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Greatly Enhanced Secretion, Soluble Expression and Characterization of an Alkaline Endoglucanase in *Escherichia coli*

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The alkaline endoglucanase gene from *B.akibai* I-1 was cloned into expression vector pET-28a(+) and expressed in *E.coli* BL21(DE3). It was found that the recombinant enzyme could be expressed in a soluble form with activity under the optimal conditions of 23-30°C and 0.2 mM IPTG. The secretion and expression level of the soluble recombinant enzyme could be greatly enhanced by addition of EDTA and lysozyme. Addition of lysozyme alone had little influence on the secretion and expression, while the combination use of 0.5% EDTA and 0.015% lysozyme at the induction time of 12h increased the secretion and expression level by about 11.5-fold and 83%, respectively. Under the optimal conditions, extracellular and total activities reached 26.5 and 40.0 U/mL, respectively. The recombinant and native endoglucanases were purified to homogeneity with column-chromatographic procedure. Both purified enzymes exhibited maximum activity at about pH 9.0 and 50°C, and were resistant to various metal ions and chelating agents examined. Unlike the native enzyme which was partly inhibited by sodium dodecyl sulfate(SDS), the recombinant enzyme had good SDS-resistance(residual activity>99%). The recombinant enzyme was very stable in the commercial detergents and no decrease in residual activity was observed in 0.2%(w/v) laundry detergent, showing great potential in detergents industry.

Key words: Alkaline endoglucanase, *Escherichia coli*, Soluble expression, SDS-resistance.

Endoglucanase (β-1, 4 - endoglucanase, EC3.2.1.4) is themajor component of cellulase system, which mainly hydrolyzes internal 1,4-β-bonds in cellulose (Kim *et al.,* 2009). Since the discovery of alkaline cellulase (mainly endoglucanase) from *Bacillus* by Horikoshi *et al.*(1984), the endoglucanases produced by bacteria have earned much attention for their successful application in detergent industry, textile biofinishing, recycled pulp and biological de-inking processes(liu *et al.,* 2008), resulting in a rapid growth in demand for bacterial endoglucanases, especially those with excellent properties such as good alkali-resistance, thermostability and resistibility to metal ions and reagents (Rastogi *et al.,* 2010).

Recombinant strains of *Escherichia coli* (*E.coli*) are widely used for the production of valuable proteins. Previous research has demonstrated that recombinant *E. coli* can produce high level of endoglucanase as a cytoplasmic or periplasmic product (Wood *et al.,*1997). However, in *E.coli* expression system, most proteins synthesized in the cytoplasm of *E.coli* are not secreted into the culture medium but remain in the cytoplasm or periplasm (Badyakina *et al.,* 2005). Especially in terms of endoglucanases, the expression products are usually accumulated in *E.coli* cell in form of non-soluble inclusion body without activity (Liu *et al.,* 2011).

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In the present study, the endoglucanase from *Bacillus akibai* **I-1** has good alkali-resistance, thermostability and resistibility to various metal ions and chelating agents examined. Unfortunately, the level of the enzyme protein in the culture supernatant of *B.akibai* **I-1** is extremely low, which limits its potential use in various industrial processes. Therefore, this study is aimed at developing an expression system for secretory production of the endoglucanase in *E.coli* and enhancing the expression level of endoglucanase as well. In this report, we primarily describes the greatly enhanced secretion and expression of the soluble endoglucanase in recombinant *E.coli* by the synergism of EDTA and lysozyme, and the improved characterization of this recombinant enzyme.

MATERIALS AND METHODS

Biological and chemical materials

Lysozyme(egg white, potencye≥20000U/mg) was purchased from NBC (USA). EDTA was from Amresco (USA). DEAE Sepharose CL-6B and Hiload 16/60 Superdex 200 pg Column were purchased from Amersham Pharmacia Biotech.,Sweden. Carboxymethylcellulose (CMC, 300-800 mpa.s), Liby® laundry detergent and all other chemicals were also from commercial sources and were of the highest purity available.

Strains and plasmids

B.akibai I-1 was used as the source of the endoglucanase and the gene of interest, which was originally isolated in a previous study(Liu *et al.,* 2008). *B.akibai* I-1 was cultured in a CMC-containing liquid medium(w/v)-1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% KH_2PO_4, 0.25% $Na_2HPO_4 \cdot 12H_2O$, 2.0% CMC-Na and 0.5% Na_2CO_3 (separately autoclaved). The organism was grown with shaking (200 rpm) at 35°C for 48h in 50mL medium in 250mL flasks. The culture of *B.akibai* I-1 was centrifuged at 14000×g for 10 min and the supernatant was stored at -20°C for purification.

Plasmid pET-28a(+) was used for the expression of the endoglucanase. The host strain used in this study was *E.coli* BL21(DE3). *E.coli* Top10F´and plasmid pMD18-T were used for plasmid construction and propagation. *E.coli* growth media were prepared according to the *E.coli* expression system manual from Novagen.

Cloning of endoglucanase gene

The endoglucanase gene EG-I-1 was amplified by PCR from the chromosomal DNA of *B.akibai* I-1 using the following degenerate primers derived from the conserved N- and C-terminal end regions of the *Bacillus* endoglucanases: 5'-GYTNM GNAARAA RACNAARC-3' and 5'-TTATTT TTTCGTAGCC TCTT-3'. PCR amplification was as follows: 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 3 min and final extension at 72°C for 7 min. The PCR product was then cloned into the pMD18-T cloning vector and transformed to *E.coli* Top10F´, which was sent to be sequenced by Takara Company of China.

Construction of the expression plasmid

The coding sequence EG-I-1, without the signal peptide coding sequence, was amplified by PCR using the primers: 5'-CCG*GAATTC*GAAGGAAACACTCGTGAAGAC-3' (containing *Eco*RI site) and 5'-CCC*AAGCTT*TTATTTTTTCGTAGCCTCTTTC-3' (containing *Hind* III site). The PCR product was gel purified, digested with *Eco*RI and *Hind* b°C, and ligated into the corresponding sites of the plasmid pET-28a(+). The recombinant plasmid pET-28a(+)-EG-I-1 was then transformed into *E.coli* BL21(DE3). The gene insert was confirmed by DNA sequencing.Endoglucanase-producing recombinants were screened on a kanamycin-containing solid plate and identified by the Congo red method. An relatively efficient endoglucanase-producing transformant was picked, containing recombinant plasmid pET-28a(+)-EG-I-1, from a single colony.

Expression of recombinant enzyme in *E.coli*

In a typical experiment, the transformant was grown overnight at 37°C in the LB medium, supplemented with 25 mg/ml kanamycin. The culture was then incubated in fresh Luria–Bertani (LB) medium containing kanamycin to an OD600nm of 0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. At the induction time of 12 h, 0.5% EDTA and 0.015% lysozyme were simultaneously added into the culture medium. The culture were incubated at 23°C for 22h induction and harvested by centrifugation at 14000×g for 10 min at 4°C. The supernatant was used for the measurement of

extracellular endoglucanase activity and SDS-PAGE analysis. In order to determine intracellular endoglucanase activity, the cells collected by centrifugation were washed, resuspended in Tris-HCl buffer (pH8.0), and disrupted by French press. The mixture was then centrifuged at 14000×g for 10 min at 4°C and the supernatant was collected for the measurement of intracellular endoglucanase activity. The total activity is the sum of the extracellular and intracellular activities.

Purification of native and recombinant enzymes

Due to the extremely low level of the enzyme protein in the culture supernatant of *B.akibai* I-1, the centrifugal supernatant was concentrated by membrane ultrafiltration. The concentrate was applied to a DEAE Sepharose CL-6B column(2.6×30 cm) pre-equilibrated with 0.01 M Tris-HCl buffer (pH 8.0). The adsorbed proteins were eluted from the column by a step-wise increasing gradient (i.e.0.4, 0.45 and 1M) of NaCl at a flow rate of 180 $ml \cdot h^{-1}$. Only one protein with CMCase activity was obtained, which was designated I-1-A. The concentrate of I-1-A was subjected to size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg Column (1.6×60 cm) in 0.01 M Tris-HCl buffer (pH8.0) at a flow rate of 80 $ml \cdot h^{-1}$. The enzyme recovered from the column was pooled and stored at 4°C for further characterization of this enzyme.

The purification of the recombinant enzyme was performed by a one-step column-chromatographic procedure on the DEAE Sepharose CL-6B column described as the native enzyme.

Assay of endoglucanase activity

Carboxymethylcellulose(CMC) was used as the substrate for the assay of the endoglucanase activity. The reaction mixture contained 1 ml 1% (w/v) CMC solvated in Tris-HCl buffer (0.05 M, pH 8.0) and 0.1ml properly diluted enzyme solution. The enzymatic reaction was carried out for 20 min in a 40°C water bath, then 2 ml 3,5-dinitrosalicylic acid (DNS) was added into the reaction mixture and incubated in boiling water for 10 min. The reducing glucose released in the enzymatic reaction was then determined by recording the absorbance at 540 nm. One unit of the enzyme activity was defined as the amount of enzyme that produced 1mg product per hour.

Protein electrophoresis

SDS-PAGE was performed basically according to the method described(Liu *et al.*,2008). The concentration of the separation gel was selected as 12.5%. The prestained protein molecular weight marker was used and protein was stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Cloning and expression of the endoglucanase gene

A 2472-bp DNA fragment of EG-I-1 gene without signal peptide was amplified by PCR from genomic DNA of *B.akibai* I-1. Comparison of the deduced amino acid sequence with those of proteins registered in the BLAST database in NCBI showed the highest identity to the endoglucanases of *Bacillus* sp.1139 (Accession number: P06564.1) and *Bacillus* sp. KSM-64 (Accession number: AAA73189.1) with 92% similarity, belonging to glycosyl hydrolase family 5 (Liu *et al.,* 2012). The coding sequence EG-I-1 was cloned into the expression vector pET-28a(+) and expressed in *E.coli* BL21(DE3). However, under the standard conditions of 37°C and 1 mM IPTG, both the extracellular activity (≤ 0.1 U/mL) in the culture supernatant and intracellular activity (≤1 U/mL) were extremely low, which indicated the formation of a non-soluble inclusion body without activity, similar to previous reports(Laymon *et al.,*1996; Tang *et al.,*2009).

The effect of the culture temperature and IPTG concentration on the expression of EG-I-1 was explored and the total EG activity was measured. As shown in Figure 1**,** a decrease in the culture temperature or IPTG concentration increased the yield of a soluble endoglucanase by slowing the rate of synthesis and preventing aggregation of folding intermediates. The optimal culture conditions of temperature and IPTG concentration were 23-30°C and 0.2 mM, respectively, under which the recombinant enzyme could be expressed in a soluble form with activity.

Greatly enhanced secretion by EDTA and lysozyme

In order to increase the secretion level of the protein product into the culture medium, we attempted to add EDTA and lysozyme to the culture medium at the induction time of 12h. As shown in

Figure 2, addition of 0.015%lysozyme alone had little influence on the secretion. Unexpectedly, based on the addition of 0.5% EDTA at the induction time of 12h, simultaneous addition of lysozyme could further increase the extracellular endoglucanase activity, which suggested that EDTA might permit lysozyme to attack the peptidoglycan layer in *E. coli*. Below a final concentraton of 0.015%, higher the concentration of extra lysozyme resulted in more extracellular endoglucanase activity. Further increase in lysozyme concentration led to little influence on extracellular endoglucanase activity. The extracellular endoglucanase activity with the simultaneous addition of 0.5% EDTA and 0.015% lysozyme was over 50% higher than that of addition of 0.5% EDTA alone, indicating existence of the synergism of EDTA and lysozyme. These results

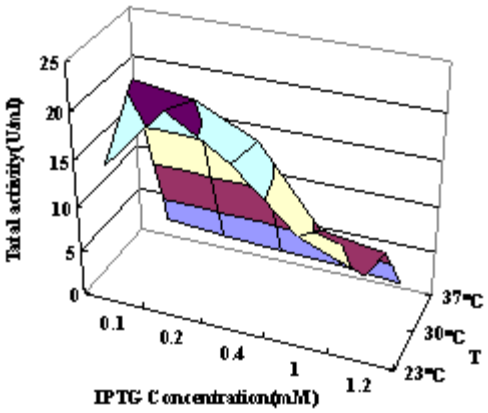

Fig. 1. Effect of temperature and IPTG concentration on the expression of EG-I-1. The expression of EG-I-1 in *E. coli* was induced by IPTG with different concentrations and incubated at different temperatures and the total activity was measured

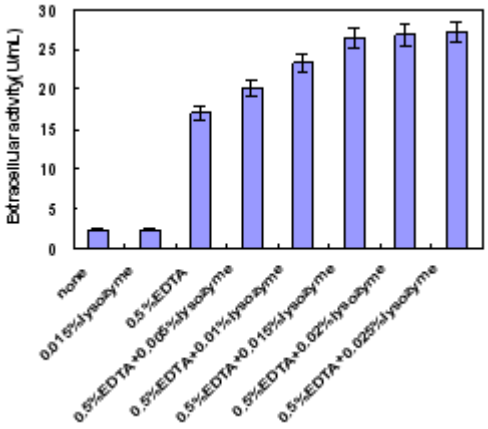

Fig. 2. Synergism of EDTA and lysozyme on the secretion of endoglucanase. 0.5%EDTA, 0.015% lysozyme, as well as 0.5%EDTA and lysozyme with different concentrations were respectively added to the medium of recombinant *E. coli*. at the induction time of 12h. Extracellular endoglucanase activities were measured after 22h induction by IPTG

demonstrated that EDTA may improve the permeability of the outer membrane of *E.coli*, leading to the entry of lysozyme through the outer membrane to act on the peptidoglycan layer, which was in accordance with the previous report on the change in outer membrane of *E.coli* caused by EDTA(Falconer *et al.,*1997). Similarly, the enhanced secretion of the endoglucanase from the cytoplasm of *E.coli* into the medium may be partly explained by the improvement of permeability of both cytoplasmic and outer membranes through the synergism of EDTA and lysozyme. Under the optimum culture conditions, about 2/3 of total endoglucanase was released into the medium and an 11.5-fold increase in secretion level was obtained.

Greatly enhanced expression by EDTA and lysozyme

To study whether the combined use of EDTA and lysozyme had any synergistic effect on the expression of the recombinant endoglucanase, 0.5%EDTA, 0.015%lysozyme, as well as 0.5%EDTA and 0.015%lysozyme were respectively added to the medium of recombinant *E.coli* at the induction time of 12h. As shown in Table1, addition of 0.5% EDTA increased total endoglucanase activity by 44.5% compared with that without EDTA, while that of lysozyme had no significant influence on the expression of recombinant enzyme. In contrast, when 0.5% EDTA and 0.015% lysozyme were simultaneously added at the induction time of 12h, an 83% increase in total endoglucanase activity

was observed, demonstrating the significant enhancement of expression of the recombinant enzyme by the synergism of EDTA and lysozyme. Under the optimum culture conditions, the extracellular and total endoglucanase activities reached 26.5 and 40 U/mL, respectively. The finding of the great enhancement of expression of the recombinant enzyme by the synergism of EDTA and lysozyme may be particularly interesting and deserve further investigation.

The results described above indicated that the synergism of EDTA and lysozyme might have two kinds of influences, namely, a selective protein release effect and a stimulatory effect on protein expression. The mechanism for the enhancement of secretion and expression by the synergism of EDTA and lysozyme is under our present investigation.

Table 1. Synergism of EDTA and lysozyme on the expression of endoglucanase

Activity (U/mL)	12h	Induction for 22h			
		Without additive	Lysozyme	EDTA	EDTA+Lysozyme
Extracellular activity	0.2	2.3	2.3	17.0	26.5
Intracellular activity	18.7	19.5	19.5	14.5	13.5
Total activity	18.9	21.8	21.8	31.5	40.0

0.5%EDTA, 0.015%lysozyme, 0.5%EDTA and 0.015%lysozyme were respectively added to the medium of recombinant *E. coli*. at the induction time of 12h. Extracellular, intracellular and total endoglucanase activities at the induction time of 12 and 22h were measured, respectively.

Purification of native and recombinant enzymes

The purification of the native endoglucanase from the culture supernatant of *B.akibai* I-1 was performed by a two-step column-chromatographic procedure including ion-exchange and gel-filtration chromatography(Figure 3A). As evident in Figure 3B, the recombinant enzyme was efficiently purified to homogeneity only by a simple one-step column-chromatographic procedure on a DEAE Sepharose CL-6B column (Lane B2). Interestingly, as depicted in Figure 3(Lane B1), the protein band of interest in SDS-PAGE was very bright while the concentration of other foreign proteins in the culture supernatant of recombinant *E. coli* was much lower, which demonstrated that the cultivation method of addition of EDTA and lysozyme selectively enhanced the secretion of endoglucanase and promoted purification of the protein of interest. This was unlike the former reports on the secretion of periplasmic proteins that, along with the proteins of interest, all other periplasmic proteins in *E. coli* were also released into the medium by the strategies of permeabilization of outer membranes(Badyakina *et al.*,2005). Additionally, the culture supernatant of *B.akibai* I-1 contained extremely low level of the protein of interest (Lane A1), whereas a clear band was observed in the crude culture supernatant of recombinant *E. coli* (Lane B1), demonstrating the efficient extracellular secretion and overexpression of the recombinant endoglucanase in *E. coli*. The recombinant enzyme had a molecular weight of about 90 kDa estimated by SDS-PAGE, which agrees with the expected molecular mass of native enzyme.

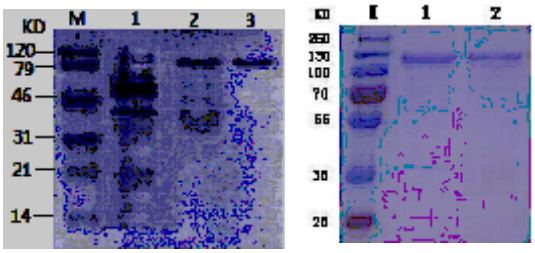

Fig. 3. SDS-PAGE of samples during purification of native(**A**) and recombinant(**B**) endoglucanases. **M:** molecular weight markers. Lane A**1**: crude culture supernatant of *B.akibai* I-1, Lane A**2**: eluate from DEAE Sepharose CL-6B column, Lane A**3**: sample of the purified native enzyme. Lane B**1**: crude culture supernatant of recombinant *E. coli*, Lane B**2**: eluate from DEAE Sepharose CL-6B column

Characterization of recombinant and native enzymes

The effect of pH on endoglucanase activity of the purified enzyme was examined at various pH values ranging from 4.0 to 11.0. As shown in Figure 4A, both the native and recombinant enzymes exhibited optimum endoglucanase activity at approximately pH 9.0, characteristic of alkaline endoglucanase. To examine enzyme stability with changes in pH value, the enzyme was incubated in 0.01M buffer of different pH values at 30°C for 30min and then residual activity was measured under standard assay conditions. As depicted in Fig. 4B, the recombinant endoglucanase was stable in the broad range of pH 6-11 and showed the excellent alkali-tolerance of the enzyme, which was the same as the native enzyme.

The effect of temperature on endoglucanase activity of the enzyme was examined at various temperatures ranging from 30 to 70°C. It was found that both the native and recombinant enzymes exhibited maximum activity at around 50°C (Fig. 5A). The thermal stability of the enzyme was determined by heating for 30min at the temperature indicated in the range of 30-80°C and then assaying the residual activity at 40°C under standard assay conditions. Figure 5B showed that, when temperature was below 55°C, the recombinant enzyme was stable and retained more than 80% activity. Further increase in temperature led to a slow decrease in residual activity and remained over 30% of residual activity even after heating at 70-80°C, indicating the good thermostability of the enzyme, which was similar to that of the native enzyme.

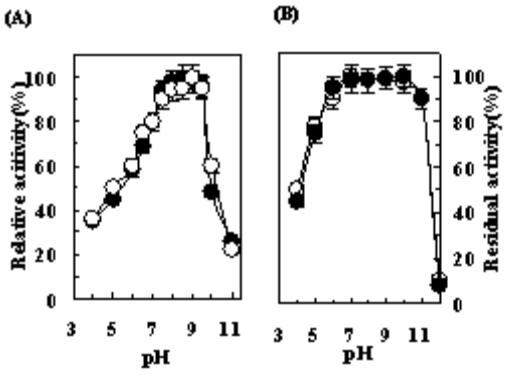

Fig. 4. Effect of pH on the activity (A) and stability (B) of the purified recombinant (´) and native(˜) enzymes. A The effect of pH on the activity was determined with CMC as substrate at 40°C for 20min in various pH values. The maximum enzyme activity at pH 9.0 was taken as 100%. B The purified enzyme was incubated at 30°C for 30 min in various buffers (0.01M) and then the residual activities were assayed at 40°C and at pH 9.0. The values are shown as percentages of the maximum activity, taken as 100%

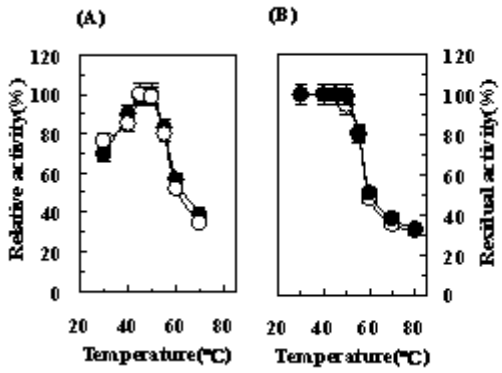

Fig. 5. Effect of temperature on the activity (A) and stability (B) of the purified recombinant (™) and native(˜) enzymes. A The purified enzymes were added to 1.0% CMC in 0.1M Tris-HCl(pH8.0), and the enzyme activity was measured at various temperatures. Each maximum activity of recombinant and native enzymes was taken as 100%. B The purified recombinant and native enzymes were preincubated in 0.1M Tris-HCl(pH8.0), respectively, for 20min at the temperatures indicated. Portions of the solution were withdrawn and the residual activities of recombinant and native enzyme were measured as described in the assay at 40°C, respectively. Each activity treated at 30° for 20min was taken as 100%

To examine the effect of metal ions and reagents on the enzyme, the purified enzyme was incubated with 1mM various metal ions, 0.05%EDTA, 0.05%EGTA, 0.01% SDS, respectively, at 30°C for 10min in Tris-HCl buffer(pH8.0) and the residual endoglucanase activity was measured under the standard conditions of the assay. As a result, the activities of both native and recombinant enzymes were not affected by metal ions including Na^+, K^+, Mg^{2+}, Cu^{2+}, $Fe^{2+}$and Ca^{2+} ions and

chelating agents such as EDTA and EGTA. As shown in Table2, the native enzyme was partly inhibited by SDS(about 80% residual activity remained), while the recombinant enzyme had good SDS-resistance(residual activity>99%). In order to examine the possibility of the application of the recombinant enzyme in detergents, 0.2% laundry detergent was added and no decrease in residual activity(residual activity>99%) was observed in 0.2%(w/v) laundry detergent, much better than the native enzyme. These results indicated that the recombinant enzyme was among some rare endoglucanases that had good SDS-resistance, which strongly inhibited the activities of many other endoglucanases (Dong *et al.,* 2010).

Table 2. Effect of SDS on the recombinant and native endoglucanases

Enzyme	Residual activity(%)		
	0.01% SDS detergent	0.1% laundry detergent	0.2% laundry
Native	80	80	71
Recombinant	>99	>99	>99

The purified recombinant and native enzymes were incubated with 0.01% SDS, 0.1% and 0.2%(w/v) Liby® laundry detergents, respectively, at 30°C for 20min in Tris-HCl buffer(pH8.0). The residual activity was measured under the standard conditions of the assay. Each activity without additives was taken as 100%.

CONCLUSIONS

The alkaline endoglucanase gene from *B.akibai* I-1 can be overexpressed in *E.coli* in a soluble form with activity under the optimal culture conditions of 23-30°C and 0.2 mM IPTG. Both secretion and expression level of the soluble recombinant enzyme could be greatly enhanced by addition of EDTA and lysozyme. Both purified native and recombinant endoglucanases have excellent properties such as good alkali-resistance, thermostability and resistibility to various metal ions and chelating agents examined, and the recombinant enzyme has improved resistibility to SDS, showing great potential in laundry detergents industry.

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