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# C-Terminal Flanking Peptide Stabilized the Catalytic Domain of a Recombinant *Bacillus subtilis* Endo-β-1, 4-Glucanase

**Yujuan Wang · Jun Wang**

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**Abstract** Three proteins, Egl330, Egl326 and Egl325, which covered the catalytic domain of a *Bacillus subtilis* endo-β-l, 4-glucanase were expressed in *Escherichia coli* and purified. Egl325 was a mutant of Egl330 with the peptide sequence Arg-Glu-Asn-Ile-Arg deleted in the C-terminus and Egl326 was another mutant of Egl330 with the peptide sequence Glu-Asn-Ile-Arg deleted in the C-terminus. These three proteins displayed same optimal reaction pH and temperature. However, the thermal stability and pH stability of Egl326 and Egl325 were diminished compared to Egl330. Results of ultra violet scanning, circular dichroism and Trp fluorescence spectrometry showed that the absence of the short peptide at the C-terminus of Egl330 resulted in the destabilization of the catalytic domain through affecting the folding of the protein.

**Keywords** *Bacillus subtilis* · Catalytic domain · Endo-β-l, 4-glucanase · Stability

**Abbreviations**

| | |
|---|---|
| CMC | Carboxymethylcellulose |
| PBS | Phosphate-buffered saline |
| Egl330 | A truncated mutant of endo-β-l, 4-glucanase Egl499 by deleting the cellulose binding domain |
| Egl325 | A mutant of Egl330 with the peptide sequence Arg-Glu-Asn-Ile-Arg deleted in the C-terminus |
| Egl326 | A mutant of Egl330 with the peptide sequence Glu-Asn-Ile-Arg deleted in the C-terminus |

Y. Wang
High Magnetic Field Laboratory, Chinese Academy of Science, Number 350 ShuShanHu Road, Hefei 230031, People's Republic of China

J. Wang (✉)
Key Laboratory of Ion Beam Bioengineering, Chinese Academy of Science, Number 350 ShuShanHu Road, Hefei 230031, People's Republic of China
e-mail: wangjun0457@yahoo.com.cn

## 1 Introduction

Cellulases are a family of enzymes which have received lots of attentions for their potential applications in biomass conversion and industrial uses such as brewing and paper making [1, 2]. Endo-β-l, 4-glucanase (EC 3.2.1.4), exo-β-1, 4-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) are three major types of cellulases. Some practical applications require cellulases with high stability so that the turnover of the enzymes can be reduced. Thermal resistance and pH tolerance are appreciable properties for cellulases [13]. They allow cellulases to work at high temperatures or under non-physiological pH values. Under these reaction conditions, the solubility of reactants can be improved, the risk of contamination by microorganisms can be decreased, and sometimes, the rate of enzymatic reaction can be increased. Different biochemical methods have been applied for obtaining cellulases with desired stabilities [5, 7, 11, 12, 14, 15, 19]. However, it remains poorly understood on how the stabilities of these cellulases are affected.

Typically, cellulases from bacteria and fungi consist of a cellulose-binding domain and a catalytic domain. The catalytic domain is responsible for the hydrolysis of the substrate, and the cellulose-binding domain endows the enzyme with affinity for cellulose. Usually, these two domains are separated by a long and flexible peptide sequence and they can function independently. Protein

engineering was employed to improve the stabilities of cellulases with industrial applications [15]. Meanwhile, several reports showed that removal of the peptide neighboring the catalytic domain affected the stability of cellulases to different extent. For example, cellulose-binding domain endowed a recombinant *Saccharomycopsis fibuligera* β-glucosidase with thermostable property [5]. The cellulose-binding domain deletion mutant of exocellulase I from *Irpex lacteus* showed narrower pH stability profile compared to its wild type counterpart [6]. Deletion of the N-terminal flanking peptide of the catalytic domain was detrimental to the thermal stability of a *Piromyces rhizinflata* cellulase [7]. Therefore, accumulated information on how flanking peptide affects the stability of catalytic domain will be of value for evolving of cellullases with expected properties.

Endo-β-1, 4-glucanase hydrolyses β-l, 4-glycosidic linkages randomly. Egl499 (GenBank accession number DQ116829) belongs to this type of cellulase formerly characterized from *Bacillus subtilis* [8]. The N-terminus region of the enzyme is the catalytic domain and a cellulose binding domain localized at the C-terminus. Egl330 is a truncated mutant of Egl499 with the cellulose binding domain deleted [18]. In the present work, the role of the C-terminus region of Egl330 in maintaining the stability of the catalytic domain was investigated and reported.

## 2 Materials and Methods

### 2.1 Materials

Restriction endonucleases, T4 DNA ligase and Taq DNA polymerase were purchased from Takara (Dalian). Ni–NTA column was from Novagen. Carboxymethylcellulose (CMC) was purchased from Fluka. All other chemicals were reagent grade. *Escherichia coli* strain DH5α was used for routine cloning and sequencing analysis. Plasmid pET22b(+)(Novagen) and *E. coli* strain BL21 (DE3) were used for the expression of recombinant enzymes.

### 2.2 Construction of Recombinant Expression Plasmids

DNA primers were synthesized in Shanghai Sangon Co. Ltd and their sequences were listed as below: P1(5′-AA C*CCATGG*ATAAACGGTCAATCTCT-3′), P2(5′-AAC*CT CGAG*GCGAATGTTTTCTCTTACGAA-3′), P3(5′-AAC*CT CGAG*TACGAATGTTCCTGAAGCAGT-3′) and P4(5′-AA C*CTCGAG*TCTTACGAATGTTCCTGAAGC-3′). Primers P1 and P2 were used to amplify the coding region corresponding to the amino acid sequence from Met1 to Arg330 (Egl330). Primers P1 and P3 were used to amplify the coding region corresponding to the amino acid sequence from Met1 to Val325 (Egl325). Primers P1 and P4 were used to amplify the coding region corresponding to the amino acid sequence from Met1 to Arg326 (Egl326). The amplified DNA fragments were digested with *Nco*I and *Xho*I, respectively. Then they were ligated into pET22b(+) plasmid predigested with the same restriction enzymes. Recombinant plasmids were transformed into *E. coli* BL21 (DE3) for expression of Egl330, Egl326 and Egl325.

### 2.3 Expression and Purification of Recombinant Enzymes

*Escherichia coli* strain BL21 (DE3) harboring recombinant plasmid was cultured in Luria–Bertani medium containing 50 μg/ml ampicillin at 37 °C until $O.D._{600}$ reached 0.8. Enzyme expression was induced at 30 °C with the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM for 10 h. Then BL21 cells were collected by centrifugation. The pellets were re-suspended in PBS for sonication. Lysate was centrifuged at 12,000 rpm and 4 °C for 20 min to remove the cell debris. The supernatant was then subjected to affinity chromatography on Ni–NTA column which was pre-equilibrated with PBS. A linear gradient of 5 mM imidazole in PBS to 150 mM imidazole in PBS was used to elute the protein. The active fractions were dialyzed against PBS and stored at 4 °C. The purity of protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All three proteins were purified without adding reducing agent.

### 2.4 Enzyme Assay

Endo-β-1, 4-glucanase activity was determined according to the rate of reduced sugar production from the substrate CMC. The reduced sugar was measured and quantified by 3, 5-dinitrosalicylic acid method with glucose as the standard [8]. Standard enzyme activity assay was performed in a 1.1-ml reaction mixture containing 1 ml 1 % CMC (w/v) in 0.2 M acetate buffer (pH 5.8) and 0.1 ml enzyme solution. After incubation at 60 °C for 10 min, 3, 5-dinitrosalicylic acid solution was added into the reaction mixture to terminate the reaction. Then the optical absorbance at 540 nm of the mixture was recorded. One unit of enzyme activity was defined as the amount of enzyme required for releasing 1 μmol of reduced sugar.

### 2.5 Enzyme Characterization

The protein concentration was determined by the method of Lowry et al. [9]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 10 %

polyacrylamide gels according to standard protocol and stained with Coomassie Brilliant Blue R-250. The molecular weight of denatured enzyme was estimated according to protein standards (14.4–97.6 kDa). Optimal enzymatic reaction pH was tested in citrate–phosphate buffer with pH value ranging from 4.0 to 8.0 at 60 °C. pH stability was detected by incubating the enzyme at 30 °C for 150 min in buffers with pH values from 3.0 to 13.0. Optimal enzymatic reaction temperature was determined at temperatures from 55 to 85 °C in citrate–phosphate buffer with pH 6.0. Thermal stability was tested by incubating enzyme at 65 °C and in citrate–phosphate buffer with pH 6.0, then the residual activity was measured at different time intervals. Activity recovery experiment was carried out as follows: enzyme was treated at 80 °C for 10 min, then cooled to and incubated at 20 °C for 10 h. Recovered activity was measured according to standard enzyme activity assay at different time intervals. All the experiments were performed in triplicate.

### 2.6 Ultra Violet and Circular Dichroism Spectrometry

The ultra violet absorption spectrum was recorded from 190 to 350 nm at 25 °C. Protein concentration used in this experiment was 0.04 mg/ml in 0.1 M PBS (pH 7.4). Circular dichroism spectrum was detected on a JASCO J-810 spectrophotometer (Japan) over the wavelength range of 190–250 nm with a cell path-length of 0.1 cm and bandwidth of 1 nm at 25 °C. The concentration of the tested enzymes was 0.15 mg/ml. The compositions of secondary structures were calculated using k2d and CDPro programs. Mean values were shown in Table 1.

### 2.7 Fluorescence Analysis of Protein Unfolding Induced by Guanidine Hydrochloride

The intrinsic fluorescence emission spectrum was collected on a Hitachi F-7000 FL Spectrophotometer (Japan) with excitation wavelength of 296 nm. Both the excitation slit and emission slit were 5 nm, and the corrected spectrum function was set at "on" during experiments. The emission spectrum was recorded in the wavelength range from 300 nm to 450 nm. Protein unfolding induced by guanidine hydrochloride (GdnHCl) was investigated by incubating the protein (0.05 mg/ml) in buffers containing GdnHCl with concentrations ranging from 0 to 6 M in PBS at 25 °C overnight. Spectrum was recorded at 25 °C and each spectrum was corrected for the background using the spectrum of the solvent recorded in identical conditions. All the experiments were performed in triplicate, and the mean value was shown in figures.

## 3 Results

### 3.1 Expression and Purification of Recombinant Enzymes

Each of Egl325, Egl326 and Egl330 covered the catalytic domain of the wild type endo-β-l, 4-glucanase. Egl325 was a mutant of Egl330 with the peptide sequence Arg-Glu-Asn-Ile-Arg deleted in the C-terminus and Egl326 was another mutant of Egl330 with the peptide sequence Glu-Asn-Ile-Arg deleted in the C-terminus. Egl325, Egl326 and Egl330 were fused to his-tag and expressed in *E. coli*, respectively. Purification of these proteins was performed through a single metal affinity chromatography step. These three proteins were eluted from the affinity column with the concentration of imidazole increased to about 0.1 M. The yields of recombinant protein were about 23 mg per liter for Egl330, 20.5 mg and 19.8 mg for Egl326 and Egl325, respectively. Molecular weights of Egl325, Egl326 and Egl330 were estimated to be about 30, 30 and 31 kDa respectively, according to the protein markers (Fig. 1).

### 3.2 Effects of pH Condition and Temperature on Hydrolysis Capacities of the Three Enzymes

Results showed that the three enzymes displayed their maximal activities at pH around 6.0. Their activities decreased obviously at pHs beyond the range of 4.5–7.0.

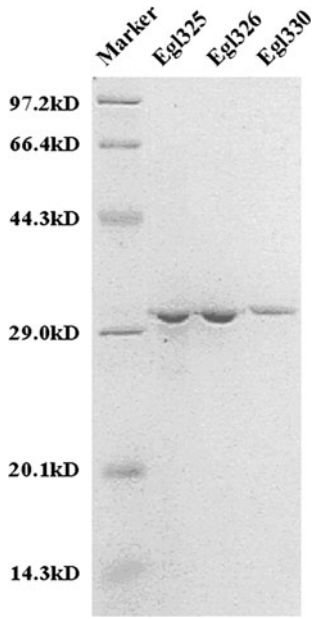


**Fig. 1** SDS-PAGE of Egl330, Egl326 and Egl325. Proteins were resolved in 10 % SDS-PAGE gel. *Lane marker* is the protein molecular weight marker from 14.3–97.2 kDa; *Lane Egl325*, *Egl326* and *Egl330* are purified protein Egl325, Egl326 and Egl330, respectively

More than 80 % of the maximal activity of Egl330 was remained after incubation in buffers with pH values from 5.0 to 11.0 at 30 °C for 150 min. As to Egl326 and Egl325, it was found that they could keep more than 80 % of their activities only when pH values of the incubation buffers were between 5.0 and 7.0 (Fig. 2). After incubation at pH 4.0, Egl330 remained 47 % of its activity, while no activity was detected for Egl326 and Egl325. These results indicated that Egl330 was more stable in buffers with pH from 4.0 to 11.0 compared with Egl326 and Egl325.

All the three enzymes displayed their maximal hydrolysis activity at 60 °C. After treatment at 65 °C for 30 min, Egl330 remained about 50 % of its original activity, Egl325 and Egl326 remained about 3.8 % of their original activities. 90 min later, Egl330 remained 15 % of activity, while Egl325 and Egl326 were totally deactivated (Fig. 3). After treatment at 80 °C for 10 min, Egl330 remained 15 % of its activity, while nearly no activity was detected with Egl325 and Egl326. Reactivation profiles of Egl330, Egl325 and Egl326 showed that Egl330 recovered more than 60 % of its original activity after 5 min at 20 °C, Egl326 recovered 16 % activity and Egl325 totally lost its activity (Fig. 4). These results showed that Egl330 was more thermally resistant than Egl326 and Egl325.

### 3.3 UV and Circular Dichroism Analysis of the Three Enzymes

The UV absorption results of Egl330, Egl325 and Egl326 were shown in Fig. 5. Egl325 and Egl326 displayed highly similar absorption spectra. However, an obvious shoulder at 220 nm was observed in the absorption spectrum of Egl330, which was not found in the spectra of Egl325 and Egl326, indicating structural difference between them. Far-UV circular dichroism spectra of Egl325, Egl326 and Egl330 were shown in Fig. 6. The calculated compositions of the secondary structures of Egl325, Egl326 and Egl330 were shown in Table 1. The Far-UV circular dichroism result was consistent with that of UV absorption. It could be concluded from above results that deletion of the C-terminus peptide destabilized the catalytic domain of the endoglucanase.

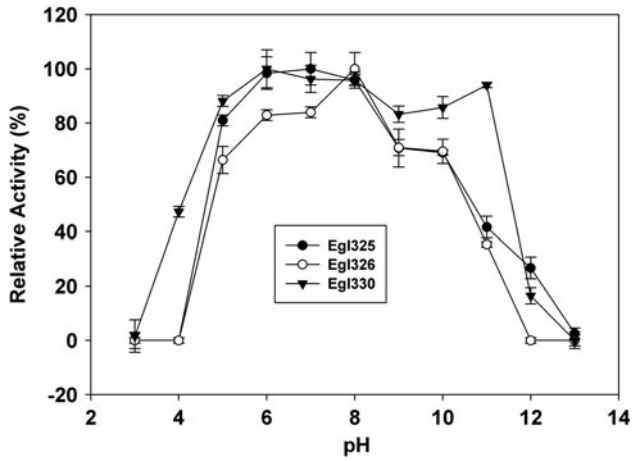


**Fig. 2** pH stability of Egl330, Egl326 and Egl325. Enzymes (0.05 mg/ml) were kept in buffers with different pH values (3.0–13.0) and incubated for 150 at 30 °C. For each of the three enzymes, maximal activity detected in this test was set as 100 %

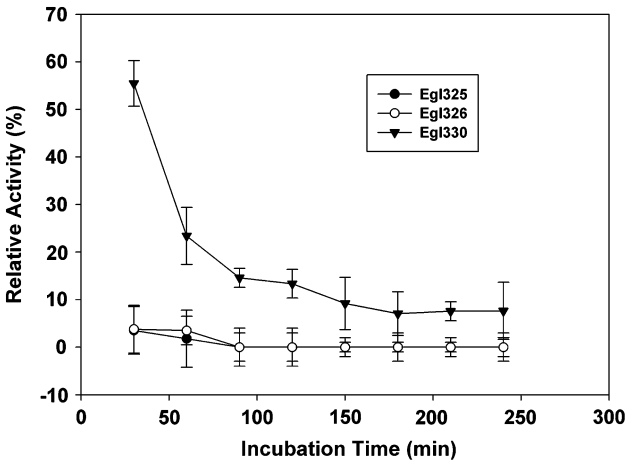


**Fig. 3** Thermal stability of Egl330, Egl326 and Egl325. Enzymes (0.05 mg/ml) were incubated in citrate–phosphate buffer (pH 6.0) at 65 °C. At different time points, the residual activity was detected at 25 °C. For each enzyme, its original activity enzyme was set as 100 %

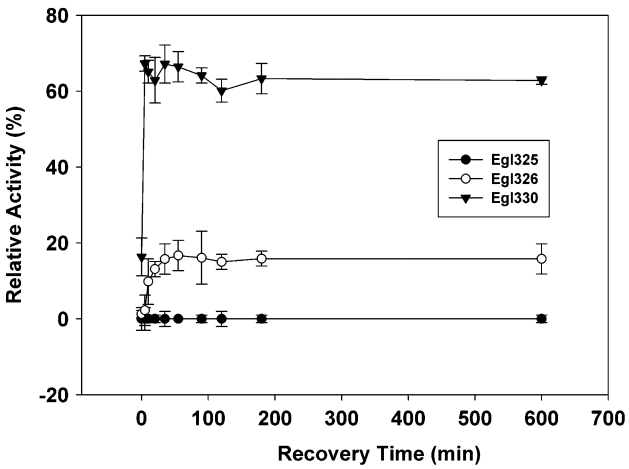


**Fig. 4** Activity recovery test of Egl325, Egl326 and Egl330. Enzyme (0.05 mg/ml) dissolved in 0.1 M PBS (pH 7.4) was treated at 80 °C for 10 min, then cooled to and kept at 20 °C. At different time points, residual activities of the heat treated enzyme were measured. Original enzyme activity was set as 100 %

### 3.4 GdnHCl-Induced Unfolding Analyzed by Fluorescence Spectrometry

The intrinsic Trp fluorescence variations of Egl325, Egl326 and Egl330 were recorded respectively to analyze GdnHCl

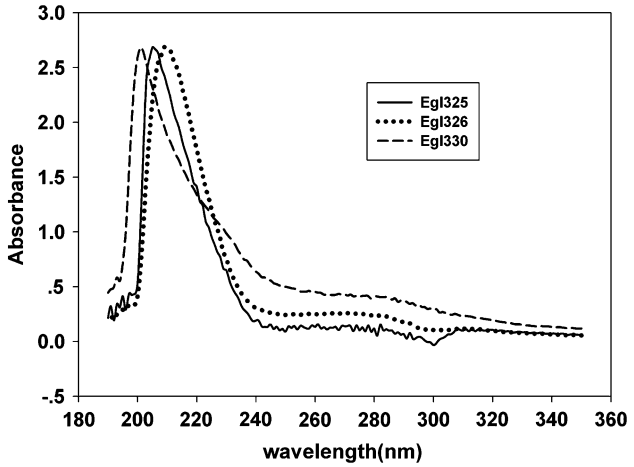


**Fig. 5** Ultra violet scanning of Egl325, Egl326 and Egl330. The concentration of each protein was 0.04 mg/ml dissolved in 0.1 M PBS (pH 7.4). The ultra violet scanning were recorded at 25 °C

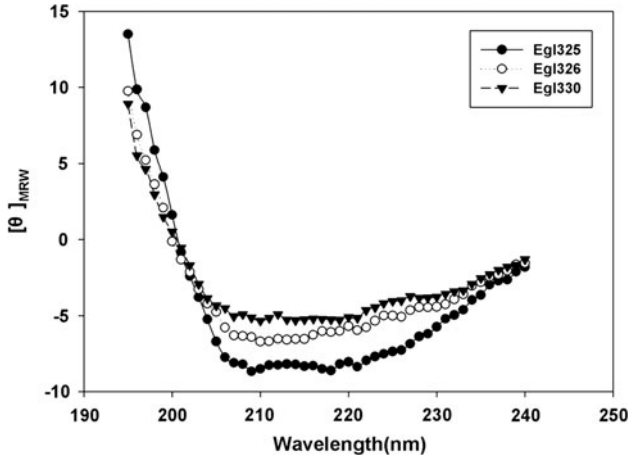


**Fig. 6** Far-UV CD analysis of Egl330, Egl326 and Egl325. The three protein (0.15 mg/ml) were dissolved in 0.1 M PBS (pH 7.4) with a concentration of 0.15 mg/ml. The CD data were recorded at 25 °C

**Table 1** The calculated compositions of the secondary structure of Egl325, Egl326 and Egl330

| | α-Helix(%) | β-Sheet(%) | β-Turn(%) | Random coil(%) |
|---|---|---|---|---|
| Egl325 | 36.0 | 18.1 | 14.5 | 31.4 |
| Egl326 | 35.9 | 15.4 | 13.7 | 35.0 |
| Egl330 | 23.8 | 30.9 | 18.5 | 26.8 |

induced conformational changes of the three proteins (Fig. 7). The fluorescence spectra of Egl330, Egl326 and Egl325 in 0.1 M PBS (pH 7.4) were presented in Fig. 8. The maximum emission wavelengths were 338.2, 341.8 and 339.4 nm for Egl325, Egl326 and Egl330 in 0.1 M PBS. The slight difference in maximum emission wavelength suggested deletion of the C-terminus peptide might

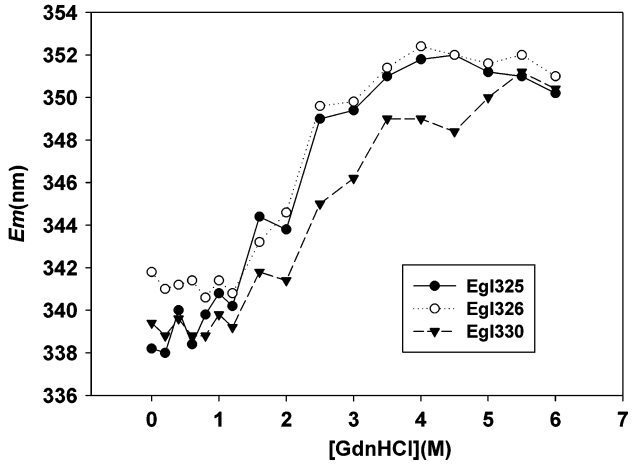


**Fig. 7** GdnHCl induced *Em* value changes for the Trp fluorescence of Egl330, Egl326 and Egl325. The concentration of each protein was 0.05 mg/ml. Protein denaturation was performed at 25 °C overnight in buffers containing GdnHCl with concentrations ranging from 0 to 6 M in PBS. Fluorescence spectra were recorded at 25 °C

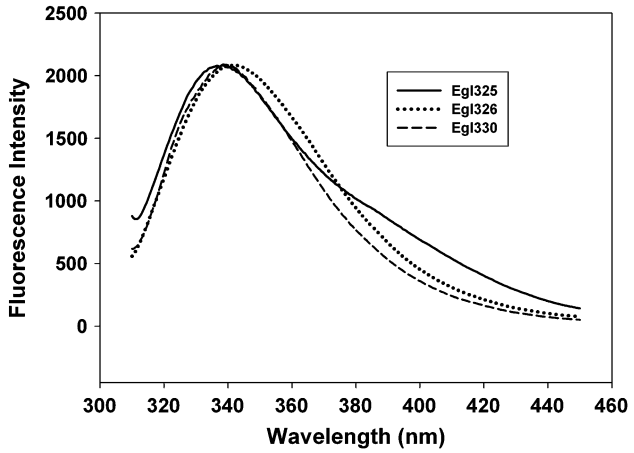


**Fig. 8** Fluorescence spectra of Egl330, Egl326 and Egl325 in 0.1 M PBS (pH 7.4). The concentration of each protein was 0.05 mg/ml. Fluorescence spectra were recorded at 25 °C

induce micro environmental changes of Trp residue. When the concentration of GdnHCl was above 1.2 M, Trp fluorescence displayed an obvious red shift for all the three proteins, indicating an unfolding process of them. Egl325 and Egl326 were almost totally unfolded with the concentration of GdnHCl increased to about 3 M. However, at the same GdnHCl concentration, Egl330 was in an intermediate unfolding state. This result reflected that the native structure of Egl330 was more resistant to the protein denaturant compared to Egl325 and Egl326. And this result further indicated that the peptide sequence located at the C-terminus of Egl330 played important role in maintaining the structural stability of Egl330.

## 4 Discussion

Rational design based protein engineering and directed evolution have been utilized for the improvements of protein stability [3, 4, 16, 20, 21], especially for those enzymes whose natural properties do not match the requirements of practical application conditions. In some occasions, extension of an enzyme with peptide fragment at its C-terminus or N-terminus will be beneficial for its thermo-stability. Matsuura et al. found that extension of the C-terminus of an enzyme with a random short peptide would lead to alteration in the thermo-stability of the enzyme [10]. By extending the N-terminal sequence of the catalytic domain, Liu et al. improved the thermo-stability of a carboxymethyl cellulase produced by *P. rhizinflata* 2301 [7]. These examples indicated that even though there were no predictable functions, peptide sequence located at the N- or C-terminus might play key roles in regulating the stability of protein domain. The effect of a flanking peptide is not constant. A peptide tail providing beneficial effect to one protein may not provide the same benefit to another. It will be of value for improving some specific enzymes by investigating how the neighboring peptide affects the stability of the catalytic domain.

Egl330, Egl326 and Egl325 all covered the catalytic domain of the wild type endo-β-l, 4-glucanase, which was confirmed by the facts that they could hydrolyze the substrate CMC and shared identical optimal reaction pH and temperature. However, deletion of peptides consisted of four to five amino acids at the C-terminus of Egl330 resulted in a significant decrease of stability of the enzyme. The results of pH stability and thermal stability indicated that Egl330 was more stable than Egl326 and Egl325. UV absorption and far-UV circular dichroism spectra showed that compared to Egl330, structural changes were observed with Egl326 and Egl325. Deletion of the peptides consisted of four to five amino acids might destabilize the enzyme through making the enzyme fold into an unstable conformation.

Protein unfolding induced by GdnHCl is commonly used to study the stability of protein. The conformational changes of Egl330, Egl326 and Egl325 under different GdnHCl concentrations reflected by intrinsic Trp fluorescence further confirmed the conclusion that the peptides consisted of four to five amino acids at the C-terminus contributed to the stability of Egl330. This result was further confirmed by the difference of residual activities of Egl330, Egl325 and Egl326 by GdnHCl treatment (data was not shown). The residual activity of Egl330 decreased from 75 to 53 % when GdnHCl concentration increased from 3 M to 6 M. However, the remained activities of Egl325 and Egl326 decreased from 60 % to nearly total deactivation when GdnHCl concentration increased from 3 to 6 M.

Stabilities of protein were studied and discussed widely. And conclusions turned out that many factors had effects on protein stabilities, such as hydrophobic interactions, hydrogen bonding, backbone strain etc. [17]. Egl325 was a mutant of Egl330 with the peptide sequence Arg-Glu-Asn-Ile-Arg deleted in the C-terminus and Egl326 was another mutant of Egl330 with the peptide sequence Glu-Asn-Ile-Arg deleted in the C-terminus. Since Arg, Glu and Asn contained polar side chain, we hypothesized that the deletion of the four to five amino acids affected the forming of salt-bridges or hydrogen bonds of the protein and therefore resulted in an unstable conformation. As we know that hydrophobic interactions are major factors in stabilizing the three-dimensional structures of proteins, the deletion of Ile might also affect the hydrophobic interactions within the amino acids of the protein and further affected the folding of the protein.

In conclusion, our study for the first time provided the information that a short peptide flanking the C-terminus played crucial role in maintaining the stability of the catalytic domain of a recombinant *Bacillus* endo-β-l, 4-glucanase. Deletion of the short peptide resulted in destabilization of the catalytic domain through affecting the folding of the protein. To this note, structure based rational design of the short peptide for this protein may be helpful for producing more stable endo-β-l, 4-glucanase.

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