Research Article

Purification and Characterization of a Thermostable β-Mannanase from Bacillus subtilis BE-91: Potential Application in Inflammatory Diseases

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β-mannanase has shown compelling biological functions because of its regulatory roles in metabolism, inflammation, and oxidation. This study separated and purified the β-mannanase from Bacillus subtilis BE-91, which is a powerful hemicellulose-degrading bacterium using a “two-step” method comprising ultrafiltration and gel chromatography. The purified β-mannanase (about 28.2 kDa) showed high specific activity (79,859.2 IU/mg). The optimum temperature and pH were 65°C and 6.0, respectively. Moreover, the enzyme was highly stable at temperatures up to 70°C and pH 4.5–7.0. The β-mannanase activity was significantly enhanced in the presence of Mn²⁺, Cu²⁺, Zn²⁺, Ca²⁺, Mg²⁺, and Al³⁺ and strongly inhibited by Ba²⁺ and Pb²⁺. \( K_m \) and \( V_{max} \) values for locust bean gum were 7.14 mg/mL and 107.5 μmol/min/mL versus 1.749 mg/mL and 33.45 μmol/min/mL for Konjac glucomannan, respectively. Therefore, β-mannanase purified by this work shows stability at high temperatures and in weakly acidic or neutral environments. Based on such data, the β-mannanase will have potential applications as a dietary supplement in treatment of inflammatory processes.

1. Introduction

Mannan consists of a series of complex polysaccharides, which are found in the cell wall of marine algae [1]. The backbone is comprised of β-1,4-linked mannose residues. Konjac glucomannan is a randomly arranged polymer of β-1,4-linked glucose and mannose residues at ratio of 1.0:1.6. Both the backbones of mannan and Konjac are modified by α-1,6-linked galactosyl residues to form galactomannan and galactoglucomannan, respectively [2].

β-mannanase (EC 3.2.1.78) is a hemicellulase that attacks the internal glycosidic bonds of mannan backbone to release the condensed β-1,4-manno-oligosaccharides [3]. β-mannanases are widely applied in pulp and paper processing [4], feed [5], food [6], pharmaceutical [7], oil, and textile industries [8] to randomly hydrolyze the β-1,4 manno-pyranose linkage in mannan, galactomannan, glucomannan, and galactoglucomannan.

β-mannanase is widely produced by bacteria [9, 10], actinomycetes [11], fungi [12], plants, and animals [13]. Among them, β-mannanase from bacteria is wildly used because of numerous advantages, including extracellular secretion, economic production and purification, and novel characteristics, such as tolerance to heat and alkaline conditions [14].

Although multiple β-mannanase-producing bacteria have been reported [15, 16], they are far from the diverse industry needs. Currently, acidic and alkaline β-mannanase has been proposed to meet the industrial demands [17]. However, the requirements of high energy in production and the environmental impact limit their development. Neutral and weakly acidic β-mannanase with lower energy for production has attracted considerable interest over the past few years; however, it has rarely been characterized. It is clarified that β-mannanase with high activity in short fermentation time confers lower costs during the production procedures. Therefore, the exploitation of strains producing high β-mannanases activity is valuable and profitable. In current study, we isolated and preserved a powerful hemicellulose-degrading bacterium (BE-91). Then we explored...
the efficient purification process and characterized the enzymatic properties of its \(\beta\)-mannanase.

2. Materials and Methods

2.1. Microorganism, Media, and Fermentation Conditions. \textit{B. subtilis} BE-91, a strain used for herbaceous fiber extraction, was identified and preserved by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science (Changsha, Hunan, China). \textit{B. subtilis} BE-91 was cultured in Petri dish containing 0.5% yeast extract, 1% NaCl, 0.5% Konjac glucomannan, 1% bacto tryptone, 0.05% trypan blue, and 1.5% agar. The seed medium was mainly composed of 0.1% glucose, 0.4% Konjac glucomannan, 0.3% beef extract, 0.2% yeast extract, 0.5% peptone, and 0.5% NaCl. The fermentation medium primarily consisted of 0.2% yeast extract, 0.7% Konjac glucomannan, 0.5% peptone, 0.3% beef extract, and 0.5% NaCl. \textit{B. subtilis} BE-91 was first activated in the seed medium at 35 ± 1°C for 5.5 h. Subsequently, the suspension was serially diluted, spread onto Petri dishes, and incubated at 35 ± 1°C for 18 h. The single colony exhibiting the largest hydrolytic halo was transferred into an Erlenmeyer flask with the seed medium and cultured at 35 ± 1°C for 6 h at 180 rpm. Consequently, 2% culture was inoculated in the fermentation medium and cultured for 6 h at 35 ± 1°C at 180 rpm [18].

2.2. Classification of Strain BE-91. The 16S rDNA of strain BE-91 was PCR amplified from genomic DNA using the Bacterial Identification PCR Kit (TaKaRa, Japan). The obtained 16S rDNA was sequenced by the ABI 3730XL 96-capillary DNA analyzer. The primers were as follows: P1 5'−AGAGTTTGATCMTGGCTCAG−3' and P2 5'−TACGGYTACCTTGTTACGACTT−3'. The resulting sequence aligned closely with the related standard sequences of other bacteria retrieved from GenBank. Neighbor-joining clusters were constructed by Mega 6.0 [19].

2.3. Enzymatic Assays. \(\beta\)-mannanase activity was estimated by initiating the reaction at 65°C for 10 min in 0.05 mol/L citric acid/0.1 mol/L Na\(_2\)HPO\(_4\) buffer (pH 6.0) with 0.2% (w/v) Konjac glucomannan as substrate. The amounts of reducing sugar in the reaction were quantified based on a standard curve generated with mannose using the 3,5-dinitrosalicylic acid (DNS) method. One unit (IU) of \(\beta\)-mannanase activity was defined as the amount of protein producing 1 \(\mu\)mol/L of reducing sugar per minute (e.g., mannose) under standard conditions [20].

2.4. Purification of \(\beta\)-Mannanase. The bacterial \(\beta\)-mannanase was purified using a two-step process involving ultrafiltration (Sartorius, Germany) and gel filtration. The fermentation liquid was fractionated orderly by 100 kDa, 50 kDa, and 5 kDa membrane thresholds. The solution filtered with 5 kDa < MW < 50 kDa was further purified on a Sephadex G-100 gel column (φ1.6 cm × 100 cm, Pharmacia). The eluate was obtained at a rate of 0.5 mL/min and collected in 5 mL fractions. \(\beta\)-mannanase activity was determined by the DNS method, whereas the protein was quantified by the Coomassie brilliant blue staining against bovine serum albumin (BSA) standard [21].

2.5. The Determination of Apparent Molecular Weight. The molecular mass of the \(\beta\)-mannanase was determined by SDS-PAGE (Bio-Rad, USA), with 3% stacking gel and 12% separating gel [22]. The protein bands were stained with 0.01% Coomassie brilliant blue R-250 and destained with a water-methanol-acetic acid (9:9:2) solvent. Zymogram analysis was performed by the method of Chanhan [17]. The molecular weight of \(\beta\)-mannanase was derived from the relative mobility of molecular weight markers resolved simultaneously.

2.6. The Effect of Temperature on the Activity and Stability of \(\beta\)-Mannanase. The activity of \(\beta\)-mannanase was assayed at a range of temperatures between 50 and 70°C in 0.05 mol/L citric acid-0.1 mol/L Na\(_2\)HPO\(_4\) buffer at pH 6.0. The thermostability was assessed by preincubating the enzyme, without a substrate, at different temperatures varying over 20–80°C for 30 min. The residual activity was promptly measured by the DNS method. The \(\beta\)-mannanase activity was considered to be 100% when preincubated at 4°C.

2.7. The Effect of pH on the Activity and Stability of \(\beta\)-Mannanase. \(\beta\)-mannanase activity was evaluated by incubating the purified enzyme at different pH conditions ranging from 4.0 to 8.0 in 0.05 mol/L citric acid-0.1 mol/L Na\(_2\)HPO\(_4\) buffer at 4°C. The stability at a particular pH was tested by preincubating the purified enzyme, without a substrate, for 30 min in various 0.05 mol/L citric acid-0.1 mol/L Na\(_2\)HPO\(_4\) buffers at pH 3.0–8.5 at 4°C. The residual \(\beta\)-mannanase activity was immediately measured after treatment by the DNS procedure.

2.8. The Effect of Metal Ions on the Activity of \(\beta\)-Mannanase. In order to examine the effects of metal ions on the activity of \(\beta\)-mannanase, the enzyme was incubated for 30 min at 4°C in the presence of various 1.0 mmol/L metal ions, CaCl\(_2\), H\(_2\)O, ZnCl\(_2\), FeCl\(_3\), PbCl\(_2\), MnCl\(_2\), CuCl\(_2\), MgCl\(_2\), AlCl\(_3\), BaCl\(_2\), and NH\(_4\)Cl. The residual \(\beta\)-mannanase activity was measured at a specific condition and that of the treatment in the absence of additives as a control.

2.9. Substrate Specificity and Kinetic Parameters. Various glycosans, such as Konjac glucomannan [23], locust bean gum from Cecotonia siliqua seeds (Sigma, G0753), carob galactomannan (Megazyme, P-GALML), guar galactomannan (Megazyme, P-GGMV), ivory nut mannann (Megazyme, P-MANIV), 1,4-beta-D-mannan (Megazyme, P-MANC), wheat arabinoxylan (Megazyme, P-120601a), beechwood xylan (Megazyme, P-14101a), and carboxymethyl cellulose (Megazyme, P-CMC4M) were examined. In brief, 0.2% (w/v) glycosans were incubated with \(\beta\)-mannanase at 65°C for 10 min in 0.05 mol/L citric acid-0.1 mol/L Na\(_2\)HPO\(_4\) buffer at pH 6.0, and the reducing sugars were measured by DNS. The Michaelis-Menten kinetic parameters, \(V_{max}\) and \(K_m\), were calculated for \(\beta\)-mannanase. The assays of the purified enzyme were carried out by the standard DNS procedure, as described.
above, using 1–5 mg/mL locust bean gum and 0.5–2.5 mg/mL Konjac glucomannan as substrates. The kinetic constants were determined from the Michaelis-Menten equation by directly inputting the initial rates from Lineweaver-Burk plots or the nonlinear regression [24].

2.10. Statistical Analysis. Each \( \beta \)-mannanase activity experiment was performed in triplicate and expressed as mean ± SD (standard deviation). The statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago IL, USA). One-way or two-way analysis of variance (ANOVA) was used to compare various treatment groups.

3. Results and Discussion

3.1. Screening of the High \( \beta \)-Mannanase Activity Producing Strain. Four bacteria were stochastically selected for the \( \beta \)-mannanase activity assay. Figure 1 exhibited the halos produced on the screening plate. Table 1 summarized the \( \beta \)-mannanase activity of the four bacteria (strain BE-23 without \( \beta \)-mannanase activity was used as a negative control). Strain BE-91 fermented for 9 h exhibited the highest activity, up to 273.7 IU/mL. Wild-type \( B. \) subtilis MA139 yielded a maximum \( \beta \)-mannanase activity of 170 IU/mL after 3 days of fermentation, and the maximum enzyme activity of \( B. \) subtilis TJ-102 was 205.3 IU/mL at 38 h [25, 26]. Notably, BE-91 secreted \( \beta \)-mannanase with higher activity in shorter time.

3.2. Classification of \( B. \) subtilis BE-91. The 1,508 bp sequence of 16S rDNA of strain BE-91 was analyzed by a phylogenetic tree (Figure 2). The homology between BE-91 16S rDNA (gi 260159552) and \( B. \) subtilis 16S rDNA (gi 530330588 and gi 341831474) was 99%. It was confirmed that the similarity of \( B. \) subtilis type strains about 16S rRNA gene sequence is higher than 98% [27, 28]. We also obtained ≥98% similarity to 16S rRNA gene sequences of \( B. \) subtilis isolates.

3.3. Isolation and Purification of \( \beta \)-Mannanase. 2,000 mL of fermentation liquor was purified by ultrafiltration and chromatography. Specific activity, recovery, and multiple purifications at each step were summarized in Table 2. The recovery of \( \beta \)-mannanase in \( B. \) subtilis BE-91 exceeded 66.0%; multiple purifications achieved 32.9-fold pure \( \beta \)-mannanase activity, and the specific activity of the purified enzyme reached 79,859.2 IU/mg. The purified \( \beta \)-mannanase was shown to be homogeneous judged by SDS-PAGE analysis (Figure 3). Compared with the previous separation and purification methods [29, 30], the two-step method has the advantages of high efficiency, high yield, and easy operation.

3.4. Apparent Molecular Weight of \( \beta \)-Mannanase. The apparent molecular weight of \( \beta \)-mannanase was 28.2 kDa (Figure 3), lower than those of the most known \( \beta \)-mannanases from \( B. \) subtilis spp. (\( B. \) licheniformis THCM 3.1, 40 kDa; \( B. \) subtilis WY34, 39.6 kDa; \( B. \) subtilis Z-2, 38 kDa; \( B. \) circulans CGMCC1554, 32 kDa) [28, 31–34]. Similarly, the molecular weights of \( \beta \)-mannanases from \( P. \) occitanis Pol6 and \( B. \) halodurans PPKS-2 were 22 and 18 kDa, respectively [30, 31]. Due to low molecular weights, these enzymes may rapidly penetrate the lignocellulose systems and depolymerize the mannans more efficiently [35].

3.5. Optimal Temperature and Thermostability of \( \beta \)-Mannanase. The purified \( \beta \)-mannanase was maximally active at 65°C (Figure 4) and remained more than 80% active at 70°C (Figure 5). Compared with the optimal temperatures obtained for other \( \beta \)-mannanases (40°C for \( P. \) occitanis Pol6; 50°C for both \( B. \) circulans TN-31 and \( B. \) subtilis B36; 60°C for \( P. \) paenibacillus sp. DZ3) [29, 31, 36], \( \beta \)-mannanase of BE-91 showed a pronounced activity at higher temperatures. As compared to the thermostability of the \( \beta \)-mannanase from wild-type \( B. \) subtilis BCC41051 (60°C for 30 min) [37], this \( \beta \)-mannanase retains 80% residual activity after incubation at 20–70°C for 30 min, indicating enhanced thermostability.

3.6. Optimal pH and Stability of \( \beta \)-Mannanase. The optimal pH and the stability of BE-91 \( \beta \)-mannanase were measured at various pHs. The optimum enzyme activity was obtained at pH 6.0 (Figure 6), and more than 80% maximal activity was retained at pH 4.5–7.0 (Figure 7). Interestingly, the optimal pH of BE-91 \( \beta \)-mannanase was the same as that of \( B. \) subtilis MA139 (pH 6.0), an enzyme that can potentially be used.
Table 2: Purification of β-mannanase by ultrafiltration and gel chromatography.

| Purification step         | Total activity (IU) | Total protein (mg) | Specific activity (IU/mg) | Recovery (%) | Purification multiple (fold) |
|---------------------------|---------------------|--------------------|---------------------------|--------------|-----------------------------|
| Fermentation liquor       | 429,650.8           | 176.7              | 2,431.4                   | 100          | 1                           |
| Ultrafiltration           | 328,317.4           | 8.6                | 38,070.2                  | 76.4         | 15.6                        |
| Gel chromatography        | 283,500.2           | 3.6                | 79,859.2                  | 66.0         | 32.9                        |

**Figure 2:** Phylogenetic tree based on 16S rDNA sequences of strain BE-91 and other bacteria by Mega 6.0 using neighbor-joining analysis with 1000 bootstrap replicates.

as a feed additive for monogastric animals [25]. At pH < 4.0, the β-mannanase activity was negligible, retaining <80% of its maximal value obtained after incubation at pH > 7.5, 4°C for 30 min. A relatively broad zone of optimum activity was observed. Therefore, BE-91 β-mannanase can be considered a weakly acidic and neutral enzyme, thereby rendering suitability for animal feed industry [38].

3.7. The Effect of Metal Ions on β-Mannanase Stability. The effect of a variety of metal ions on β-mannanase activity was measured (Table 3). The highest induction was achieved with Mn²⁺, which showed 168% baseline activity, followed by Al³⁺, Ca²⁺, Cu²⁺, Zn²⁺, Mg²⁺, and NH₄⁺, respectively. K⁺ and Fe³⁺ had no obvious effects on β-mannanase activity in these conditions. Ba²⁺ and Pb²⁺ greatly inhibited the enzyme activity to a final rate of 83% and 74%, respectively. This suggests that BE-91 β-mannanase should not be contaminated by Ba²⁺ and Pb²⁺.

Table 3: Effects of different metal ions (1 mmol/L) on β-mannanase activity.

| Metal ions     | Relative activity (%)² |
|----------------|------------------------|
| Blank          | 100                    |
| K⁺             | 99 ± 3.2               |
| NH₄⁺           | 103 ± 2.7              |
| Ca²⁺           | 117 ± 3.6              |
| Zn²⁺           | 115 ± 2.9              |
| Mn²⁺           | 168 ± 4.5              |
| Cu²⁺           | 116 ± 2.1              |
| Mg²⁺           | 107 ± 2.8              |
| Ba²⁺           | 83 ± 3.1               |
| Pb²⁺           | 74 ± 2.9               |
| Fe³⁺           | 99 ± 3.6               |
| Al³⁺           | 121 ± 4.3              |

²Data are mean ± SD, n = 3.
3.8. Kinetic Parameters. The purified enzyme hydrolyzed Konjac glucomannan but only slightly hydrolyzed ivory nut mannan, guar galactomannan, and 1,4-beta-D-mannan. Wheat arabinoxylan, beechwood xylan, and CMC were barely hydrolyzed, as shown in Table 4. This beta-mannanase exhibited the highest activity with Konjac glucomannan, enriched in glucose units. This finding suggests that beta-mannanase of BE-91 preferentially hydrolyzes the beta-1,4-linkage of the glucosylated mannan backbone.

Table 4: Hydrolytic activity of the purified enzyme on different polysaccharides.

| Substrate (0.5%, w/v) | Relative activity (%) |
|-----------------------|-----------------------|
| Konjac glucomannan    | 100                   |
| Locust bean gum       | 88.15 ± 1.8           |
| Carob galactomannan   | 91.85 ± 1.7           |
| Guar galactomannan    | 35.70 ± 0.6           |
| Ivory nut mannan      | 32.74 ± 0.3           |
| 1,4-Beta-D-mannan     | 46.22 ± 0.4           |
| Wheat arabinoxylan    | 0                     |
| Beechwood xylan       | 0                     |
| Carboxymethyl cellulose| 0                     |

Assays were carried out at 65°C at pH 6.0 for 10 min in 0.05 mol/L citric acid-0.1 mol/L Na₂HPO₄ buffer.

Data are mean ± SD, n = 3.

$K_m$ and $V_{max}$ values of this beta-mannanase estimated by the Lineweaver-Burk plot were 7.14 mg/mL and 107.5 μmol/min/mL, respectively, for locust bean gum, versus 1.749 mg/mL and 33.45 μmol/min/mL for Konjac glucomannan.
respectively. These results displayed higher affinity of $\beta$-mannanase towards natural Konjac glucomannan ($V_{\text{max}}/K_m$, 19.1 $\mu$mol/min/mg) than the locust bean gum ($V_{\text{max}}/K_m$, 15.0 $\mu$mol/min/mg), similar to the values obtained for Penicillium pinophilum Cl and Penicillium freii F63, hence constituting it as an adequate candidate in food industry for the production of oligosaccharides [17, 18, 39].

4. Conclusion

B. subtilis bacteria are abundant, moderately stable, and mostly nonpathogenic microorganisms. Our results indicated that B. subtilis BE-91 could be considered a prominent candidate for the production of extracellular $\beta$-mannanase. In addition, this study developed an advanced purification approach, “two-step method,” with high efficiency, high yield, and easy operation. Furthermore, the $\beta$-mannanase purified from BE-91 was extremely stable at relatively high temperatures and various weak acidic or neutral pHs. Finally, the enzyme showed a higher affinity towards natural Konjac glucomannan, a major functional food material. Therefore, this $\beta$-mannanase, purified and characterized from B. subtilis BE-91 for the first time, is suitable for inflammatory diseases.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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