Cold adaptation of a mesophilic cellulase, EG III from *Trichoderma reesei*, by directed evolution

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Abstract  Cold-active enzymes have received little research attention although they are very useful in industries. Since the structure bases of cold adaptation of enzymes are still unclear, it is also very difficult to obtain cold-adapted enzymes for industrial applications using routine protein engineering methods. In this work, we employed directed evolution method to randomly mutate a mesophilic cellulase, endoglucanase III (EG III) from *Trichoderma reesei*, and obtained a cold-adapted mutant, designated as w-3. DNA sequence analysis indicates that w-3 is a truncated form of native EG III with a deletion of 25 consecutive amino acids at C-terminus. Further examination of enzymatic kinetics and thermal stability shows that mutant w-3 has a higher $K_{cat}$ value and becomes more thermolabile than its parent. In addition, activation energies of w-3 and wild type EG III calculated from Arrhenius equation are 13.3 kJ/mol and 26.2 kJ/mol, respectively. Therefore, the increased specific activity of w-3 at lower temperatures could result from increased $K_{cat}$ value and decreased activation energy.

Keywords: cold adaptation, directed evolution, endoglucanase III, *Trichoderma reesei*.

Although a large proportion of our planet is in cold environment, little research has been devoted to the studies of psychrophilic microorganisms, especially of cold enzymes produced by psychrophilic microorganisms. Only recently has the investigation on the molecular basis of cold-active enzymes from psychrophiles received concerted interest[1]. Many features are proposed to explain the molecular basis of cold adaptation, including reduction of salt bridges in intra-domain and inter-domain positions, increased hydrophobicity in core regions[2], loops of increased length carrying more charge and less proline residues, increase in solvent-exposed hydrophobic residues[3], poorer van der Waals packing interactions to the molecule[4], increases in flexibility in small areas of the molecule affecting the mobility of adjacent active-site structures[5], and so on. Hence, it seems to be generally accepted that more flexible structure is the main structural adaptation of the cold enzymes regardless of the principal determinants of the enzyme flexibility. However, the structure basis of adaptation of enzymes to low temperature is poorly understood[3]. Since all the researches mentioned above are based on the sequence and structure analyses of the naturally existing homologues, it is hard to identify special mutations responsible for cold adaptation. Error-prone PCR and DNA shuffling have recently shown great power in creating new protein homologues[6]. In both cases, mutations can be generated rapidly by evolution in the labora-
tory and proteins with certain changes in properties can be quickly identified. However, little work was reported on artificial cold adaptation of mesophilic enzymes.

Endoglucanases (EGs) are used at low dosage for various surface treatments in paper, textile, and laundry industries. However, EG III from *Trichoderma reesei* exhibits maximal activity at around 50°C. This property must be genetically altered to fit low temperature environments in some industries. In this case, artificial cold adaptation of such mesophilic cellulase as EG III by means of directed evolution is not only advantageous in elucidating cold adaptive mechanisms, but also beneficial to industrial applications.

In this work, we employed an experimental error-prone PCR to randomly mutate EG III entire gene followed by screening for clear-halo-forming colonies on carboxymethyl cellulose (CMC) plates at low temperature. With the resultant EG III mutant, we have explored the possible molecular mechanism of its cold adaptation.

1 Materials and methods

1.1 Strains and plasmids

*Saccharomyces cerevisiae* H158 was used as a host. Plasmid pAJ401 with *Ura3* gene was employed as expression vector (VTT Biotechnology, Finland). *S. cerevisiae* H158 (eg3) (from this laboratory) was the strain transformed with the wild type EG III cDNA, while *S. cerevisiae* H158 (w-3) (in the present paper) with the mutant EG III cDNA.

1.2 Enzymes and reagents

Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were purchased from Promega (Madison, WI, USA). Agarose gel extraction kit was from Boehringer Mannheim (Indianapolis, IN, USA).

1.3 PCR-based random mutagenesis

Error-prone PCR was performed using the full-length EG III cDNA sequence to induce random mutagenesis. The PCR solution mixture was identical to that used by Moore Jeffrey et al.\[6\] with the exception of 2% DMSO. The two primers, P-1 (5'-GAAGTTCGGAATTCCGGCA-3') and P-2 (5'-TGTGGAAATTGTGAGCGGA-3') were engineered with *Eco*RI and *Xho*I sites, respectively. Mutagenized libraries of EG III were cloned into the expression vector pAJ401 through the same restriction sites, transformed into *S. cerevisiae* H158 competent cells\[7\], and spread onto SC-URA plates for further screening as described below.

1.4 Screening for cold adaptive mutants—CMC Congo Red assay

Yeast cells containing EG III mutant genes were plated on SC-URA and the cells harboring wild type EG III gene were used as controls. After 3 d incubation at 30°C, the plates were covered with 5 mL of top medium consisting of 0.5% CMC-Na and 0.8% agar in 50 mmol/L acetate buffer, pH 5.0. Then 0.1% Congo Red was added and plates were incubated at 15°C for 20 min
for CMC hydrolysis and Congo Red staining. At the end of the incubation, plates were washed with 1 mol/L NaCl, the positive colonies with visualized hydrolysis zones in colder conditions were picked and subjected to a second round of the assay to confirm the result.

1.5 Enzyme purification

*S. cerevisiae* H158 (eg3) or H158 (w-3) was grown in flask at 120 r/min at 30°C for 3 d in SC-URA. After being separated by centrifugation at 5000 g for 5 min, the supernatant of the culture was filtered and concentrated by ultrafiltration. EG III proteins were purified using Bio-gel P100 chromatography followed by CM-Sepharose Fast Flow columns.

1.6 Determination of enzyme activity

0.5 mL of diluted enzyme solution was added to 0.5 mL of 0.5% CMC-Na in 50 mmol/L acetate buffer, pH 5.0. After 1 h incubation at various temperatures, reducing sugars liberated were determined according to Somogyi[8].

1.7 Thermal stability of EG III

Enzyme solution was incubated at different temperatures for 60 min and then residual activity was determined as described in sec. 1.6.

1.8 Sequence analysis of EG III mutant genes

Selected mutant plasmids were digested with *Eco*RI and *Xho*I and the fragments containing EG III gene were recovered and then subcloned into the plasmid pUC19 through *Eco*RI and *Sal*I sites. The recombinant plasmids were amplified into *E. coli* DH5α, and DNA sequencing was carried out according to Sambrook et al.[9].

2 Results

2.1 Cold adaptive mutant obtained by mutagenesis and screening

After random mutagenesis and screening, one mutant out of a mutant library containing 2 × 10⁵ colonies showed a clear CMC hydrolysis zone at 15°C. As control, wild type EG III did not show any CMC hydrolysis activity under the same condition. To confirm the novel activity, this mutant was selected and spread onto fresh SC-URA plates and incubated for 3 d at 30°C. Then single colonies were randomly picked and spread onto fresh SC-URA plates again for another 3 d incubation. The hydrolysis activity at 15°C was screened again using CMC Congo Red assay. The uniform hydrolysis zones on all plates suggested that the activity of the mutant, named w-3, was stable. DNA sequence and amino acid analyses (fig. 1) revealed that w-3 gene has lost only a single base of C at the position 1180 (with the A of start codon ATG as +1). This single nucleotide deletion causes a frame shift with the immediately following TGA as an earlier termination codon. As a result, the mature w-3 protein contains the first 372 residues of the 397 residues from the matured EG III, with the last 25 consecutive amino acids missing from its C-terminus.
2.2 Enzyme purification

To characterize the kinetic properties of the cold adaptive mutant w-3, wild type and mutant enzymes were purified by successive column chromatography as described in sec. 1. SDS-PAGE analysis (fig. 2) showed that both purified w-3 and native EG III proteins migrated as a homogenous band with anticipated molecular weight, indicating a successful purification.

2.3 Catalytic activities of cold adaptive mutant w-3

To analyze the properties of w-3, the activity of w-3 and parent EG III enzyme at various temperatures was determined. As shown in table 1, w-3 is more active than EG III when the temperature is lower than 40°C, whereas EG III is more active than w-3 at 50—80°C. The relative activities (the ratio of activity at various temperatures to that at 50°C \( \text{per se} \)) of w-3 were significantly higher than those of parent enzyme at low temperatures (fig. 3). The maximum difference was found at 30°C. The data showed that w-3 acquired cold-adaptive property with higher activity at low temperatures.
Table 1  The activities of w-3 and parent EG III at different temperatures

<table>
<thead>
<tr>
<th>Temperature/°C</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>w-3/IU • mg⁻¹</td>
<td>3.99</td>
<td>8.86</td>
<td>13.71</td>
<td>24.21</td>
<td>35.60</td>
<td>22.07</td>
<td>3.45</td>
<td>0.71</td>
</tr>
<tr>
<td>EG III/IU • mg⁻¹</td>
<td>2.67</td>
<td>6.46</td>
<td>10.21</td>
<td>21.03</td>
<td>39.90</td>
<td>29.21</td>
<td>8.42</td>
<td>2.04</td>
</tr>
</tbody>
</table>

2.4 Thermal stability of evolved enzyme

Furthermore, the thermal stability of the mutant enzyme w-3 was examined by measuring the original and mutant EG III activities at 50°C after 1 h incubation at various temperatures, as shown in fig. 4. The thermostability of w-3 was significantly decreased, especially at higher temperatures, indicating that w-3 becomes more thermolabile than the wild type EG III.

Fig. 3. Relative activities (the ratio of activity at appropriate temperatures to that at 50°C) of w-3 and its parent. ◆, % parent; ▲, % w-3.

Fig. 4. Thermal stability of w-3. ▲, w-3; ◆, parent.

2.5 Enzymatic kinetics of w-3

Enzymatic parameters with CMC as substrate at different temperatures are listed in table 2. Because CMC-Na is a kind of polymer, $K_m$ value is calculated as mg • mL⁻¹. The specific activity of w-3 at 30°C is significantly improved. The mutation in w-3 leads to a decrease in $K_{cat}$ value at 50°C compared to its parent. When temperature switches from 50 to 30°C, $K_m$ values for both parental and w-3 enzymes decrease by 52%, whereas $K_{cat}$ value for w-3 decreases much less than that of the parent enzyme. Consequently, $K_{cat}/K_m$ at 30°C relative to 50°C of w-3 is increased by

Table 2  Enzymatic parameters of w-3 and its parent for hydrolysis of CMC at different temperatures

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction temperature/°C</th>
<th>$K_{cat}$/min⁻¹</th>
<th>$K_m$/mg • mL⁻¹</th>
<th>$K_{cat}/K_m$</th>
<th>Specific activity/IU • mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native EG III</td>
<td>30</td>
<td>5.478</td>
<td>1.151</td>
<td>4.759</td>
<td>27.739</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10.433</td>
<td>2.368</td>
<td>4.406</td>
<td>39.907</td>
</tr>
<tr>
<td>w-3</td>
<td>30</td>
<td>6.217</td>
<td>1.274</td>
<td>4.881</td>
<td>32.913</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.621</td>
<td>2.679</td>
<td>3.218</td>
<td>35.556</td>
</tr>
</tbody>
</table>
41% compared to its parent.

2.6 Activation energy of enzymes

According to Arrhenius equation, activation energy ($E_a$) is given by

$$E_a = \ln \frac{K_1}{K_2} \frac{R^T T_1}{T_2 - T_1}$$

where $K$ is $K_{cat}$, $R$ is the universal gas constant, and $T$ is the absolute temperature.

Activation energies of native EG III and w-3 at 30°C are 26.2 kJ mol$^{-1}$ and 13.3 kJ mol$^{-1}$, respectively. The activation energy of w-3 is decreased to 50% of that of its parent, possibly accounting for an increase in velocity of the hydrolytic reaction.

3 Discussion

A simple and powerful screening strategy is critical for successful application of directed evolution method. Plate assay with visible hydrolytic zones, which is one of the best choices for selecting enzyme with specific properties, is available for analyzing EG III activity. However, the temperature used in searching for cold active mutants is generally at 10—20°C, different from the incubation temperature (around 30°C) for the yeast libraries. To solve this problem, mutant library was first plated and incubated at 30°C for 3 d for enzyme secretion, then the plates were overlaid with top medium containing 0.5% CMC, and CMC Congo Red assay was performed. The short incubation time of 20 min was good enough to distinguish the cold-adapted mutant from the wild type.

Protein molecules require both flexibility and rigidity for its function. The delicate balance of stabilizing and destabilizing interactions allows enzymes to fit special environments. Adjustment of conformational flexibility is a key event in the thermal adaptation of protein\textsuperscript{10}. Highly flexible structure provides enhanced abilities for conformational changes during catalysis. Thermal instability of cold-adapted enzymes is therefore regarded as a consequence of their conformational flexibility\textsuperscript{11}.

The cold adaptive EG III mutant w-3, obtained by directed evolution methods from its mesophilic parent, lacks a 25-amino acid-long peptide chain located in the catalytic domain of the enzyme at C-terminus (fig. 1). As a result, the mean hydrophobicity index of w-3 is decreased from $-0.30$ of native enzyme to $-0.32$, indicating that mutant w-3 is more hydrophilic. Additionally, there is a Cys residue among the 25 deleted amino acids. With the EG III structure yet unknown, any Cys residue in the molecule could be potentially involved in the formation of disulfide bond, and might thus play a role in protein stability. From the molecular structure point of view, reduction in $K_{cat}$ value of mutant w-3, compared to the original enzyme at 50°C, likely results from both an increase in the hydrophilicity and a decrease in the number of cysteine residues of the enzyme, which may reduce the compactness of the active site and increase conformational
flexibility.

The more flexible structure might also account for an increase in thermal sensitivity of w-3. Although Miyazaki et al.\cite{12} successfully improved the thermostability of the psychrophilic protease subtilisin S41 without a cost of activity, mutations that increase enzyme thermostability while maintaining low temperature activity are rare. Nonetheless, unlike the conclusion by Taguchi et al. that cold adaptation was achieved mainly by the decrease in the $K_m$ value\cite{13}, the improved specific activity of w-3 at 30°C compared with that of mesophilic counterpart mostly results from increased $K_{cat}$ value rather than decreased $K_m$ value at low temperature.

In the catalytic process, the enzyme-substrate complex must be converted into an active complex first, and then turned into products. To generate the active complex, the free energy of activation must be added to the enzyme-substrate complex. The less the activation energy needed, the higher the velocity of the reaction. In the present paper, activation energy ($E_a$) of the mutant w-3 calculated from the Arrhennius equation is about 50% lower than that of the native EG III, which may lead to an increase in activity of w-3 at low temperatures.

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References