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ORIGINAL PAPER

Enhanced secreting expression and improved properties of a recombinant alkaline endoglucanase cloned in *Escherichia coli*

Sen-Lin Liu · Wei-Zhao Chen · Gang Liu · Miao Xing

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Abstract An alkaline endoglucanase from *Bacillus akibai* III-3A was successfully expressed in *Escherichia coli* in active form, and secretion was greatly enhanced by addition of 5 g/l ethylenediamine tetraacetic acid (EDTA) to the culture medium at the induction time of 12 h. Under the optimal culture conditions, extracellular and total endoglucanase activities were 18.5 and 31.2 U/ml, respectively. Both the recombinant and native enzymes exhibited similar properties with respect to broad pH stability, good thermostability, and resistibility to various metal ions and reagents examined. However, unlike the native endoglucanase that was partly inhibited by sodium dodecyl sulfate (SDS), the recombinant enzyme had good resistibility to SDS, being very stable in the commercial detergents, and no decrease in residual activity was observed in 0.2% (w/v) laundry detergent, indicating that it was suitable for application in detergents industry.

Keywords Alkaline endoglucanase · Recombinant enzyme · Resistibility · *Escherichia coli* · Secretion

Introduction

Cellulases produced by fungi have been thoroughly investigated in recent years [1, 2, 12, 17, 19]. In contrast to this, study of bacterial cellulases lags behind that of fungal enzymes [11, 20, 26], due to the fact that bacteria lack the complete cellulase system and the main activity is endoglucanase (endo-1,4-β-glucanase, EC 3.2.1.4) which only randomly hydrolyzes internal 1,4-β-bonds in cellulose and does not cleave crystalline cellulose [15, 18]. Since the discovery of alkaline cellulase (mainly endoglucanase) from *Bacillus* by Horikoshi et al. in 1984 [9], the alkaline endoglucanases produced by bacteria have attracted much attention for their revolutionary application in laundry detergents [10], and there has been a rapid growth in demand for alkaline endoglucanases, especially those with excellent properties such as broad pH stability, good thermostability, and resistibility to metal ions and reagents [6, 8, 14].

We recently purified an alkaline endoglucanase from *Bacillus akibai* III-3A that had broad pH stability, good thermostability, and resistibility to various metal ions and chelating agents examined. Unfortunately, this enzyme was partly inhibited by SDS (only 70% residual activity remained), which might be an obstacle for the enzyme to become an effective additive in detergents, and the level of the enzyme protein in the culture supernatant of *Bacillus akibai* III-3A was extremely low. Therefore, we attempted to develop a system, including expression of endoglucanase in *Escherichia coli* (*E. coli*), for secreting sufficient enzyme protein and improving resistibility to SDS as well. However, in the *E. coli* expression system, the quantity of enzyme secreted to the culture medium was very low and the expression products were usually accumulated in *E. coli* cells in the form of inclusion bodies without activity [22, 23, 25]. For the first time, we found that secretion of endoglucanase to the culture medium in active form could be greatly enhanced by addition of EDTA to the culture medium of recombinant *E. coli* and that the recombinant enzyme had good resistibility to SDS. In this report, we primarily describe the enhanced secreting expression in active

S.-L. Liu (✉) · W.-Z. Chen · G. Liu · M. Xing
Shenzhen Key Laboratory for Microbial Gene Engineering, College of Life Science, Shenzhen University, Shenzhen 518060, People's Republic of China
e-mail: liulsl@szu.edu.cn

form and the improved properties of this recombinant alkaline endoglucanase.

Materials and methods

Strains and plasmids

Bacillus akibai III-3A was used as the source of the gene of interest (III-3a), which was originally isolated from an alkaline soil sample. *Bacillus akibai* III-3A was cultured in a carboxymethylcellulose (CMC)-containing liquid medium (w/v) of 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, 0.5% Na_2CO_3 (separately autoclaved). The organism was grown with shaking (200 rpm) at 35°C for 48 h in 50 ml medium in 250-ml flasks. The culture of *Bacillus akibai* III-3A was centrifuged at 14,000 × *g* for 10 min, and the supernatant was stored at −20°C for purification.

E. coli BL21 (DE3) and vector pET-28a(+) were used for expression of endoglucanase. *E. coli* Top10F′ and vector pGEM-T were used for plasmid construction and propagation. *E. coli* growth media were prepared according to the *E. coli* expression system manual from Invitrogen. Carboxymethylcellulose (CMC, 300–800 mPa·s), Liby® laundry detergent, and all other chemicals were also from commercial sources and of analytical grade.

Cloning and sequence analysis

DNA extraction and plasmid DNA preparation were carried out according to the established methods [21]. The gene of interest was amplified by polymerase chain reaction (PCR) from the chromosomal DNA of *Bacillus akibai* III-3A using the following degenerate primers derived from the conserved N- and C-terminal end regions of the known endoglucanases from KSM-64 [23] and *Bacillus* sp. 1139 [5]: 5′-GAYCCNGTNTAYGCNGGNGC-3′ and 5′-TTA TTTTTTCGTAGCCTC-3′. PCR amplification was as follows: 94°C for 5 min, followed by 30 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 pGEM-T vector and transformed to *E. coli* Top10F′, which was sequenced by Takara Company of China. The protein sequences were aligned using BlastP (http://www.ncbi.nlm.nih.gov/Blast/).

Construction of the expression plasmid and screening of recombinant colony

The coding sequence III-3a, excluding the signal peptide, was amplified by PCR using the following primers: 5′-GAT*GA ATTC*GAAGGAAACACTCGTGAAGAC-3′ (containing a *Eco*RI site) and 5′-GTT*AAGCTT*TTATTTTTTCGT AGCCTCTTTC-3′ (containing a *Hin*dIII site). The PCR product was gel-purified, digested with *Eco*RI and *Hin*dIII, and ligated into the corresponding sites of the vector pET-28a(+). The recombinant plasmid pET-28a(+)-III-3a was then transformed into *E. coli* DL21 (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 as described by Teather and Wood [24]. A relatively efficient endoglucanase-producing transformant was picked, containing recombinant plasmid pET-28a(+)-III-3a, from a single colony.

Expression of recombinant enzyme in *E. coli*

The transformant was grown overnight at 37°C in LB medium supplemented with 25 mg/ml kanamycin. The culture was then incubated in fresh LB medium containing kanamycin to OD_{600} of 0.6. Isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) was added to final concentration of 0.2 mM. At the induction time of 12 h, EDTA with final concentration of 5 g/l was added to the culture medium. The culture were incubated at 23°C for 22 h induction and harvested by centrifugation at 14,000 × *g* for 10 min at 4°C. The supernatant was used for measurement of extracellular endoglucanase activity and further purification. To determine intracellular endoglucanase activity, cells collected by centrifugation were washed, resuspended in Tris–HCl buffer (pH 8.0), and disrupted by French press [16]. The mixture was then centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatant was collected for measurement of intracellular endoglucanase activity.

Purification of native and recombinant enzymes

The centrifugal supernatant of culture broth of *Bacillus akibai* III-3A was dialyzed against distilled water. The solution was directly applied to a DEAE Sepharose fast flow 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 stepwise increasing gradient (i.e., 0.4, 0.45, and 1 M) of NaCl at flow rate of 180 ml/h. The fractions containing CMCase activity were dialyzed and concentrated by PEG-20000. The concentrate 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 (pH 8.0) at flow rate of 60 ml/h. The enzyme recovered from the column was pooled and stored at 4°C.

Purification of the recombinant enzyme was performed by a one-step column-chromatographic procedure on the

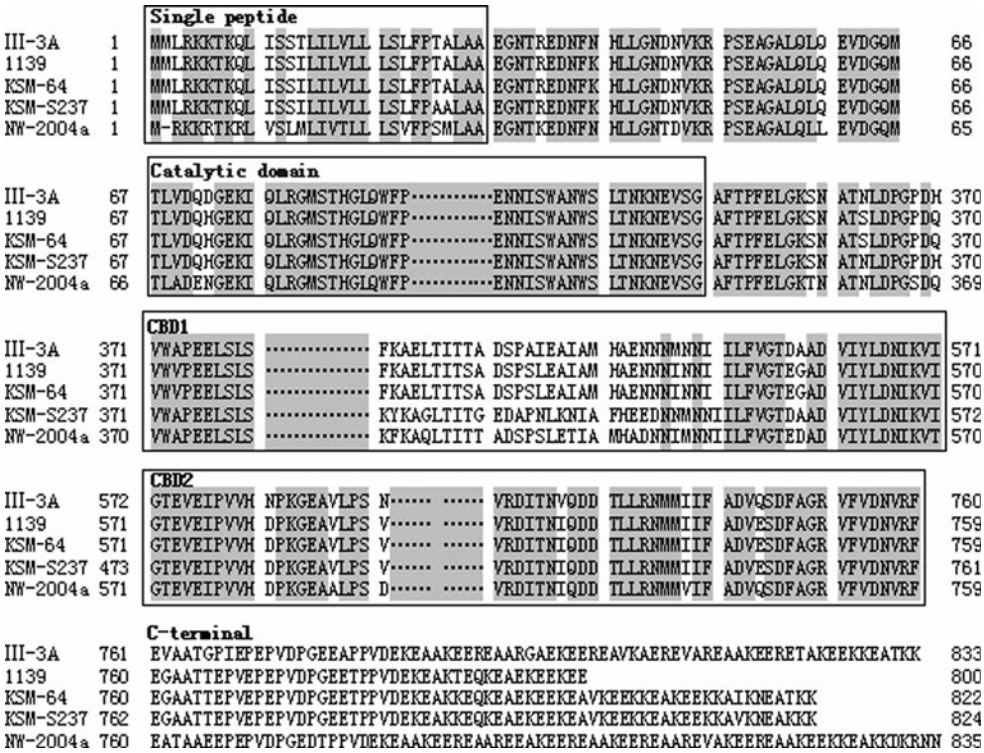

Fig. 1 Partial amino acid sequence alignment of deduced peptide sequence of *Bacillus akibai* III-3A with those of family A5 alkaline endoglucanases. The GenBank accession numbers of amino acids from *Bacillus* sp. 1139, *Bacillus* sp. KSM-64, *Bacillus* sp. KSM-S237, *Bacillus* sp. NW-2004a are P06564.1, AAA73189.1, JC7532, and AAT97264.1, respectively. *CBD* cellulose-binding domain

DEAE Sepharose fast flow column as described for the native enzyme.

Assay of enzyme activity

Carboxymethylcellulose (CMC) was used as the substrate for the endoglucanase activity assay. The reaction mixture contained 1 ml 1% (w/v) CMC solvated in Tris–HCl buffer (0.05 M, pH 8.0) and 0.1 ml 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 to 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 enzyme activity was defined as the amount of enzyme that produced 1 mg product per hour.

Protein electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed basically according to the method described by Sambrook et al. [21]. The concentration of the separation gel was selected as 12.5%. For molecular mass determination, the prestained protein molecular weight marker was used and protein was stained with Coomassie Brilliant Blue R-250.

Results and discussion

Amino acid sequence analysis

An open reading frame (ORF) of 2,502 nucleotides that started from ATG and ended with TAA was successfully amplified by PCR from chromosomal DNA of *Bacillus akibai* III-3A, which encoded 833 amino acid residues. As shown in Fig. 1, the deduced amino sequence contained a 30-amino-acid signal peptide composed of many hydrophobic amino acids, which was similar to the signal peptides of alkaline endoglucanases from *Bacillus* sp. 1139 [5] and *Bacillus* sp. KSM-64 [23].

Amino acid sequence analysis showed that it contained three conserved domains with distinct structures and functions, including a catalytic domain at the N-terminal end belonging to glycoside hydrolase family 5 and two cellulose-binding domains (CBD) both belonging to the carbohydrate-binding modules of family 17/28. A basic local alignment search tool (BLAST) search showed homology between the alkaline endoglucanase from *Bacillus akibai* III-3A and those from *Bacillus* sp. 1139 (92%), *Bacillus* sp. KSM-64 (92%), *Bacillus* sp. KSM-S237 (91%), and *Bacillus* sp. NW-2004a (90%) [5, 7, 23]. However, low similarity was found in the C-terminal amino acid sequence, which might be partly responsible for the superior characteristics of the alkaline endoglucanase from *Bacillus akibai* III-3A.

Enhanced secreting expression of recombinant endoglucanase by addition of EDTA

The coding sequence without the N-terminal signal peptide was amplified by PCR and subcloned into the vector pET-28a(+). The resulting expression recombinant vector pET-28a(+)-III-3a with a C-terminal His-tag coding sequence was then introduced into *E. coli* DL21 (DE3) strain to express the endoglucanase. However, only very low endoglucanase activity was detected in the culture supernatant of recombinant *E. coli*, similar to the previous report [13, 22]. To enhance secretion of the protein product into the culture medium, for the first time, we attempted to add EDTA to the culture medium after 12 h induction by IPTG. As shown in Fig. 2, extracellular endoglucanase activity was greatly enhanced by addition of EDTA, and below final concentration of 8 g/l, the higher the concentration of EDTA added, the more extracellular endoglucanase activity was obtained. However, further increase of the added EDTA concentration led to a drop in extracellular enzyme activity.

To further investigate the influence of addition of EDTA on the secretion, a time-course study and SDS-PAGE analysis of extracellular endoglucanase secreted by recombinant *E. coli* were carried out (Fig. 3). As can be seen in Fig. 3, before the addition of EDTA, low extracellular endoglucanase activity and low level of secreting protein were detected. In contrast, after the addition of EDTA with final concentration of 5 g/l at the induction time of 12 h, the extracellular endoglucanase activity and the level of secreting protein increased sharply, which implied great enhancement of secretion of recombinant enzyme into the culture medium.

The effect of addition of EDTA on the extracellular, intracellular, and total endoglucanase activities is outlined in Table 1. As shown in Table 1, addition of 5 g/l EDTA to the culture medium at the induction time of 12 h increased the secretion level by about 3.5-fold and also increased the total activity by about 26.3%. Under the optimum conditions, the extracellular and total endoglucanase activities were 18.5 and 31.2 U/ml, respectively. The mechanism for the enhancement of secretion by addition of EDTA is now under investigation.

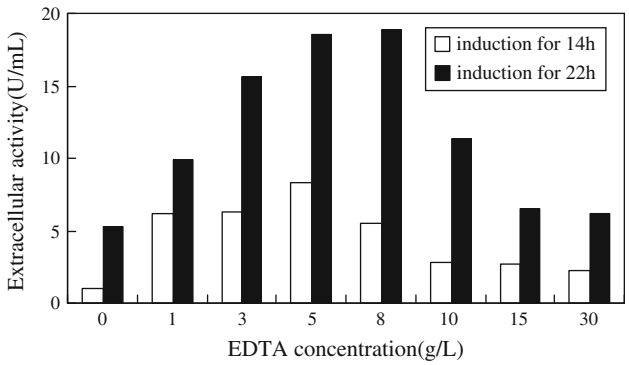

Fig. 2 Effect of EDTA concentration added on the extracellular endoglucanase activity. EDTA was added at the induction time of 12 h. The extracellular endoglucanase activity was measured directly from the supernatant

Purification of native and recombinant endoglucanases

Purification of native endoglucanase from culture supernatant of *Bacillus akibai* III-3A was carried out by a two-step column-chromatographic procedure involving ion-exchange and gel-filtration chromatography (Fig. 4a), while that of recombinant enzyme from *E. coli* was done only by a simple, one-step column-chromatographic procedure on a DEAE Sepharose fast flow column (Fig. 4b). This ion-exchange chromatography procedure is highly efficient in providing large amounts of pure enzyme. The culture supernatant of *Bacillus akibai* III-3A contained extremely low level of the enzyme protein (lane A3), whereas a clear band was observed in the crude culture supernatant of recombinant *E. coli* (lane B1), demonstrating efficient secretion of the recombinant enzyme to the culture medium. The molecular mass of recombinant endoglucanase was estimated to be about 90 kDa, which agrees with the expected molecular mass.

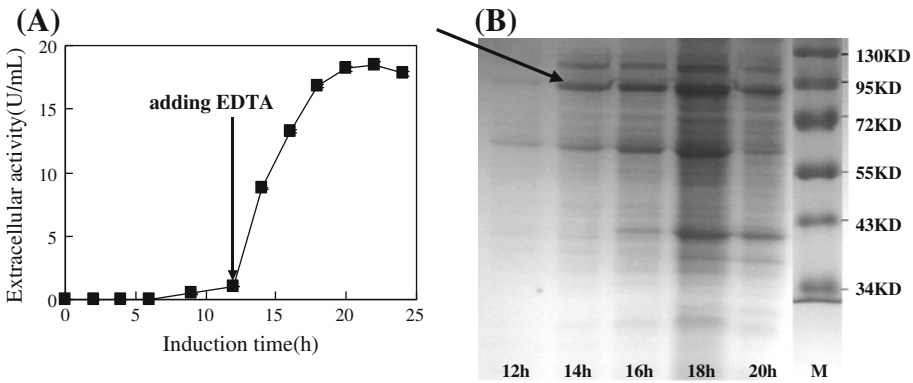

Fig. 3 **a** Time course of extracellular endoglucanase produced by recombinant *E. coli*. EDTA of final concentration of 5 g/l was added at the induction time of 12 h. **b** SDS–PAGE analysis of recombinant endoglucanase. Lanes correspond to samples of 12, 14, 16, 18, and 20 h culture supernatants following induction with IPTG; *M* molecular weight markers. The recombinant endoglucanase is marked by the *arrow*

Properties of the recombinant endoglucanase

The effect of pH on CMCase activity of the purified enzymes was examined at various pH values ranging from 4.0 to 11.0. As can be seen in Fig. 5a, the purified recombinant endoglucanase exhibited optimal CMCase activity at about pH 9.0, similar to that of the native enzyme. To explore enzyme stability with changes in pH value, the purified enzymes were incubated in 0.01 M buffer of different pH values at 30°C for 30 min, and then residual activities were measured under standard assay conditions. As shown in Fig. 5b, recombinant endoglucanase had a broad pH stability range from 5.0 to 11.0, which was the same as native enzyme.

The effect of temperature on CMCase activity of the purified enzymes was examined at various temperatures ranging from 30°C to 80°C. As shown in Fig. 6a, both the recombinant and native enzymes exhibited maximum activity at around 45°C. The thermal stability of the purified enzymes was determined by heating for 20 min 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 6b shows that both the recombinant and native enzymes were stable up to 50°C.

Table 1 Effect of addition of EDTA on extracellular, intracellular, and total endoglucanase activities

Activity (U/ml)	Induction for 12 h	Induction for 22 h	
		Without EDTA added	With EDTA added
Extracellular activity	0.7	5.3	18.5
Intracellular activity	19.3	19.4	12.7
Total activity	20	24.7	31.2

EDTA at final concentration of 5 g/l was added at the induction time of 12 h

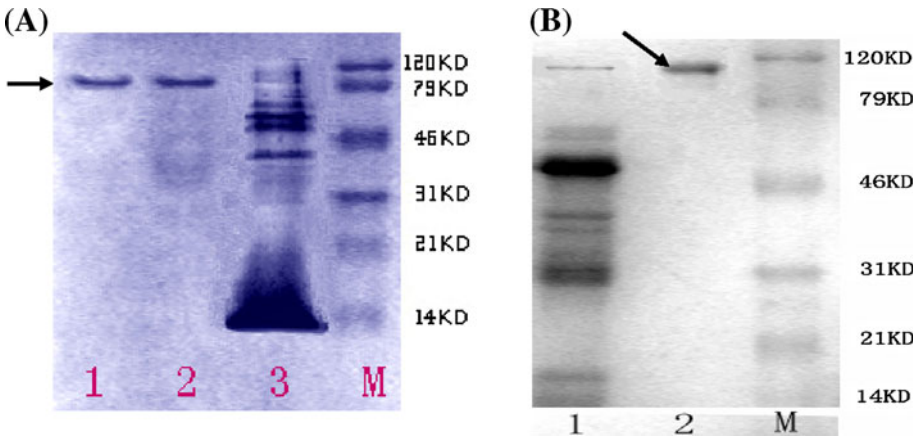

Fig. 4 SDS-PAGE of samples during purification of **a** native and **b** recombinant endoglucanases. *M* molecular weight markers. *Lane A1* sample of the purified native enzyme, *lane A2* eluate from DEAE Sepharose fast flow column, *lane A3* crude culture supernatant of *Bacillus akibai* III-3A. *Lane B1* crude culture supernatant of recombinant *E. coli*, *lane B2* sample of the purified recombinant enzyme. The proteins of interest are marked by *arrows*

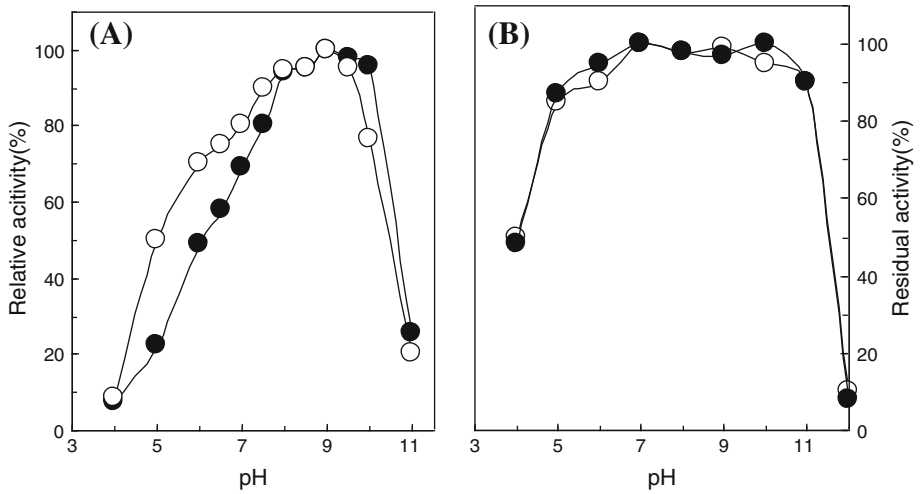

Fig. 5 Effect of pH on the activity (**a**) and stability (**b**) of the purified recombinant (*opened circles*) and native (*filled circles*) enzymes. **a** The effect of pH on the activity was determined with CMC as substrate at 40°C for 20 min at various pH values. The maximum enzyme activity at pH 9.0 was taken as 100%. **b** The purified enzyme was incubated at 30°C for 30 min in various buffers (0.01 M), and then the residual activities were assayed at 40°C and at pH 9.0. Values are shown as percentages of maximum activity. The buffers used were citric acid (pH 4 to 6), phosphate (pH 7), Tris-HCl (pH8), glycine-NaCl (pH 9–11), and phosphoric acid-NaOH (pH 12)

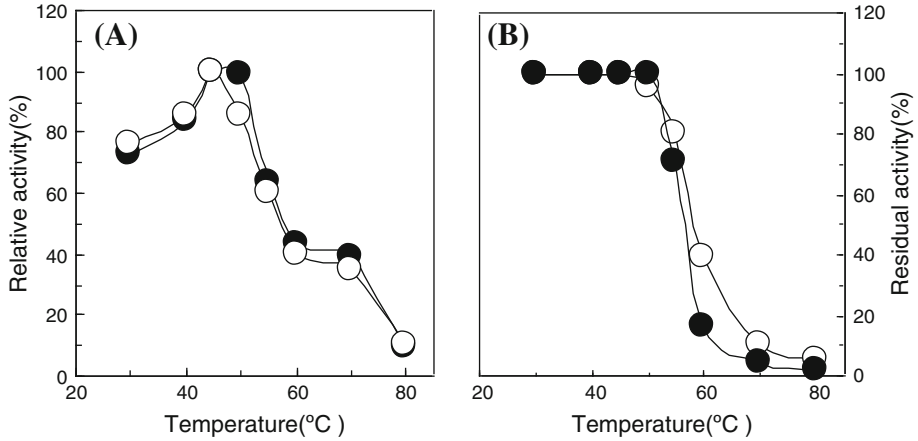

Fig. 6 Effect of temperature on the activity (**a**) and stability (**b**) of the purified recombinant (*opened circles*) and native (*filled circles*) enzymes. **a** The purified enzymes were added to 1.0% CMC in 0.1 M Tris–HCl (pH8.0), and the enzyme activity was measured at various temperatures. Each maximum activity of recombinant and native enzymes was taken as 100%. **b** The purified recombinant and native enzymes were preincubated in 0.1 M Tris–HCl (pH8.0), respectively, for 20 min 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°C for 20 min was taken as 100%

To examine the effect of metal ions and reagents on the recombinant enzyme, the purified enzymes were incubated with 1 mM of various metal ions, 0.05% EDTA, 0.05% ethylene glycol tetraacetic acid (EGTA), and 0.01% SDS, respectively, at 30°C for 20 min in Tris–HCl buffer (pH8.0), and the residual endoglucanase activity was measured under the standard conditions of the assay. It was found that both recombinant and native enzymes were resistant to metal ions including Na^+, K^+, Mg^{2+}, Cu^{2+}, Fe^{2+}, and Ca^{2+} ions and chelating agents such as EDTA and EGTA. However, unlike the native endoglucanase which was partly inhibited by SDS (only 70% residual activity remained), the recombinant enzyme had good resistibility to SDS (Table 2). Recombinant endoglucanase was very stable in the commercial detergents, and no decrease in residual activity was observed in 0.2% (w/v) laundry detergent, much better than the native enzyme. These results indicate that the recombinant enzyme is among the rare endoglucanases that have good resistibility to SDS, which is the major component of laundry detergents and strongly inhibited many other endoglucanases [3, 4, 8, 18]. Amino acid sequencing analysis of the native enzyme showed that low similarity was found in the C-terminal amino acid sequence, and thus the additional C-terminal His-tag in the recombinant enzyme might be partly responsible for this improved resistibility to SDS.

Table 2 Effect of SDS on the recombinant and native endoglucanases

Enzyme	Residual activity (%)		
	0.01% SDS	0.1% laundry detergent	0.2% laundry detergent
Native	70	80	72
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 20 min in Tris–HCl buffer (pH 8.0). The residual activity was measured under the standard conditions of the assay. Each activity without additives was taken as 100%

Conclusions

An alkaline endoglucanase from alkaliphilic *Bacillus* was successfully expressed in *E. coli* in active form, and secretion was greatly enhanced by addition of 5 g/l EDTA to the culture medium at the induction time of 12 h. The recombinant endoglucanase had improved resistibility to SDS and was very stable in commercial detergent, having broad pH stability, good thermostability, and resistibility to various metal ions and reagents examined, and thus showed great potential for application in detergents industry.

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