkaliphilic characteristics [12]. Based on the unique insertion at the flexible loop of the N-terminal domain of *B. lehensis* ClpC (*Bl*-ClpCN), there is a strong probability that this loop of alkaliphilic ClpC has some unique functions that are relevant to the Hsp100 family. However, a question that remains to be answered is whether these additional residues influence the folding, structure and function of *B. lehensis* ClpC (WT).

In this study, the role of the α4-β2 loop in retaining WT structure and function was scrutinised by making loop deletion. Further insight was obtained by performing physicochemical characterisations of the mutant. It was found that the deletion of a portion of the flexible loop resulted in severe and glaring consequences in folding and activity of the said protein.

## 2.0 METHODOLOGY

### 2.1 Cloning and Deletion Mutant

The deletion mutant (*Δ76-79*) of ClpC was generated via overlapping-extension (OE) polymerase chain reaction (PCR) amplification and was cloned into pET28b (+) (Novagen). Forward and reverse PCR fragment were generated using primers P1 5'-GCCATATGATGATGTTTGGAAGATTTTCTGAGCTG-3' and P2 5'- GTGTGGCGTGCTTTGTCCTTCTTGACC-3', as well as P3 5'-GGTCAAGAAGGACAAAGCACGCCACAC-3' and P4 5'- GCGAGCTCAATTACGCTTGTTTCTTCTTTTGC-3', respectively. pDEST-17 WT plasmid was used as the template [15]. Final full length mutant ClpC gene was generated using outer primer P1 and P4, and forward and reverse PCR fragments. The PCR product was digested using restriction enzymes *Nde*I/*Sac*I and cloned in pET28, resulting in the plasmid pET28-ClpC. This enabled the production of modified ClpC which has a *Nde*I recognition site at the 5' end, a *Sac*I recognition site at the 3' end and a DNA fragment deletion at the target location. The deletion was verified by DNA sequencing.

### 2.2 Protein Expression and Purification

*Escherichia coli* (*E. coli*) BL21 (DE3) was transformed using pET-28b(+) plasmids with *Δ76-79*. The proteins were expressed and purified as follows: 1L of Luria-Bertani medium supplemented with kanamycin (34 µg/mL) was inoculated with 10 mL of overnight bacterial culture, and then cultured at 37 °C and 220 rpm, until the optical density (OD at 600 nm) was 0.5 - 0.7. The induction of *Δ76-79* expression was initiated

by adding 0.5M isopropyl-beta-D-thiogalactopyranoside (IPTG). The culture was then grown for 12 hr at 18 °C, and the cells were harvested by centrifugation at 8,000 x g for 20 min. The cell pellets were resuspended in 50 mL of lysis buffer [10 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 10% glycerol, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 20 mM imidazole] and gently mixed for 1 hr at room temperature. The lysates were immersed in ice and dispersed by sonication (3 x 40 s bursts at 40% amplitude). The lysates which contained soluble proteins, were separated from the pellet by centrifugation at 10,000 x g and 4 °C for 30 min. They were then placed in the Nickel-Sepharose High Performance (Amersham Bioscience) for affinity chromatography, before being washed with a five-column volume of buffer A [10 mM Tris-HCl (pH 8.0), and 150 mM sodium chloride] with the addition of 30 mM imidazole. The soluble and correctly folded recombinant $His_6$-tagged proteins were then eluted in buffer A with the addition of 350 mM imidazole, and detected by SDS-PAGE. The soluble protein was further purified by size-exclusion chromatography in a calibrated 16/60 Superdex 200 prep-grade column (Amersham Pharmacia, Uppsala, Sweden) in buffer B [10 mM Tris-HCl (pH 8.0), 10% glycerol and 150 mM sodium chloride]. The highly purified mutant protein was concentrated to 5 mg/mL in buffer B, by using a Centricon device (Millipore). The protein concentration was determined spectrophotometrically (Nanodrop 1000; Thermo Scientific) at 280 nm and calculated at an extinction coefficient of 36 330 $M^{-1}$ $cm^{-1}$.

### 2.3 Circular Dichroism Spectroscopy

Far-UV circular dichroism (CD) measurement was used to analyse the secondary structure of the proteins. The UV CD spectra (200–260 nm) were recorded with the JASCO J-810 CD instrument (JASCO); at a bandwidth of 0.5 nm and scan speed of 50 nm/min. The cell length was 10 mm. In all measurements, the concentration of the proteins was kept at 0.2 µmol/mL. All CD measurements were performed at 25 °C. To prepare the proteins, samples were incubated for 30 min in 10 mM sodium acetate (pH 6.0) and, 250 mM sodium chloride. For both the baseline scan (with a buffer) and protein-containing samples the three spectra were recorded and averaged. Following the subtraction of buffer spectrum from that of averaged baseline, the results are presented in millidegrees (mdeg). The sample compartment was purged with nitrogen [16].

### 2.4 Differential Scanning Colorimetry

Differential scanning calorimetry (DSC) experiments were performed using a VP-DSC instrument (Microcal; Northhampton, MA) for protein samples containing 1mg/mL protein in 10 mM sodium acetate buffer (pH 6.0) and, with 250 mM sodium chloride. The samples were vacuum-degassed for 10 min prior to the calorimetric analysis. Prior to scanning of the samples, at least three buffer–buffer scans were performed to obtain the baseline values and establish the thermal history of the instrument. Thermal scans were performed over a temperature range of 20 – 80 °C at a scan rate of 60 °C/hr. The reversibility of DSC endotherms was indirectly assessed by partial thermal scans, in which samples were scanned in DSC to approximate the ends of a given endotherm (T end) when the thermogram approaches a baseline value. The samples were immediately cooled in the DSC (nom. cooling rate ~6 °C/min) at 25 °C and rescanned to the same trend in DSC [17].

### 2.5 Measurement of ATPase Activity

The ATPase activity of the purified proteins was determined by using the ATPase assay kit (Innova Biosciences) according to the protocol provided by the manufacturer. In short, the reaction was carried out by mixing 100 µl of ATP buffer (Buffer A, 5 mM magnesium chloride, 0.5 mM ATP) and 100 µl of purified proteins (1 µM) in buffer B [10 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 10% glycerol] for 10 min at room temperature. Then 50 µl of 'stop solution' was added to stop further reactions. After 2 min, 20 µl of stabilizer solution was added to the mixture and incubated for 30 min to facilitate the development of colour. The absorbance of the mixture at 630 nm was then measured using a 96-well plate reader. To correct for the effects of phosphate (which was not produced during the hydrolysis of ATP by proteins) on the measurement (i.e. contamination of the mixture), a simultaneous reaction was set up, in which 'stop solution' was added to the purified proteins prior to the addition of ATP buffer to the mixture. In this case, the content of the mixture was the same, but proteins were not able to hydrolyse ATP since they were denatured by the 'stop solution'. The absorbance of the 'denatured' proteins in the mixture was subtracted from that of the 'intact' proteins (this should reflect the apparent absorbance of the phosphate released by the proteins), and the released phosphate was then estimated using the calibration curve generated by known concentrations of inorganic phosphate. All reactions were carried out at room temperature. We performed three independent measurements, and the averaged values with their standard deviations were reported.

### 2.6 Biochemical Characterisation

The optimum pH for ATPase activity was determined by incubating the reagents in buffers of varying pH ( 10 mM of sodium acetate for pH 3.0 - 6.0; 10 mM MOPS fpr pH 7.0; 10 mM Tris-HCl for pH 8.0 - 9.0; 10 mM glycine-sodium hydroxide for pH 10; sodium hydroxide for pH 11 -12) at room temperature. The salt tolerability was evaluated by incubating the

reagents (pH 6.0) in buffers of varying concentrations (0 - 300 mM) of sodium chloride at room temperatures. The optimum temperature for ATPase activity was determined by incubating the reagents (pH 6.0, 250 mM NaCl) at temperatures ranging from 20 °C - 80 °C. The release of free phosphates was monitored spectrophotometrically at 630 nm (UV-120-02 spectrophotometer; Shimadzu). All reactions were carried out in triplicates. The standard errors of the means were calculated using the Sigma plot software.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Construction and Expression of ClpC Δ76-79 Mutant

The required mutated WT gene, which carries deletion in α4-β2 loop, was generated by using the OE PCR approach. To simplify the purification procedures, the mutant coding sequence was cloned into an expression vector that encodes amino-terminal histidine extensions. The proteins were initially purified from *E. coli* using $Ni^{2+}$-NTA affinity resin, and further purified by Sephacryl S-200 gel filtration chromatography. The purified proteins were analysed on SDS-PAGE, which indicated their molecular weights, which were indistinguishable from that of the WT (Figure 1).

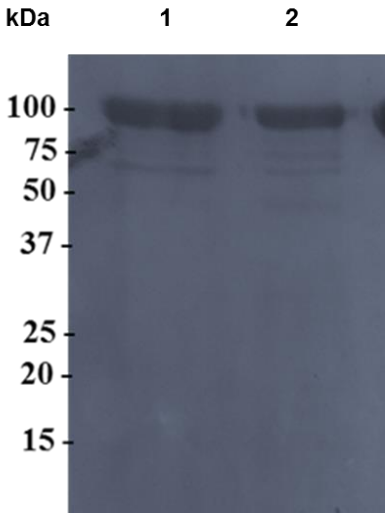



**Figure 1** SDS-PAGE analysis of purified WT and Δ76-79. The purified proteins were the eluted proteins after gel filtration chromatography. 1: WT, 2: Δ76-79. The size of WT and Δ76-79 are approximately 91 kDa

### 3.2 Analysis of the α4-β2 Surface-Exposed Loop of *Bl*-ClpCN

The structure of the *Bl*-clpCN (PDB: 4P15) was obtained, to allow the comparison of its folding and interaction interface with those of a previously solved neutralophilic ClpC. The overall structure of the *Bl*-ClpCN is an α-helical globular fold that serves as a rigid protein-protein interaction domain. The domain has seven α-helices and two β-bridges, but only the first of these is large enough to be classified as β-sheet. The structure of the solved *Bs*-ClpCN [18] and that of the *Bl*-ClpCN are similar (root mean squared deviation of 0.6Å$^2$ over 137 Cα). The *Bl*-ClpCN shares 82% of sequence identity with the model organism of *B. subtilis*. Of all the substitutions present, 83 amino acids were conservative. The theoretical pI (5.6 and 5.8) and amino acid usage of the two orthologues are similar. Despite the similarities, the largest structural difference was found in the loop connecting α-helix 4 to β-strand 2, whereby the most significant sequence change was present between *Bl*-ClpCN (PDB:4P15) and *Bs*-ClpCN[18] (PDB:2Y1Q) (Figure 2A). In the isolated N-terminal domain structures of both *Bl*- and *B. subtillis* ClpC, five residues of the α4-β2 loop (including those within *Bl* insertion) were not visible in the electron density, despite being solved at high resolution (1.85Å and 1.5Å respectively). The comparison of both protein structure and structure based-sequence alignment showed that the N-terminal ClpC-interacting and hexamerisation domain of *B. lehensis* housed a unique insertion which had four amino acids and an unknown function. This was located at the loop between helix 4 and strand 2, which were situated outside the barrel-shaped molecule. The similar four-amino acid insertion is found in the closely-related alkaliphilic *Bacillus clausii,* but not in other alkaliphilic *Bacillus* species (Figure 2B).

The *Bl*-ClpCN flexible loop surface consists of 13 amino acids which are made up of combination polar, positively-charged, and hydrophobic residues (Figure 3). The flexible loop of ClpCN in *B. subtilis* contains less positively-charged residues and is four residues shorter compared to the *Bl*-ClpCN loop. The deletion of four amino acids (Lys-76, Ser-77, Ile-78 and His-79), may result in changes in the conformational stability. In fact, the loop is surface-exposed, thus, the shortening of this loop is expected to break the structural integrity of the protein [19]. This concurs with the results of the study done by Collinet *et al*. [20] which, demonstrated that relatively small modifications (insertions/deletions) can sometimes results in large consequences on the protein structure.

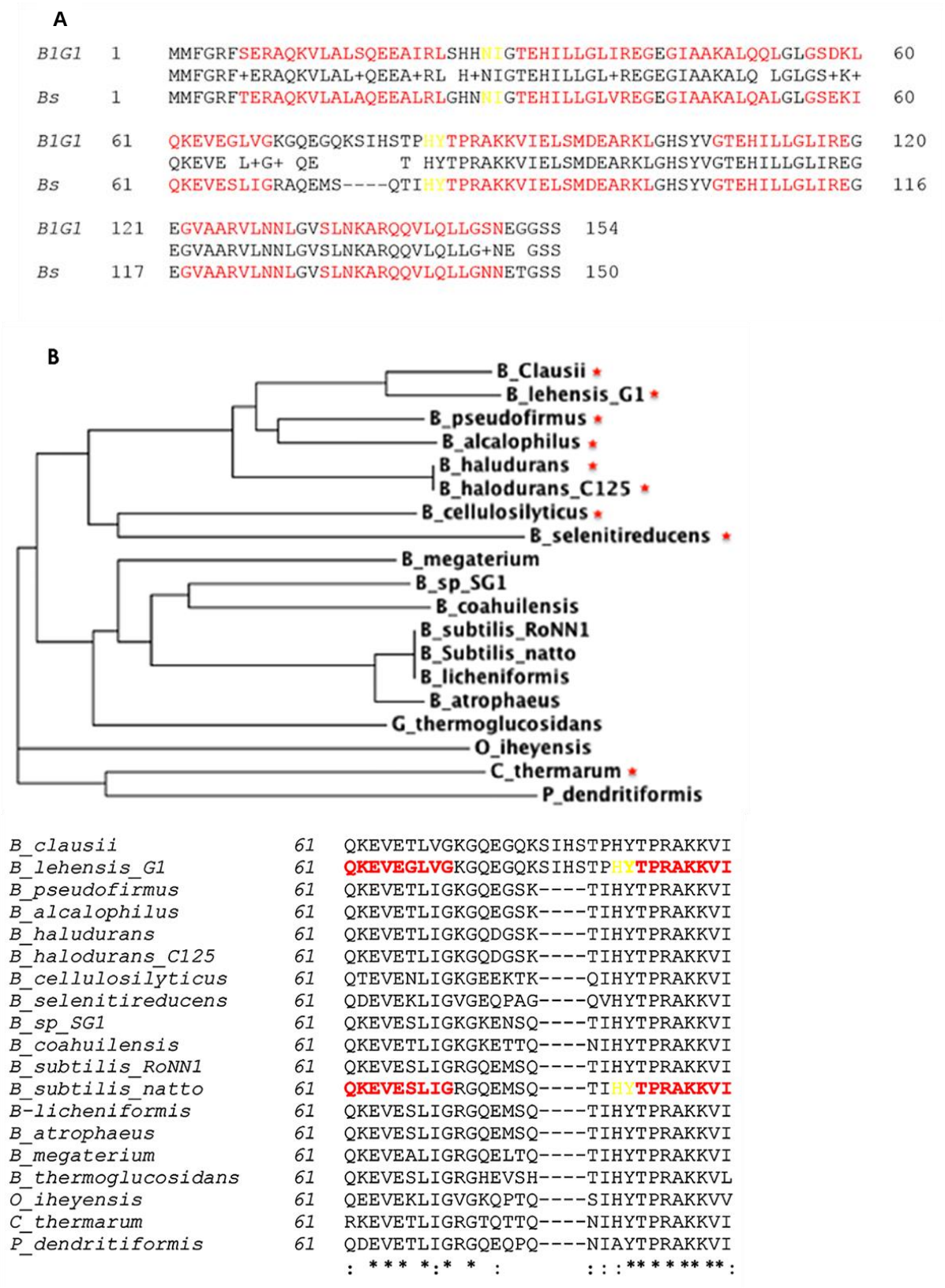



**Figure 2 A**. Structure-based sequence alignment of *B. lehensis and B. subtilis* ClpCN-terminal domains. **B.** *Top:* A ClustalW cladogram depicts the conservation of ClpC chaperone in a number of *Bacillus* species, *red stars* denote alkaliphily. *Bottom:* A sequence alignment of the divergent h4-s2 loop

70 82

Val. Gly. Lys. Gly. Gln. Glu. Gly. Gln. **Lys. Ser. Ile. His**. Ser. Thr. Pro. His. Tyr

**Figure 3** Loop residues of ClpC of *B. lehensis* G1. The 13 mobile loop residues are indicated in brackets and the four deleted residues bold

### 3.3 Effects of 76-79 Deletion on pH and Salt Tolerability

WT is shown to be active in a broad range of pH, (pH 3 – 12) and exhibits maximum activity at pH 6. The deletion of four amino acids did not change the pH profile as both WT and Δ76-79 exhibited similar optimal pH. However, there was a decrease in ATPase activity in Δ76-79 as compared to WT, which was observed during pH profiling (Figure 4A). This demonstrated that deletion of residues 76 - 79 decreased the activity of WT, and that loop length was also important to retain the ATPase activity of ClpC. Different sodium chloride concentrations were used to monitor the halotolerance of WT and Δ76-79 (Figure 4B). Sodium chloride actively increases the activities of both WT and its mutant as the ATPase activities were proportionally increased with increasing buffer ionic strength from 0 to 250 mM (Figure 4B). The Δ76-79 had the highest salt tolerability, by retaining ~ 90% of protein activity in 250 mM sodium chloridel solution as compared to WT (100%). WT and its mutant had a similar tolerability to salt owing to the fact that both had a similar number of Asp and Glu residues which resisted the action of electrolytes [21].

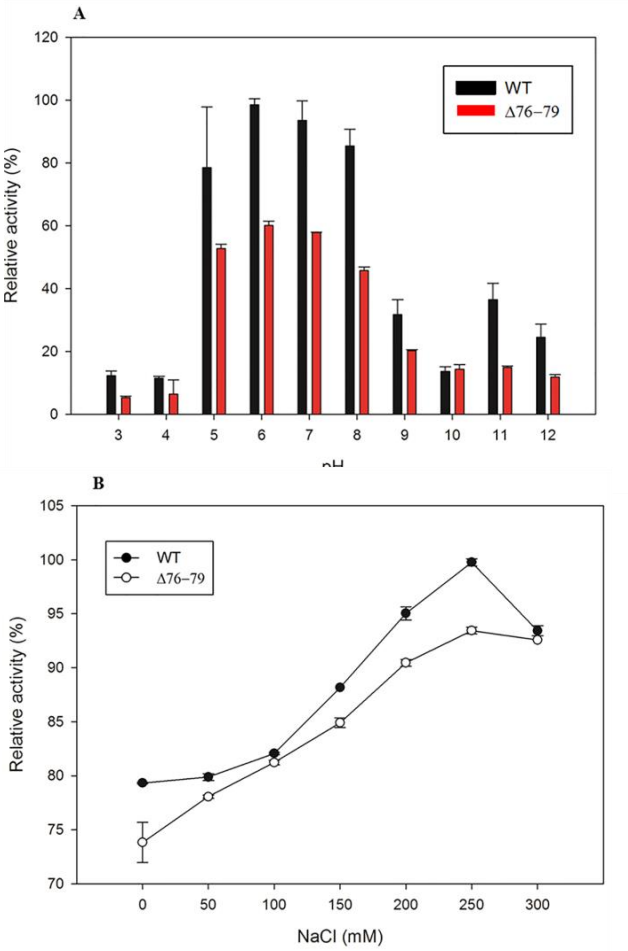



**Figure 4 A.** The ATPase activity of WT and Δ76-79, measured at various pH values. The released $P_i$ was measured by spectrophotometer at 630 nm. **B.** The effect of salt concentration on WT and Δ76-79. ATPase activity started to decrease at concentration above 250 mM. The error bars represent the standard deviations of triplicate experiments

### 3.4 Structural Disturbances Caused by Δ76-79

To investigate the significance of structural changes due to deletion of the four amino acids, CD spectral analysis was performed to study the secondary structure of Δ76-79, the results of which were compared with WT. At physiological pH (pH 6), WT showed a CD spectrum with maximal negative ellipticity at 208 and 222 nm, indicating the presence of a substantial amount of α-helical content in its structure. The CD profile clearly showed that Δ76-79 was highly destabilised and had the characteristics of highly unfolded polypeptide with a drastic decrease in its secondary structure (Figure 5). This could be due to the dominance of hydrophobic residues in protein folding [22–24]. Evidently, hydrophobic interactions are the major forces involved in the initialisation of protein folding and maintenance of the three-dimentional stability of proteins [25]. Thus, the deletion of the hydrophobic amino acids greatly affected the conformation and stability of the loop, that could alter the whole protein structure in this case the *Bl*-ClpC. Nevertheless, the presence of some degree of residual helical structure was suggested in the spectrum of the mutant at a wavelength of 222 nm. This could result from the formation of strand structure and/or transient helical structure either at different protein sites or at a small core or region. This in turn resisted the destabilising effects and retained a well-ordered structure in the said areas, when the rest of the protein was fully unfolded[16].

In general, mutations can disrupt protein function through various mechanisms. In this case, the protein conformation was locally altered; although it did not lead to complete unfolding, the dynamic and structural properties that were essential for protein function were affected. As shown in the CD spectra of Δ76-79, the degree of conformational

changes implied that the primary cause of the function loss was associated with the loss of protein's native structure. This was in line with the fact that ATPase efficiency was affected by the deletion of short amino acids. Also, the inefficiency of Δ76-79 *in vitro* demonstrated that the α2-β4 domain loop in ClpC had a great impact on the functional structure of the protein.

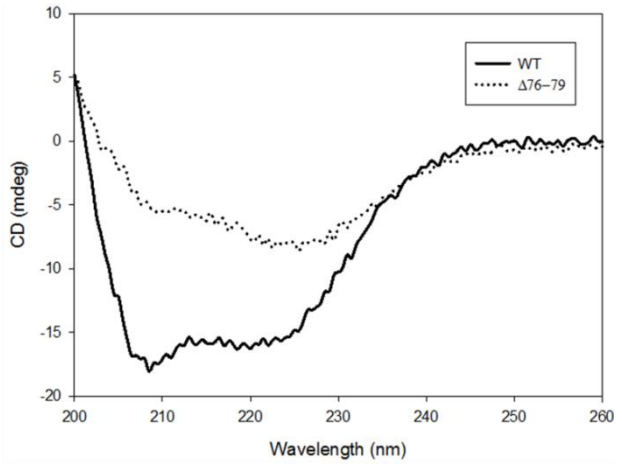



**Figure 5** structural validations of WT and Δ76-79 by CD spectroscopy

### 3.5 The Effect of Deletion on Thermostability and Activity of Δ76-79 at Various Temperatures

The effects of loop deletion are an important consideration in the process of improving protein thermostability [27]. In order to evaluate whether Δ76-79 has similar functions as WT, the effects of activity measurements and improper folding (as described by CD) on temperature sensitivity and stability of the mutant was compared to those of WT. The temperature-denaturation curve (Figure 6A) of Δ76-79 demonstrated that it was less heat-stable than the parent protein. WT had a higher optimal temperature (45 °C), whereas that of the mutant 25 °C, beyond which its activity declined gradually and stopped at 80 °C. This indicated that Δ76-79 was thermosensitive and worked better at lower temperatures (20 and 25 °C). The mutant has a relative activity of ~13 °C higher than WT. Raghunathan *et al*. [28] reported that lower temperatures tend to favour appropriate folding, so the results of this study regarding the thermosensitivity of the mutant supported the aforementioned fact. In addition, the mutant's thermostability was also analysed (Figure 6B). The deletion of four amino acid residues resulted in decreased in protein stability, whereby the melting temperature $T_m$, was 50.89 °C, a decline of ~5 °C compared to the previously characterised WT, ($T_m$ 55.39 °C) (unpublished data). It seemed that the low thermostability was attributed to the deletion of the four residues. This in turn led to the loss of the alpha-helical structure of the protein as indicated by CD spectra, which otherwise contributed to the thermophilic property of WT.

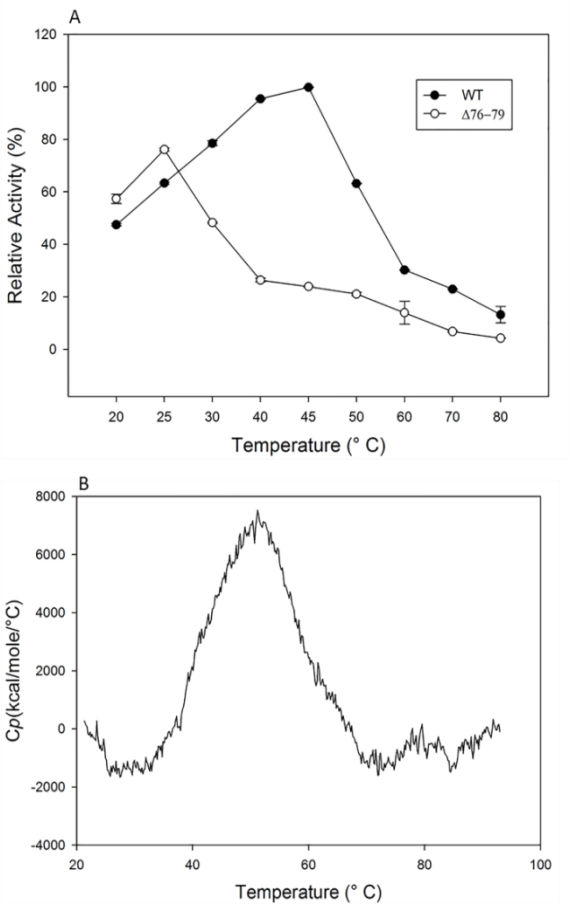



**Figure 6 A**. The effect of temperature on ATPase activity of WT and Δ76-79. **B**. Thermogram of Δ76-79 obtained at scan rate of 1.5K/min at pH 6. The DSC peak shows $T_m$ = 50.89° C

## 4.0 CONCLUSION

In conclusion, from the deletion studies, the four amino acids at the α4-β2 loop enable appropriate protein folding, and the loss of these residues significantly affects the functional properties of WT. When the structure changes, so does the protein activity. This study in fact supports the significance recognition of the loops, in which it has crucial function rather being regarded as distinct from the mere existence of mobile loop near the enzyme active sites.

## Acknowledgement

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