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Prokaryotic Expression, Purification and Characterization of *Aspergillus sulphureus* β-Mannanase and Site-Directed Mutagenesis of the Catalytic Residues

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Abstract Wild type (WT) DNA sequence, which encoded a mature β-mannanase of *Aspergillus sulphureus*, composed of 1,152 nucleotides (nt), was amplified from pUCm-T-mann by polymerase chain reaction (PCR). Based on this DNA fragment, mutants designated as $E^{206}G$ and $E^{314}G$ were constructed by overextension PCR (OE-PCR). Glutamic acids of the 206th and 314th sites in the amino acid sequence of β-mannanase were separately replaced by glycine in these two mutants. The WT and mutant genes were ligated into prokaryotic vector pET-28a (+) and transformed into the *Escherichia coli* BL21 strain, respectively. The recombinant enzyme proteins were expressed by IPTG induction and detected by Western blot. The recombinant proteins purified with Ni-NTA column were dialyzed to correctly refold. The WT recombinant β-mannanase showed optimal activity at 50 °C and pH 2.4. The kinetic parameters of K_m and V_{max} for purified β-mannanase were 1.38 mg/ml and 72.99 U/mg, respectively. However, the mutant proteins did not show any activity. It was demonstrated that E^{206} and E^{314} were the catalytic residues of β-mannanase.

Keywords *Aspergillus sulphureus* · β-mannanase · Prokaryotic expression · Site-directed mutagenesis

Introduction

Mannan, as a major component of hemicellulose in plant cell wall, is a polysaccharide consisting of β-1, 4-linked D-mannopyranose residues [1, 2]. Degradation of mannan mainly depends on β-mannanase, which can be produced by many organisms, such as bacteria, fungus, and plant seeds. β-mannanases from different sources are variable in enzymic characterization. β-mannanase has been classified into two families, namely, glycosyl hydrolase family 5 and family 26 [3]. The nucleotide sequences of β-mannanase that belonged to family 5 of *Trichoderma reesei* and *Aspergillus aculeatus* have been

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cloned and the properties of the enzymes were well studied. It has been demonstrated that amino acid residues of E^{169} and E^{276} were the catalytic sites of the *T. reesei* β-mannanase [4]. In our laboratory, β-mannanase gene of *A. sulphureus* has been cloned and expressed in *Pichia pastoris* [5]. The open reading frame (ORF) of the enzyme gene consisted of 1,152 nt and encoded a protein with a molecular weight of 41 kDa. The amino acid sequence of the *A. sulphureus* β-mannanase shared identities of 72.2% and 45.9% to that of *A. aculeatus* and *T. reesei*, respectively.

In this paper, the wild type (WT) β-mannanase gene of *A. sulphureus* was expressed in *Escherichia coli* and the presumed catalytic residues were identified by site-directed mutagenesis.

Material and Methods

Vectors and Strains

pUCm-T-mann plasmid containing the full-length cDNA sequence of β-mannanase gene of *A. sulphureus* was constructed in our laboratory (not published). *E. coli* Top10 and BL21 strains were used as host cells for genes cloning and expression.

Construction of Expression Vectors

With pUCm-T-mann as template, wild type (WT) β-mannanase gene was amplified by primers PB-F-1 (5′-CCG*GAATTC*CTGCCCAAAGCCTCTCCTGC-3′, italicized letters indicate the *Eco*RI site) and F2 (5′-CC*AAGCTT*TTAGGCGCTATCAATAGC-3′, italicized letters indicate the *Hin*dIII site). Polymerase chain reaction (PCR) was performed with 35 cycles of 94 °C 30 s, 63 °C 30 s, and 72 °C 2 min. The PCR products were recovered by 0.8% agarose gel and purified with UNIQ-10 DNA purification kit (Sangon, Shanghai, China). To introduce $E^{206}G$ mutant in the β-mannanase gene, WT DNA was amplified with primers PB-F-1/E1-CG-R (5′-ACATCTCGGCCCATTCGCCA-3′) and F2/E1-CG-F (5′-TGGCGAATGGGCCGAGATGT-3′), respectively. The purified PCR products were mixed and amplified by primers PB-F-1/F2 with the procedure of 94 °C 30 s, 63 °C 30 s, and 72 °C 2 min for 35 cycles. The same procedure was conducted for the $E^{314}G$ mutant with primers PB-F-1/E2-CG-R (5′-TGACTCCGTATCCCTCCAGT-3′) and F2/E2-CG-F (5′-ACTG GAG*GGA*TACGGAGTCA-3′) amplifying 5′ and 3′ termini fragments of the *WT* gene.

The purified DNA fragments of WT, $E^{206}G$, and $E^{314}G$ digested by *Eco*RI and *Hin*dIII were ligated into corresponding sites of pET28a (+) and transformed into *E. coli* Top10. The resulting recombinant plasmids, pET-mann, $pE^{206}G$, and $pE^{314}G$, were sequenced with T7 promoter primer in a Model 377 Automated DNA sequencer (Applied Biosystems, CA, USA) with dideoxy chain terminator chemistry.

Expression and Purification of Recombinant Proteins

The recombinant expression vectors pET-mann, $pE^{206}G$, and $pE^{304}G$ were transformed into *E. coli* BL21 strain, respectively. The transformants were incubated in LB medium (containing 50 μg/ml kanamycin) at 37 °C overnight. One milliliter of the aliquots was transfer-inoculated into 100 ml LB medium (containing 50 μg/ml kanamycin) and shaken at 37 °C for 2–3 h. The recombinant proteins were induced with treatment by final concentration of 1 mmol/l isopropyl β-D-thiogalactopyranoside (IPTG) for 5–6 h. The total

proteins of the bacteria were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-II apparatus (BioRad, USA). The recombinant proteins were purified with Ni-NTA column (Zhuoguan, Beijing, China) according to the manufacturer's instruction. The concentrations of the recombinant proteins were determined using Micro-BCA Protein Assay Reagent (Pierce, USA).

Western Blot Assay of Recombinant Proteins

Western blot detection of the expressed proteins was performed according to the method of Sambrook et al. [6]. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA) with 200 mA for 1 h using the Mini Trans-Blot cell (BioRad). The primary antibody and detection kit used in this experiment were in accordance with that of Chen et al. [5].

Denaturation, Refolding, and Enzyme Assay

The purified recombinant protein was pretreated with 0.2 mol/l of PBS containing 8 mol/l of urea (pH 7.4) at room temperature for 1 h. The denatured protein was step-by-step dialyzed against 0.2 mol/l of PBS containing 6, 4, 2, and 0 mol/l of urea at 4 °C for 24 h.

Enzyme assay was performed according to the method of Lu et al. [7]. One unit of β-mannanase activity (U) was defined as the amount of enzyme that liberated 1 μmol reducing sugar (mannopyranose) from the substrate solution per minute at the conditions of pH 2.4 and 50 °C. Na_2HPO_4–citric acid buffers with different pH values were used in our experiments. The reaction mixture containing 2 ml of 0.6% (*w*/*v*) locust bean gum (Sigma G-0753) solution and 2 ml diluted enzyme was incubated at 50 °C for 30 min, and the reducing sugar (mannopyranose) was measured by the dinitrosalicylic acid method [8]. The kinetics parameters were determined in accordance with the method of Chen et al. [5].

Results and Discussion

Construction of Expression Vectors

The ORF of *A. sulphureus* β-mannanase, which was composed of 1,152 nt, encoded a protein of 384 amino acids. The amino acid sequence of the *A. sulphureus* β-mannanase shared identities of 72.2% and 45.9% to that of *A. aculeatus* and *T. reesei*, respectively. It has been speculated that glutamic acid (E) at positions 206 and 314 of β-mannanase served as active residues [5]. To verify this proposition, the mutants of $E^{206}G$ and $E^{314}G$ were obtained with OE-PCR (Fig. 1a and b). In these two mutants, E^{206} and E^{314} were replaced by glycine, respectively. WT and mutants of $E^{206}G$ and $E^{314}G$ of β-mannanase were located downstream of the T7 promoter of pET28a (+) for inducible expression in *E. coli*. Compared with the WT amino acid sequence of the β-mannanase, no other mutant, except for E^{206} and E^{314}, was found by DNA sequencing.

Expression, Purification and Western-blot Assay of the Recombinant Proteins

pET-mann, $pE^{206}G$, and $pE^{314}G$ were transformed into *E. coli* BL21 strain, respectively. The transformants were induced with IPTG and approximately 43 kDa of proteins were detected by SDS-PAGE (Fig. 2). The recombinant proteins were in fusion with a peptide

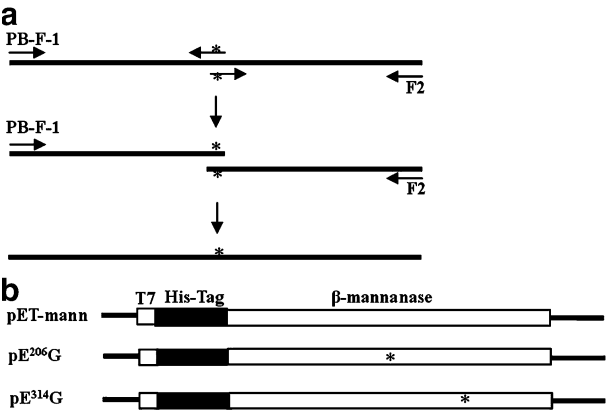

Fig. 1 **a** Illustration of site-directed mutation of *A. sulphureus* β-mannanase with overlap-extension PCR. $\overleftarrow{*}$ represents primer E1-CG-R or E2-CG-R. $\overrightarrow{*}$ represents primer E1-CG-F or E2-CG-F. **b** Organization of the expression vectors of WT mannanase and its mutants for catalytic residues. The *asterisk* shows the mutant sites

containing His–tag in the vector. No activity of β-mannanase was detected in the soluble cell lysate of the WT transformant, from which it was assumed that the recombinant protein was presented as inclusion body. The recombinant proteins purified with the Ni-NTA column were assayed by Western blot with rabbit anti-*A. sulphureus* antibody (Fig. 2). It was shown that WT β-mannanase and its mutants have no difference in antibody specificity.

Enzyme Assay

To analyze the activity difference between WT and mutant β-mannanase, the recombinant proteins were refolded by gradient dialysis. The purified WT β-mannanase showed relative activity of 5.2 U/ml toward the locust bean gum at 50 °C and pH 2.4. The specific activity of the WT enzyme was 208 U/mg. The kinetic parameters of K_m and V_{max} for purified β-mannanase were 1.38 mg/ml and 72.99 U/mg, respectively. No catalytic activity was observed on the mutant recombinant proteins. These results suggested that the glutamic acids at positions 206 and 314 of the *A. sulphureus* β-mannanase might serve as its active residues.

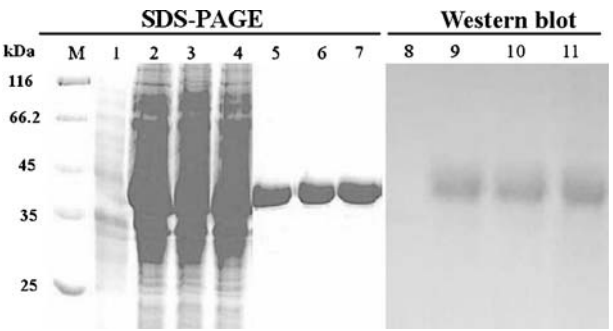

Fig. 2 Expression analysis of *A. sulphureus* β-mannanase in *E. coli*. *Lane M*: protein molecular mass marker. *Lane 1*: total proteins of the pET-mann transformant induced without IPTG. *Lane 2*: total proteins of the pET-mann transformant induced with IPTG. *Lane 3*: total proteins of the $pE^{206}G$ transformant induced with IPTG. *Lane4*: total proteins of the $pE^{314}G$ transformant induced with IPTG. *Lane 5*: purified WT recombinant β-mannanase. *Lane 6*: purified mutant $E^{206}G$. *Lane 7*: purified mutant $E^{314}G$. *Lane 8*: total proteins of the pET-mann transformant induced without IPTG. *Lane 9*: total proteins of the pET-mann transformant induced with IPTG. *Lane 10*: total proteins of the $pE^{206}G$ transformant induced with IPTG. *Lane 11*: total proteins of the $pE^{314}G$ transformant induced with IPTG

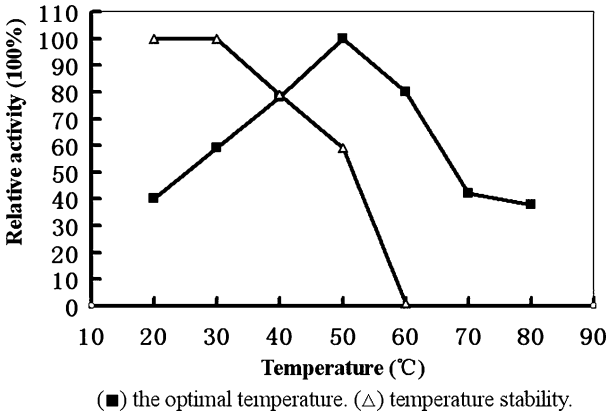

Fig. 3 Effect of temperature on activity and stability of the WT β-mannanase expressed in *E. coli*. The optimal temperature was determined using the standard activity assay at various temperatures. To estimate thermal stability, the recombinant enzyme was incubated at various temperatures for 30 min at pH 2.4 and the residual mannanase activity was then determined at pH 2.4 and 50 °C

The WT β-mannanase gene has been expressed in *P. pastoris* with a yield of 96 U/ml in shake flask. The recombinant enzyme showed optimal activity at 50 °C and pH 2.4. It has been demonstrated that the activity of β-mannanase was significantly affected by glycosylation [5]. However, proteins expressed in *E. coli* are not glycosylated. The enzymic properties of the recombinant WT purified with the Ni-NTA column were determined. The optimal temperature was determined at various temperatures from 20 to 80 °C by the standard enzyme activity assay method. It was showed that the recombinant β-mannanase had optimal activity at 50 °C (Fig. 3). The result corresponds with that of the enzyme expressed in *P. pastoris*. To estimate thermal stability, the enzyme was preincubated in disodium hydrogen phosphate–citric acid buffer (pH 2.4) at the various temperatures (20–80 °C) for 30 min and then assayed for residual activity at 50 °C and pH 2.4. The results were shown in Fig. 3. No any residual activity was observed after the recombinant protein had been incubated at 60 °C for 30 min. But the enzyme expressed by *P. pastoris* showed about 50% residual activity after incubating at 60 °C for 50 min.

The effects of different pH values on the activity of the recombinant β-mannanase expressed in *E. coli* were measured at 50 °C. It was shown that the enzyme displayed a pH optimum at 2.4 (Fig. 4). To estimate the pH stability of the recombinant protein, the enzyme was preincubated in different pH buffers for 1 h at 50 °C and then assayed for residual activity at pH 2.4 (Fig. 4). The results about optimal pH and pH stability of the enzyme

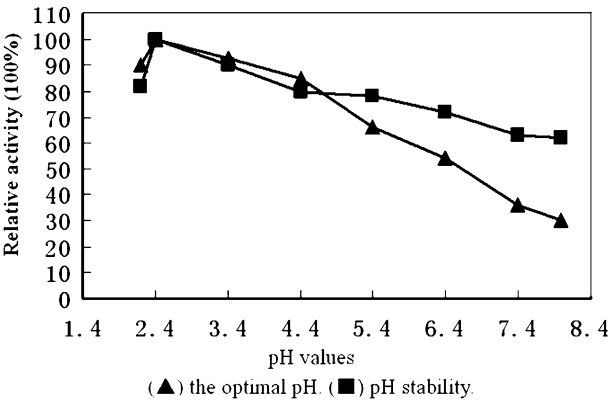

Fig. 4 Effect of pH on activity and stability of the WT β-mannanase expressed in *E. coli*. The optimal pH was determined using the standard activity assay at 50 °C with different pH values buffers. To estimate pH stability, the recombinant enzyme was incubated in the different pH buffers at 50 °C for 1 h, and then the residual mannanase activity was determined at pH 2.4 and 50 °C

have no obvious difference to that of β-mannanase expressed in *P. pastoris*. It was concluded that optimal pH and pH stability of the *A. sulphureus* β-mannanase were not affected by glycosylation.

Conclusions

We hereby present the expression and purification of *A. sulphureus* WT β-mannanase and its mutants in which the presumed catalytic residues E^{206} and E^{314} were replaced by glycine, respectively. From the results that the recombinant proteins designated $E^{206}G$ and $E^{314}G$ lost the activity of catalytic degradation of the locust bean gum, it was deduced that E^{206} and E^{314} were the catalytic residues of the β-mannanase. The optimal temperature and pH value of the recombinant enzyme expressed in *E. coli* were consistent with that of the enzyme secreted and expressed by *P. pastoris*. That is to say the optimum temperature and pH of the *A. sulphureus* β-mannanase was not affected by glycosylation. But glycosylation had a relatively large effect on the temperature stability of the recombinant enzyme.

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References

1. Matheson, N. K., & McCleary, B. V. (1985). In G. O. Aspinall (Ed.), *The Polysachharides* (vol. 3, p. 1). New York: Academic.
2. McCleary, B. V., & Matheson, N. K. (1986). Enzymic analysis of polysaccharide structure. *Advances in Carbohydrate Chemistry and Biochemistry, 44*, 147–276.
3. Henrissat, B., & Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal, 293*, 781–788.
4. Stålbrand, H., Saloheimo, A., Vehmaanperä, J., Henrissat, B., & Penttilä, M. (1995). Cloning and expression in *Saccharomyces cerevisiae* of a *Trichoderma reesei* β-mannanase gene containing a cellulose binding domain. *Applied and Environmental Microbiology, 61*, 1090–1097.
5. Chen, X. L., Cao, Y. H., Ding, Y. H., Lu, W. Q., & Li, D. F. (2007). Cloning, functional expression and characterization of *Aspergillus sulphureus* β-mannanase in *Pichia pastoris*. *Journal of Biotechnology, 128*, 452–461.
6. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual (*2nd ed., p. 808). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
7. Lu, W. Q., Li, D. F., & Wu, Y. B. (2003). Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solidstate fermentation by *Aspergillus sulphureus*. *Enzyme and Microbial Technology, 32*, 305–311.
8. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry, 31*, 426–428.