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# Cloning of a β-1,3-1,4-Glucanase Gene from *Bacillus subtilis* MA139 and its Functional Expression in *Escherichia coli*

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**Abstract** A gene encoding β-1,3-1,4-glucanase was cloned by polymerase chain reaction (PCR) from *Bacillus subtilis* MA139. Sequencing result showed 97% homology to the corresponding gene from *Bacillus licheniformis*. The open reading frame (ORF) of the gene contained 690 bp coding for a 226 amino-acid matured protein with the estimated molecular weight of 24.44 kDa. The β-1,3-1,4-glucanase gene was subcloned into an expression vector of pET28a and expressed in *Escherichia coli* BL21 and then purified by metal affinity chromatography using a nickel–nitrilotriacetic acid (Ni–NTA) column. The purified β-1,3-1,4-glucanase demonstrated 24.05 and 12.52 U $ml^{-1}$ activities for the substrates of barley β-glucan and lichenan, respectively, and the specific activities were 728.79 and 379.1 U $mg^{-1}$ for them, respectively. The optimal temperature and pH of the purified enzyme were 40°C and 6.4, respectively. When barley β-glucan was used as the substrate, $K_m$ was 5.34 mg $ml^{-1}$, and $K_{cat}$ showed 7,206.71 $S^{-1}$, thus the ratio of $K_{cat}$ and $K_m$ was 1,349.67 ml $s^{-1}$ $mg^{-1}$. The activity of β-1,3-1,4-glucanase was affected by a range of metal ions or ethylenediaminetetraacetic acid (EDTA).

**Keywords** *Bacillus subtilis* · β-1,3-1,4-glucanase · Cloning · Expression

## Introduction

β-1,3-1,4-glucan is a major linear polysaccharide in endosperm cell wall of barley, wheat, rye, rice, etc., which accounts for more than 5.5% of the grain dry weight [1]. β-1,3-1,4-glucanase (lichenase, EC 3.2.1.73) can hydrolyze the β-1,4-glycosidic bond adjacent to the β-1,3-glycosidic bond of barley β-glucan and lichenan, which results in the hydrolysate mainly comprised of cellobiosyltriose and cellotriosyltetraose [2].

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Bacterial β-1,3-1,4-glucanase belongs to the glycosyl hydrolase family 16 (GH16) characterized by a conserved "EIDIEF" motif [3] in which the two glutamic acid residues showed functional effect on the hydrolytic activity [4].

Feed supplementation of β-glucanase can improve average daily gain, feed intake and feed conversion rate [5] by enhancing ileum digestibility [6], as β-glucanase can decrease the chyme viscosity in the small intestine.

The genus of *Bacillus* is the source of extracellular hydrolytic enzymes [7], and *Bacillus subtilis* is popularly applied as a host system in genetic engineering studies [8]. β-1,3-1,4-Glucanase gene has been cloned from numerous bacteria, such as, *Bacillus licheniformis* [9], *Bacillus halodurans* [10], *B. subtilis* [11], and the fungi, *Orpinomyces* [2]. Expression of β-1,3-1,4-glucanase gene had also been conducted in *Escherichia coli* [9] and *Pichia pastoris* [12]. The specific activity were 7,980 U $mg^{-1}$ after expression in *E. coli* of the truncated β-1,3-1,4-glucanase (TF-glucanase) [12]. The glycosylated TF-glucanase was the most active known β-1,3-1,4-glucanase, with a specific activity of 10,800 U $mg^{-1}$ after expression in *P. pastoris* [12].

*B. subtilis* MA139 has been successfully isolated in our previous study [13] which contains β-1,3-1,4-glucanase. The objective of this study was to clone the β-1,3-1,4-glucanase gene from *B. subtilis* MA139 and heterologously expressed in *E. coli* BL21. Subsequently, the enzyme was characterized in the biochemical aspect.

## Materials and Methods

### Strains, Vectors, and Chemicals

*B. subtilis* MA139 isolated from pig intestine exhibited high antimicrobial activity [13]. Further study showed that β-1,3-1,4-glucanase could be secreted by this bacteria (unpublished). *E. coli* DH5α and BL21 were used as the host strains. pUCm-T was purchased from Sangon (Shanghai, China), and pET28a (Novagen, Madison, WI, USA) was stored in our lab. Yeast extract and Tryptone were purchased from OXOID (Hampshire, England). All other chemicals used in this study were of analytical grade commercially available.

### Cloning and Sequencing of the β-1,3-1,4-Glucanase Gene

The *B. subtilis* MA139 was grown overnight at 37°C in Luria–Bertani (LB) medium (5 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ tryptone, and 10 g $l^{-1}$ NaCl). Genomic DNA was isolated according to the manual of Genome DNA Extraction Kit (Sangon, Shanghai, China). Two primers, (BS-glu1, 5′-GGATTGTTTATGAGTTTGT-3′, corresponding to the 5′ end of the cloned gene, and BS-glu2, 5′-TTATTTTTTT(G/C)TATAGCGCA-3′, complementary to the 3′ end of the cloned gene) were designed by alignment of three *Bacillus* β-1,3-1,4-glucanase genes with the GenBank accession no. U60830, AF490978, and Z46862, respectively. Polymerase chain reaction (PCR) consisting of 40 cycles amplification was performed denaturation at 94°C for 1 min, annealing at 40°C for 30 s and extension at 72°C for 1.5 min. An additional extension step was at 72°C for 10 min. The PCR products were purified by UNIQ-10 Column Collection Tube Kit (Sangon, Shanghai, China) and ligated with the pUCm-T vector, and the ligation products were transformed into *E. coli* DH5α-competent cells. Transformants were screened by colony PCR using BS-glu1 and BS-glu2 as two primers. Positive transformants were further confirmed by sequencing using dideoxy chain-termination method [14].

## Construction of Expression Plasmid

The signal peptide of the full β-1,3-1,4-glucanase gene was removed by PCR using the forward primer (Glu-pro-F) designed from the first codon downstream of the signal peptide and then inserted into pUCm-T. Recombinant pUCm-T plasmid was amplified by PCR with the primers by adding *Eco*RI and *Hin*dIII sites at two ends of the β-1,3-1,4-glucanase gene (Glu-pro-F, 5′-CG*GAATTC*CAAACAGGCGGATCGTTTTTTG-3′ and Glu-pro-R, 5′-CCC*AAGCTT*TTATTTTTTTGTATAGCGCA-3"). The PCR amplification was performed as 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A final extension step was at 72°C for 10 min. The amplified β-1,3-1,4-glucanase gene was purified by UNIQ-10 Column Collection Tube Kit and digested with *Eco*RI and *Hin*dIII (TaKaRa, Tokyo, Japan) and ligated with similarly digested pET28a with corresponding restriction enzymes. The ligation product was transformed into *E. coli* DH5α [15] followed by incubation overnight at 37°C on LB plate supplemented with 100 μg $ml^{-1}$ kanamycin (Invitrogen, San Diego, CA, USA). Transformants were screened by colony PCR using Glu-pro-F and Glu-pro-R primers. Positive transformants were further confirmed by sequencing using dideoxy chain-termination method [14].

## Expression, Purification, Denaturation, and Renaturation

Recombinant expression plasmid was transformed into *E. coli* BL21. Transformed single colony was inoculated in 10 ml kanamycin selection LB medium and cultivated overnight at 37°C. The cell culture was inoculated in 100 ml LB medium by 1:100 dilution and grown until $OD_{600}$ reached 0.6, then 0.5 mmol $l^{-1}$ isopropyl-β-D-thiogalactoside (IPTG; Sangon, Shanghai, China) was added to induce the β-1,3-1,4-glucanase gene expression for 5 h at 30°C.

Cells were harvested by centrifugation, and the pellet was resuspended followed by ultrasound to release misfolded β-1,3-1,4-glucanase protein in inclusion body. After centrifugation at 12,000 rpm for 3 min, the supernatant containing 0.1 mmol $l^{-1}$ phenylmethanesulphonyl fluoride passed through a 0.2-μm membrane filter (Sartorius AG, Goettingen, Germany) and was applied to Ni-NTA (Novagen, Madison, WI, USA) equilibrated with 500 mmol $l^{-1}$ phosphate buffer solution (pH 7.4). After washing with a buffer containing 50 mmol $l^{-1}$ imidazole, the recombinant protein was eluted with elution buffer containing 400 mmol $l^{-1}$ imidazole. Collected fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

As part of the modification of the methods by Patel et al. [16], for unfolding the misfolded protein, 4,700 μl 8 mol $l^{-1}$ urea solution was added into 300 μl purified protein solution, incubated for 30 min. By dialysis against a series of buffers of 6, 4, and 2 mol $l^{-1}$ urea solutions and phosphate buffer (0.1 mol $l^{-1}$, pH 7.0), urea was removed from the solution and the protein realized its refolding.

Protein concentration was determined using BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) as described by the specification by Sunrise Absorbance Reader (Tecan, Mannedorf, Switzerland) with bovine serum albumin as a standard. Purified protein was used for its characterization.

## β-1,3-1,4-Glucanase Activity Assay

The reaction mixture containing 1 mL of 0.8% (*w*/*v*) barley β-glucan (Sigma, St Louis, MO, USA) solution and 1 mL diluted enzyme was incubated at 40°C for 30 min, and the reducing sugar was measured by the dinitrosalicylic acid method [17]. $OD_{540nm}$ was

measured of the reactant after each reaction was finished. One unit β-1,3-1,4-glucanase activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar from the β-glucan solution per minute at 40°C and pH 6.4. Specific activity was defined as activity unit per milligram of protein.

### Characterization of the β-1,3-1,4-glucanase

The substrate was diluted in the range of 0.08–0.8% (*w*/*v*) in 0.1 mol $l^{-1}$ $Na_2HPO_4$–citric acid buffer (pH 6.4). The enzyme concentration was 0.033 mg $ml^{-1}$. The values of the Michaelis–Menten constant ($k_m$) and maximal velocity ($V_{max}$) were determined using Lineweaver–Burk plot. The kinetic parameters, turnover number ($k_{cat}$) and catalytic efficiency value ($k_{cat}/k_m$), were calculated from initial velocities using concentrations of 0.8% (*w*/*v*) of barley β-glucan.

The optimal pH of the β-1,3-1,4-glucanase was determined over various pH ranges of $Na_2HPO_4$–citric acid buffer (pH 4.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0). For pH stability determination, the β-1,3-1,4-glucanase was incubated in different buffer solutions for 1 h at room temperature, and the remaining activity was measured under the standard assay condition.

The optimal temperature was determined by incubating the enzyme for 30 min at the temperatures ranging from 20°C to 80°C at pH 6.4. For determination of the heat stability, the β-1,3-1,4-glucanase was incubated at the temperatures ranging from 40°C to 70°C for 2, 5, 10, 30, 60, 120 min. Samples were withdrawn after different time intervals and immediately cooled on ice. The residual activity of each sample was determined as described above.

### Effect of Metal Ions and Chemicals on β-1,3-1,4-Glucanase Activity

To study the effects of different metal ions and chemical reagents on the β-1,3-1,4-glucanase activity, different metal ions or chemicals, $FeSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, KI, $Na_2SeO_3$, $Na_2MoO_4$, NaCl, $CaCl_2$, $MgCl_2$, ethylenediaminetetraacetic acid (EDTA), arginine and sucrose, were individually added with a final concentration of 10 mmol $l^{-1}$ to the reaction solution. β-1,3-1,4-glucanase activities were determined under the standard assay. The reaction mixture without the metal ions or chemicals was used as control.

### Nucleotide Sequence Accession Number

The nucleotide sequence of *B. subtilis* β-1,3-1,4-glucanase gene was assigned to the GenBank accession no. EU082110 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=157326988).

## Results and Discussion

### Cloning of the β-1,3-1,4-Glucanase Gene

Partial cDNA sequence of β-1,3-1,4-glucanase gene was amplified from *B. subtilis* MA139 by PCR. As shown in Fig. 1, the gene is composed of 690 bp, which encodes a deduced protein of 229 amino acids. The conserved motif "EIDIEF" of GH16 is located from amino acids 120 to 125, with a molecular weight of 25.5 kDa and a theoretical isoelectric point of

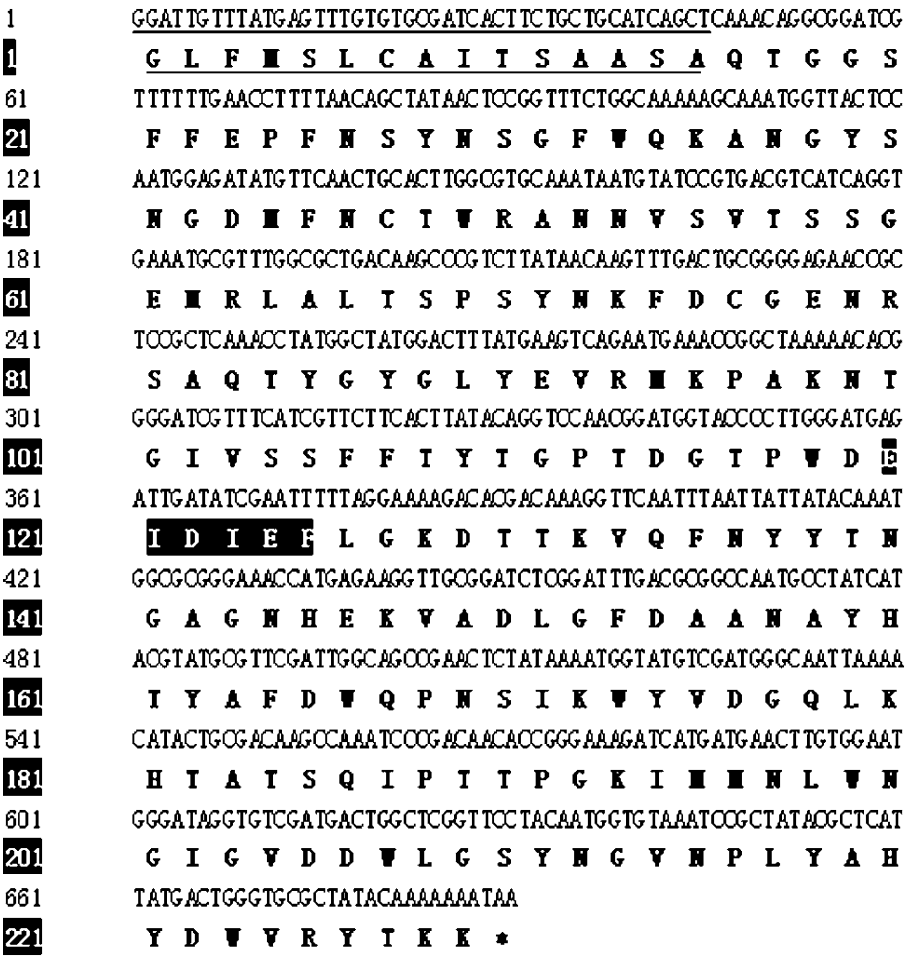


**Fig. 1** The nucleotide sequence and the deduced amino acid of β-1,3-1,4-glucanase gene from the *B. subtilis* MA139. *Number on the left* indicated the position of the first base or amino acid in the row; the putative signal peptide sequence was shown *underlined*; the conserved motif of GH16 was demonstrated *shadowed*; the stop codon was indicated by an *asterisk* (*)

5.55 predicted at http://au.expasy.org/tools/pi_tool.html. The 15 amino acids located at the N-terminus of the deduced amino acid showed the typical attributes of a signal peptide [18].

The cloned β-1,3-1,4-glucanase gene showed 97% homology to the gene from *B. licheniformis* (GenBank accession no. AY160109 and AF546871). The amino acid sequence of the β-1,3-1,4-glucanase showed similarities with those from *B. subtilis* (93%, GenBank accession no. AF490978) and *B. licheniformis* (97%, GenBank accession no. AAO18342).

## Expression, Purification, and Enzyme Assay of the Recombinant Protein

As shown in Fig. 2, IPTG can induce β-1,3-1,4-glucanase gene expression in *E. coli* BL21 compared to the un-induced treatment, but the expressed protein is a little larger than the estimated protein size which should be 24.44 kDa. This difference in molecular mass is

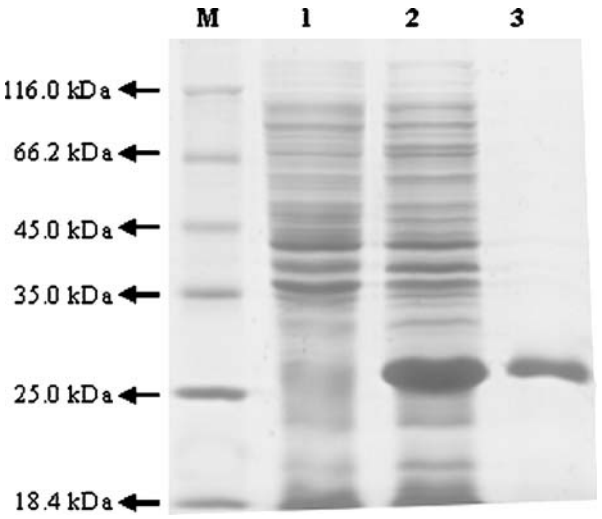


**Fig. 2** Expression of β-1,3-1, 4-glucanase in *E. coli* BL21. SDS-PAGE analysis of β-1, 3-1,4-glucanase protein present in cell pellets. *Lane M* Low molecular mass standard protein: β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), restriction endonuclease *Bsp* 981 (25.0 kDa), β-lactoglobulin (18.4 kDa), *Lane 1* soluble cell lysate without induction, *Lane 2* soluble cell lysate after induction, *Lane 3* affinity purified β-1, 3-1,4-glucanase

tentatively interpreted as caused by additionally fused 34 N-terminal amino acids including His-tag encoded by a fragment from 270 to 287 upstream of the former restriction enzyme site *Eco*RI.

The innocent strain of *E. coli* BL21 was free of endogenous β-1,3-1,4-glucanase expression (data not shown). The transformed *E. coli* BL21 expressed the exogenous glucanase in inclusion body under our experimental condition. The recombinant protein purified with Ni-NTA column was refolded by dialysis. The exogenous expressed β-1,3-1,4-glucanase in *E. coli* BL21 showed the activity of 24.05 and 12.52 U $ml^{-1}$ when barley β-glucan and lichenan were used as the substrates, and the specific activities were 728.79 and 379.1 U $mg^{-1}$ for them, respectively.

## Characterization of the β-1,3-1,4-glucanase

The $K_m$ value of β-1,3-1,4-glucanase was 5.34 mg $ml^{-1}$, and the maximal velocity was 47.85 μmol $min^{-1}$ when barley β-glucan was used as the substrate. The turnover number and the catalytic efficiency value were 7,206.71 $s^{-1}$ and 1,349.67 ml $s^{-1}$ $mg^{-1}$, respectively, according to the $K_m$ and $V_{max}$.

As shown in Fig. 3, the optimal pH of the β-1,3-1,4-glucanase was 6.4, and β-1,3-1,4-glucanase activity decreased rapidly when pH value was less than 5.4 or more than 7.4 showing that the β-1,3-1,4-glucanase functioned better under neutral pH. The residual activity remained above 70% when incubated at the pH range of 5.4 to 9.0 for 1 h. The recombinant enzyme showed excellent activity and stability in nearly neutral buffer, which suggested that it functions well in animal digestive tract.

As shown in Fig. 4, 40°C was the optimal temperature for the β-1,3-1,4-glucanase. Relative β-1,3-1,4-glucanase activity at 70°C was less than 10% of the maximal enzyme activity and decreased remarkably at higher temperature. When the β-1,3-1,4-glucanase was incubated at 70°C for 5 min, the residual activity was only about 10% of the maximal enzyme activity (Fig. 5), which indicated that supplementation of this enzyme in animal feed was not facilitated in feed temperature processing.

To investigate the specificity of the β-1,3-1,4-glucanase to glycosidic bonds, multiple substrates of lichenan, laminarin, cellulose, xylan, and mannan (Sigma, St Louis, MO,

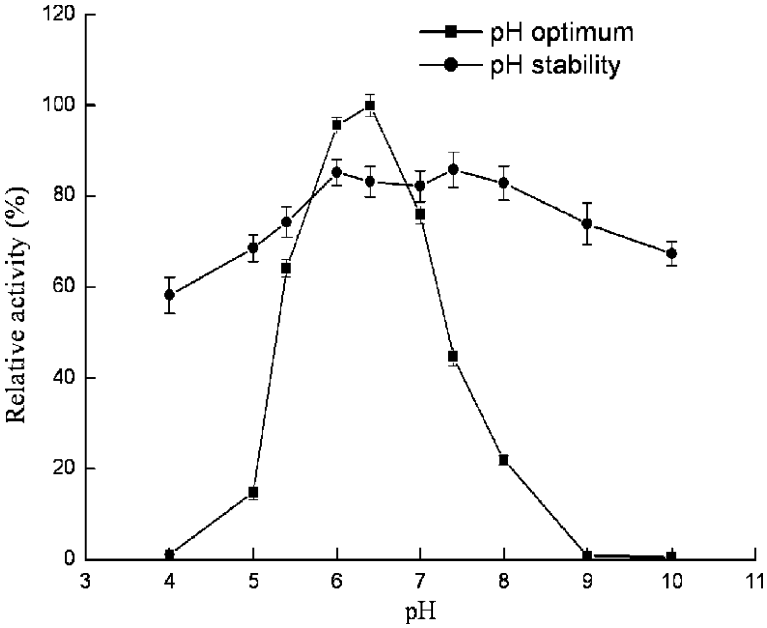


**Fig. 3** pH effect on the β-1,3-1,4-glucanase activity. pH optimum was determined by measuring the relative activity using $Na_2HPO_4$–citric acid buffer (pH 4.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0) at 40°C; pH stability was determined by measuring the residual activity after 1 h incubation at room temperature using buffers above. All determination were repeated in triplicate. *filled squares*: pH optimum; *filled circles*: pH stability

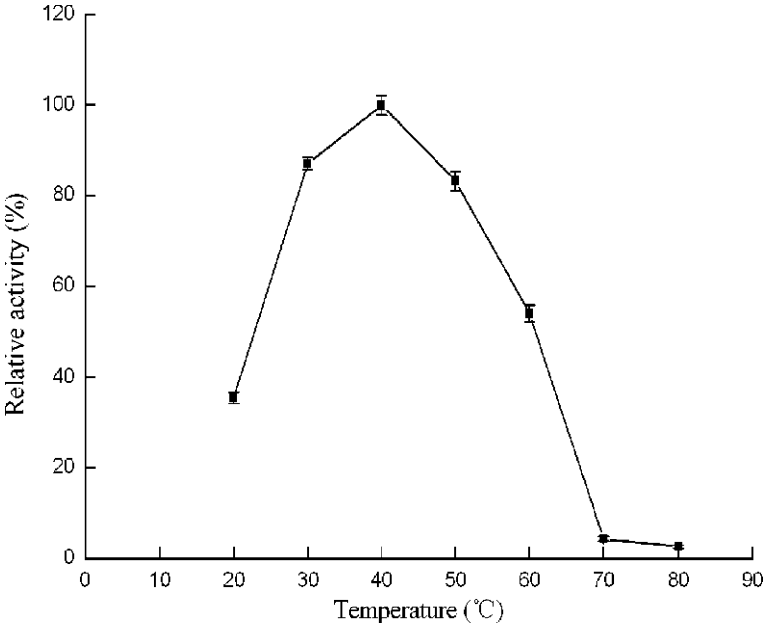


**Fig. 4** Temperature effect on the β-1,3-1,4-glucanase activity. Temperature optimum was determined by incubating the enzyme for 30 min at the temperatures ranging from 20°C to 80°C at pH 6.4. All determination were repeated in triplicate

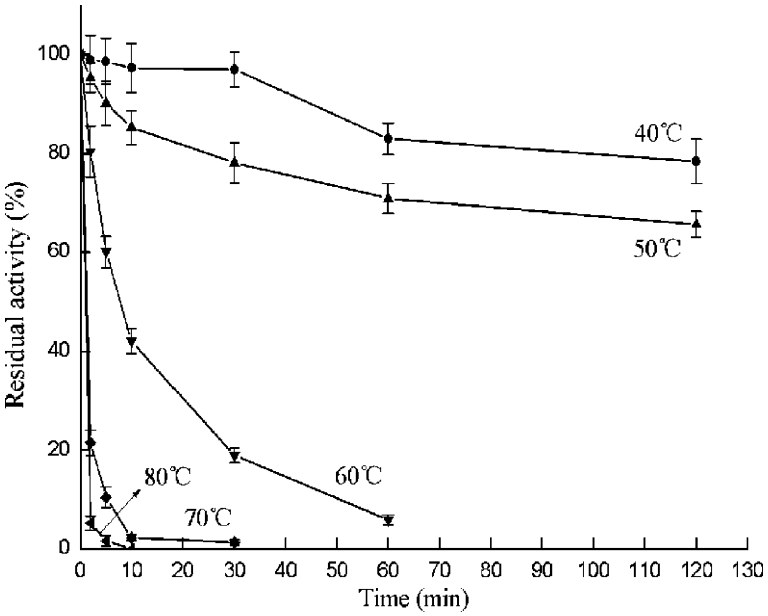


**Fig. 5** Thermostability of the recombinant β-1,3-1,4-glucanase. For determination of the thermostability, the recombinant β-1,3-1,4-glucanase was incubated at the temperatures ranging from 40°C to 70°C for 2, 5, 10, 30, 60, 120 min. Samples were withdrawn after different time intervals and immediately cooled in an ice bath. All determination was repeated in triplicate

USA) were selected, in which lichenan consists of both β-1,3 and β-1,4 glucose glycosidic bond, and laminarin and cellulose are β-1,3 and β-1,4 glucose glycosidic bond, respectively. However, xylan comprises β-1,4-xylose glycosidic bond, and mannan consists of β-1,4-mannose glycosidic bond. As a result, the β-1,3-1,4-glucanase showed inactivity on laminarin, cellulose, xylan, or mannan, as this enzyme can only hydrolyze the β-1,4-glycosidic bond which is adjacent to β-1,3-glycosidic bonds.

**Table 1** Effects of metal ions or chemical reagents on the β-1, 3-1-glucanase activity.

| Reagents | Relative activity[a] (%) |
|---|---|
| None | 100 |
| $FeSO_4$ | 109.19±1.88[a] |
| $CuSO_4$ | 2.76±0.15 |
| $MnSO_4$ | 45.83±2.04 |
| $ZnSO_4$ | 93.38±4.95 |
| KI | 103.68±2.38 |
| $Na_2SeO_3$ | 109.01±3.08 |
| $Na_2MoSO_4$ | 80.27±2.04 |
| NaCl | 112.56±0.61 |
| $MgCl_2$ | 100.98±0.62 |
| $CaCl_2$ | 100.43±1.36 |
| EDTA | 71.51±2.36 |
| Arginine | 108.33±1.73 |
| Sucrose | 101.23±1.62 |

[a] Mean±SE. Experimental data were determined in triplicate. Metal ions or chemicals were individually added up to a final concentration of 10 mmol $l^{-1}$

Effect of Metal Ions or Chemicals on β-1,3-1,4-Glucanase Activity

As shown in Table 1, compared to the activity of the standard β-1,3-1,4-glucanase, the enzyme activity declined to 2.76%, 45.83%, 80.27%, and 71.51% when supplemented with $CuSO_4$, $MnSO_4$, $Na_2MoO_4$ and EDTA, respectively, in the reaction systems. The values, however, were raised to 112.56% in the presence of NaCl in the reaction mixtures.

## Conclusions

We hereby presented a sequence of β-1,3-1,4-glucanase from *B. subtilis* MA139 which was expressed in *E. coli*. The partial properties of the recombinant enzyme were studied. However, heterogenous protein expressed by *E. coli* easily forms inclusion body, which is inactive. Expression system of *P. pastoris* was broadly used in recent years. Further study will focus on enhancement of enzyme thermostability and expression in *P. pastoris*.

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