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# Purification and characterization of a novel alkaline β-1,3-1,4-glucanase (lichenase) from thermophilic fungus *Malbranchea cinnamomea*

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**Abstract** A novel alkaline β-1,3-1,4-glucanase (McLic1) from a thermophilic fungus, *Malbranchea cinnamomea*, was purified and biochemically characterized. McLic1 was purified to homogeneity with a purification fold of 3.1 and a recovery yield of 3.7 %. The purified enzyme was most active at pH 10.0 and 55 °C, and exhibited a wide range of pH stability (pH 4.0–10.0). McLic1 displayed strict substrate specificity for barley β-glucan, oat β-glucan and lichenan, but did not show activity towards other tested polysaccharides and synthetic *p*-nitrophenyl derivates, suggesting that it is a specific β-1,3-1,4-glucanase. The $K_m$ values for barley β-glucan, oat β-glucan and lichenan were determined to be 0.69, 1.11 and 0.63 mg $mL^{-1}$, respectively. Moreover, the enzyme was stable in various non ionic surfactants, oxidizing agents and several commercial detergents. Thus, the alkaline β-1,3-1,4-glucanase may have potential in industrial applications, such as detergent, paper and pulp industries.

**Keywords** β-1,3-1,4-Glucanase · *Malbranchea cinnamomea* · Alkaline · Purification · Characterization

## Introduction

β-1,3-1,4-Glucans, the major cell wall component of cereal endosperm, are the linear glucans of up to 1,200 β-D-glucosyl residues linked by β-1,4-linkages (three to four) interrupted by a single β-1,3-linkage [1, 2]. β-1,3-1,4-Glucanases (EC 3.2.1.73, lichenases) is a type of hydrolytic enzymes catalyzing the hydrolysis of β-1,4-linkages adjacent to a β-1,3 glycosidic moiety on the non-reducing end of β-1,3-1,4-glucans to yield mainly trisaccharide and tetrasaccharide [3]. They have gained considerable attention in recent years due to their potential application in various industrial processes, including clarification of juices and beer in food industry [4, 5], digestibility improvement of animal feed in poultry industry [6], reducing the amount of chlorine used for bleaching in paper and pulp or detergent industries [7] and bioconversion of lignocellulosic materials into bio-ethanol or other fermentable products in energy industry [8].

β-1,3-1,4-Glucanases are widely distributed in various organisms, including bacteria, fungi and plants [9]. To date, a number of microbial β-1,3-1,4-glucanases have been identified and characterized [3, 7, 9–18], while most of them are from bacteria, *Bacillus* species, such as *Bacillus brevis* [10], *B. licheniformis* [9], *Bacillus* sp. [7, 11, 19] and *B. subtilis* [3, 13, 20]. Only several β-1,3-1,4-glucanases have been reported from fungi such as *Cochiobolus carbonum* [21], *Aspergillus terreus* [15], *Paecilomyces thermophila* [16], *Laetiporus sulphureus* var. *miniatus* [17], *Rhizomucor miehei* [18] and *Penicillium occitanis* [5]. Furthermore, most reported fungal β-1,3-1,4-glucanases showed acidic or neutral pH values [1, 5, 18, 22, 23], and no alkaline β-1,3-1,4-glucanases from fungal species have ever been reported. The thermophilic fungi from *Malbranchea* sp. are known as potential industrial-enzyme producers as they have been reported to secrete multiple kinds of hydrolytic enzymes, such as xylanase [24] and α-amylase [25]. However, no β-1,3-1,4-glucanase has been reported from the fungal species of this genus.

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The newly isolated *Malbranchea cinnamomea* S168 has been found to produce an α-amylase in our previous study [25]. Furthermore, it also secreted a β-1,3-1,4-glucanase in the fermentation broth (data not shown). In the present study, we described the purification, identification and biochemical characterization of a novel β-1,3-1,4-glucanase from the strain. The potential application of the enzyme in laundry detergent was further evaluated. To the best of our knowledge, this is the first report on a novel alkaline β-1,3-1,4-glucanase from a fungus.

## Materials and methods

### Materials

Barley β-D-glucan, oat glucan, CMC (low viscosity), locust bean gum (LBG), soluble starch, pullulan (from *Aureobasidium pullulans*), cellobiose and *p*-nitrophenyl derivates were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). β-1,3-Glucan (from *Euglena fracilis*) was obtained from Fluka Chemical Co. (Steinheim, Germany). Lichenan (from *Cetraria islandica*), laminarin (from *Laminaria digitata*) and xyloglucan (from Tamarind seed) were the products of Megazyme International Ireland Ltd. (Bray, Wicklow, Ireland). Avicel was from Merck Co. Ltd. (Darmstadt, Germany). Q-Sepharose Fast Flow resin and Phenyl-Sepharose™ 6 Fast Flow resin were obtained from Pharmacia (Pharmacia, Uppsala, Sweden). Corncobs and other biomass were obtained locally and were ground into small pieces (0.45–0.9 mm) in a hammer mill. All other chemicals used were of analytical grade unless otherwise stated.

### Fungal strain and growth conditions

The thermophilic fungus, *M. cinnamomea* S168 used in the present study has been deposited at the China General Microbiological Culture Collection Center (CGMCC) under accession number 6022. It was maintained on potato dextrose agar (PDA) plate, and stored at 4 °C until use.

For β-1,3-1,4-glucanase production, a piece (1 $cm^2$) of agar medium covered with 4-day-old mycelium was inoculated into 50 mL basal culture medium (in 250 mL-Erlenmeyer flask) containing (g $L^{-1}$): 15 corncob (0.45–0.9 mm), 10 beef peptone, 10 yeast extract, 1 $KH_2PO_4$, 0.3 $(NH_4)_2SO_4$, 0.3 $CaCl_2$, 0.3 $MgSO_4 \cdot 7H_2O$, 0.3 $FeSO_4$, and 5 Tween-80 with initial pH of 5.5, and incubated at 45 °C in a shaker at a rotation speed of 200 rpm. After 7 days of cultivation, the culture broth was centrifuged at 10,000*g* for 10 min, and the supernatant was collected and used as the crude enzyme for subsequent experiment.

The fermentation conditions for β-1,3-1,4-glucanase production by *M. cinnamomea* S168 were further optimized by a "single-factor experiment". The key parameters: carbon sources and its content (0.5–5.0 %, w/v), nitrogen sources and its content (0.5–5.0 %, w/v), culture pH (pH 4.0–8.0), cultivation temperature (30–50 °C) and incubation time (1–10 days) were optimized step by step.

### Purification of a β-1,3-1,4-glucanase

Unless otherwise stated, all the purification processes were performed at 4 °C. The crude enzyme (300 mL) was firstly subjected to 40–60 % saturated ammonium sulfate precipitation. The precipitates were collected by centrifugation at 10,000*g* for 15 min, re-dissolved in 20 mM phosphate buffer pH 7.0 (buffer A), and dialyzed against buffer A overnight. The dialyzed sample was then applied onto a Q-Sepharose Fast Flow column (10 × 1.5 cm) pre-equilibrated with buffer A. After washing with 5 column volumes of buffer A, the bound proteins were eluted with a linear gradient (0–500 mM) of NaCl at a flow rate of 1.0 mL $min^{-1}$. Active fractions were concentrated and dialyzed against 20 mM pH 6.0 citrate buffer (buffer B), and then loaded onto a Q-Sepharose Fast Flow column (10 × 1.5 cm) again. The bound proteins were eluted with 100 mM NaCl and 200 mM NaCl in buffer B, respectively. The fractions showing β-1,3-1,4-glucanase activity were collected and buffer changed with buffer B. Then the sample was loaded onto a Phenyl-Sepharose™ 6 Fast Flow column (10 × 1.5 cm). The bound proteins were eluted with a 0–1 M gradient of ammonium sulfate in buffer B at a flow rate of 1 mL $min^{-1}$. The active fractions were collected, concentrated and checked for purity by SDS-PAGE.

### Enzyme assay and protein determination

For β-1,3-1,4-glucanase activity determination, a reaction mixture containing 50 μL of 1 % (w/v) barley β-glucan in 50 mM glycine–NaOH buffer (pH 10.0) and 150 μL properly diluted enzyme solution was incubated at 55 °C for 10 min, and then the released reducing sugars were estimated by the DNS method using glucose as the standard [26]. One unit (U) of β-1,3-1,4-glucanase activity was defined as the amount of enzyme that required to produce 1 μmol reducing sugar per minute under the above conditions. Specific activity was expressed as units per milligram protein.

Protein concentration was measured by the Lowry method [27] using bovine serum albumin (BSA) as the standard.

### SDS-PAGE, zymogram and molecular mass determination

SDS-PAGE was performed as described by Laemmli [28] using 12.5 % (w/v) separation gel and 4.5 % (w/v) stock

gel. Protein bands were visualized by Coomassie brilliant blue R-250 staining. The low molecular mass calibration kit used contains: phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonicanhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.3 kDa). Zymogram analysis of β-1,3-1,4-glucanase was carried out according to the method of Yang et al. [16].

The native molecular mass of β-1,3-1,4-glucanase was determined by gel filtration method on a Sephacryl S-100 HR column (1 × 40 cm) pre-equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. The purified β-1,3-1,4-glucanase (0.5 mL, 2 mg $mL^{-1}$) was loaded onto the column and eluted with the equilibration buffer at a flow rate of 0.3 mL $min^{-1}$. The protein standards used for molecular mass calibration consisted of phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), albumin from chicken egg white (45.0 kDa), α-chymotrypsinogen A (25.7 kDa) and cytochrome *c* (12.3 kDa).

### Analysis of internal peptide sequences

To determine the internal peptides' amino acid sequence of purified β-1,3-1,4-glucanase, the single protein band indicated on SDS-polyacrylamide gel was excised and hydrolyzed by trypsin in 20 mM phosphate buffer (pH 7.0). The hydrolysate was then submitted to the National Center of Biomedical Analysis (Beijing, China) for sequencing the internal peptides using high-performance liquid chromatography-electrospray tandem mass spectrometry (HPLC–ESI–MS/MS). Mass spectral sequencing was performed on a Q-TOF II mass analyzer (Micromass Ltd., Manchester, UK). Peptide sequencing was performed using a palladium-coated borosilicate electrospray needle (Protana, Denmark).

### Effects of pH and temperature on the purified β-1,3-1,4-glucanase

The optimal pH of the purified β-1,3-1,4-glucanase (McLic1) was determined in different buffers (50 mM) within pH 3.0–12.0. Buffers used were: citrate (pH 3.0–6.0), MES (pH 5.5–7.0), MOPS (pH 6.5–8.0), CHES (pH 8.0–9.5), glycine–NaOH (pH 8.5–10.5), CAPS (pH 9.5–11.0) and $Na_2HPO_4$–NaOH (pH 11.0–12.0). To determine the pH stability, the residual activity of the purified enzyme was assayed after it was incubated in different buffers mentioned above at 37 °C for 30 min.

The effect of temperature on β-1,3-1,4-glucanase's activity was evaluated at different temperatures (30–80 °C) in 50 mM glycine–NaOH buffer (pH 10.0). For determination of thermostability, McLic1 was incubated in 50 mM glycine–NaOH buffer (pH 10.0) at different temperatures (30–80 °C) for 30 min, and then the residual β-1,3-1,4-glucanase activities were measured by the standard enzyme assay.

To investigate the effects of various metal ions and reagents on the β-1,3-1,4-glucanase activity, the enzyme was treated with individual metal ion or reagent (4 mM) in 50 mM glycine–NaOH buffer (pH 10.0) at 25 °C for 30 min. The residual β-1,3-1,4-glucanase activities were then measured at 55 °C in 50 mM glycine–NaOH (pH 10.0) by the standard enzyme assay.

### Substrate specificity and kinetic parameter of the purified β-1,3-1,4-glucanase

The substrate specificity of McLic1 was determined by measuring the enzyme's activity at 55 °C in 50 mM glycine–NaOH (pH 10.0) with various polysaccharides (1 %, w/v) including oat β-glucan glucan, lichenan, CMC, Avicel, laminarin, birchwood xylan, LBG, pullulan, soluble starch, and *p*NP-glycoside substrates (5 mM) including *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside and *p*NP-β-D-xylopyranoside. The reactions were performed at 55 °C for 10 min in 50 mM glycine–NaOH buffer (pH 10.0). The released reducing sugars were estimated by DNS method [26], while the *p*NP formed was detected by spectrophotometry at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that required to produce 1 μmol reducing sugar or *p*NP per minute under the above assay conditions.

To determine the kinetic parameters, the reaction was performed at 55 °C for 5 min in 50 mM glycine–NaOH buffer (pH 10.0) using different substrate concentrations. $K_m$ and $V_{max}$ values were calculated by the software "GraFit".

### Hydrolysis property of the purified β-1,3-1,4-glucanase

The hydrolysis property of the β-1,3-1,4-glucanase was investigated by analyzing the products of barley β-glucan and lichenan. 5 U of β-1,3-1,4-glucanase was added to 1 mL of 1 % (w/v) of each substrate in 50 mM glycine–NaOH buffer (pH 10.0) and incubated at 50 °C for 8 h, separately. Aliquots were withdrawn at different time intervals, boiled for 5 min to terminate the added enzyme and then analyzed by thin layer chromatography (TLC). For TLC analysis, the samples were spotted on a silica gel plate 60F 254 (Merck, Darmstadt, Germany). The plate was developed with two runs in a butanol:acetic acid:water (2:1:1, v/v) solvent system. After spraying with methanol:sulfuric acid (95:5, v/v) solvent, the sugars on the plate were visualized by heating for a few minutes at 130 °C. A mixture consisting of glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4) and cellopentaose (G5) was used as the standard.

**Table 1** Purification of the McLic1 from *M. cinnamomea*

| Purification step | Total activity[a] (U) | Total protein[b] (mg) | Specific activity (U $mg^{-1}$) | Purification factor (-fold) | Recovery (%) |
|---|---|---|---|---|---|
| Culture supernatant | 27,489.4 | 1,398.6 | 16.9 | 1.0 | 100 |
| $(NH_4)_2SO_4$ precipitation | 18,352.8 | 515.5 | 31.2 | 1.8 | 66.8 |
| Q-Sepharose Fast Flow (pH 7.0) | 8,148.7 | 208.3 | 34.0 | 2.0 | 29.6 |
| Q-Sepharose Fast Flow (pH 6.0) | 1,242.2 | 21.3 | 50.7 | 3.0 | 4.5 |
| Phenyl-Sepharose™ Fast Flow | 1,006 | 16.7 | 52.3 | 3.1 | 3.7 |

[a] Activity was measured in 50 mM glycine–NaOH buffer (pH 10.0) at 55 °C for 10 min, using barley β-D-glucan as the substrate

[b] The protein was measured by the Lowry method [27], using BSA as the standard

### Effect of detergent additives and commercial detergents on the stability of McLic1

The effect of detergent additives on the stability of McLic1 was investigated by measuring the residual activities after the enzyme (1 mg $mL^{-1}$) was incubated in the presence of 1 and 5 % of surfactants (Tween 20, Tween 80, Triton ×100 and SDS) or 1 and 5 % of oxidizing agent ($H_2O_2$) at 40 °C for 1 h.

The compatibility of McLic1 with some commercial liquid and solid detergents was studied according to the method of Maktouf et al. [7]. The commercial detergents were diluted with distilled water to a final concentration of 7 mg $mL^{-1}$ to simulate the washing conditions, and then boiled for 5 min. Equal volumes of enzyme (1 mg $mL^{-1}$) and detergent solution were mixed and incubated at 40 °C for 1 h. Then, the residual β-1,3-1,4-glucanase activity was measured at 55 °C in 50 mM citrate buffer (pH 10.0) by the standard enzyme assay. The enzyme incubated at the same conditions without the presence of detergents was used as control.

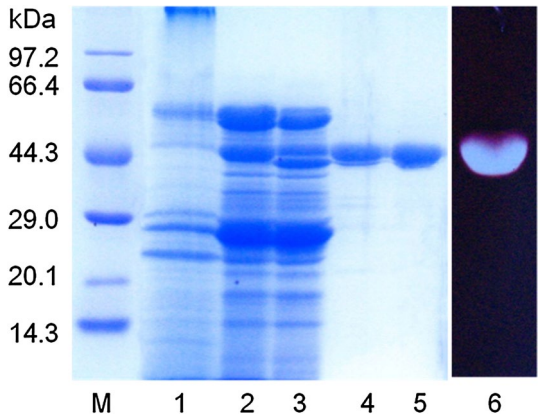


**Fig. 1** SDS-PAGE of the proteins during the purification process of β-1,3-1,4-glucanase McLic1 from the culture broth of *M. cinnamomea* S168. *Lane M* low molecular weight standard proteins, *lane 1* crude enzyme, *lane 2* 40–60 % ammonium sulphate precipitation, *lane 3* after QSFF column (pH 7.0), *lane 4* after QSFF column (pH 6.0), *lane 5* after Phenyl-Sepharose™ 6 Fast Flow column, *lane 6* zymogram of the purified McLic1

## Results

### Production of β-1,3-1,4-glucanase by *M. cinnamomea* S168

The optimal fermentation conditions for the production of β-1,3-1,4-glucanase by *M. cinnamomea* S168 in submerged culture were obtained, viz. 2.5 % (w/v) corncob as carbon source, 2.5 % (w/v) yeast extract as nitrogen source, initial pH of 5.5, cultivation temperature of 45 °C and incubation time of 7 days. Under the optimal conditions, the highest β-1,3-1,4-glucanase activity of 100.3 U $mL^{-1}$ was obtained (data not shown).

### Purification of β-1,3-1,4-glucanase from *M. cinnamomea* S168

The β-1,3-1,4-glucanase (McLic1) was purified to homogeneity as indicated on SDS-PAGE with a purification fold of 3.1 and a recovery yield of 3.7 % (Table 1). The specific activity of the enzyme was increased from 16.9 to 52.3 U $mg^{-1}$. The denatured molecular mass of the purified enzyme was estimated to be 44.7 kDa by SDS-PAGE (Fig. 1), while the native molecular mass of the enzyme was determined to be 46.9 kDa by gel filtration (data not shown), indicating that McLic1 is a monomer.

Three amino acid sequences of internal peptides derived from the purified β-1,3-1,4-glucanase (McLic1) were obtained. They are peptide I (GESDGTSDTSAER), peptide II (AAAASNGEFSLED) and peptide III (AAAASNGEFS DNSAA), respectively (Table 2). The sequences were subjected to the NCBI-BLAST database to compare with that of other reported β-glucanases. The results revealed that peptide I displayed 100 % identities with the putative β-glucanases from *Malbranchea cinnamomea* and *Aspergillus terreus* NIH2624, and peptide II showed 100 % of identity with the putative β-glucanase from *M. cinnamomea*. However, peptide III showed only 76 % identity with the β-glucanase from *Thielavia terrestris* NRRL 8126 (Table 2).

**Table 2** Three internal peptides derived from McLic1

| Sources | Position[a] | Sequence[b] | Identity (%) | Accession No. |
|---|---|---|---|---|
| Peptide I | | GESDGTSDTSAER | | |
| *Malbranchea cinnamomea* | 342 | GESDGTSDTSAER | 100 | CDF76449.1 |
| *Aspergillus terreus* NIH2624 | 353 | GESDGTSDTSAER | 100 | XP_001210279.1 |
| Peptide II | | AAAASNGEFSLED | | |
| *M. cinnamomea* | 122 | AAAASNGEFSLED | 100 | CDF76449.1 |
| Peptide III | | AAAASNGEFSDNSAA | | |
| *Thielavia terrestris* NRRL 8126 | 213 | AAAASNGEFSIANNGAA | 76 | XP_003648546.1 |

[a] Position is the first amino acid of the peptide sequence

[b] Identity amino acids are underlined

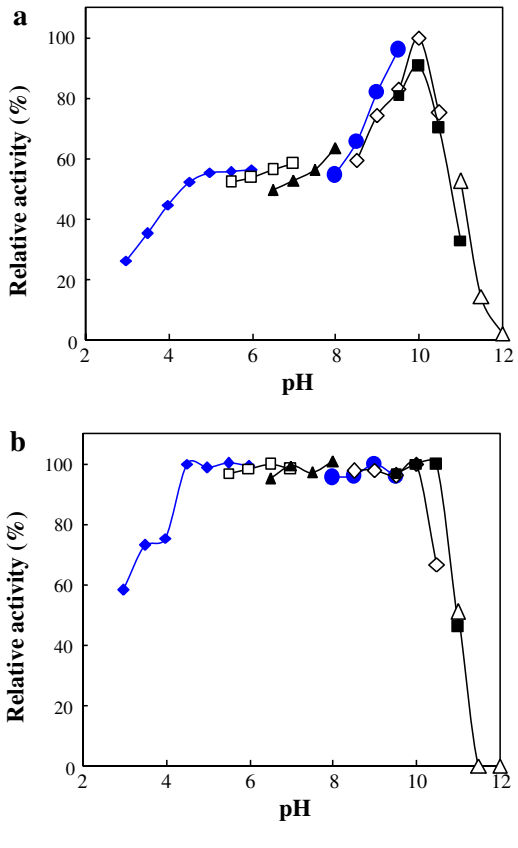


**Fig. 2** Optimum pH (**a**) and pH stability (**b**) of the purified McLic1 from *M. cinnamomea* S168. The optimal pH was determined at 55 °C in different buffers (50 mM) with pH ranging from 3.0–12.0. The buffers used were citrate buffer (*filled diamond*) (pH 3.0–6.0), MES buffer (*open square*) (pH 5.5–7.0), MOPS buffer (*filled triangle*) (pH 6.5–8.0), CHES buffer (*filled circle*) (pH 8.0–9.5), glycine–NaOH buffer (*open diamond*) (pH 8.5–10.5), CAPS buffer (*filled square*) (pH 9.5–11.0) and $Na_2HPO_4$–NaOH buffer (*open triangle*, pH 11.0–12.0). To determine pH stability, McLic1 was incubated at 37 °C for 30 min in various buffers mentioned above, and then the residual activities were measured

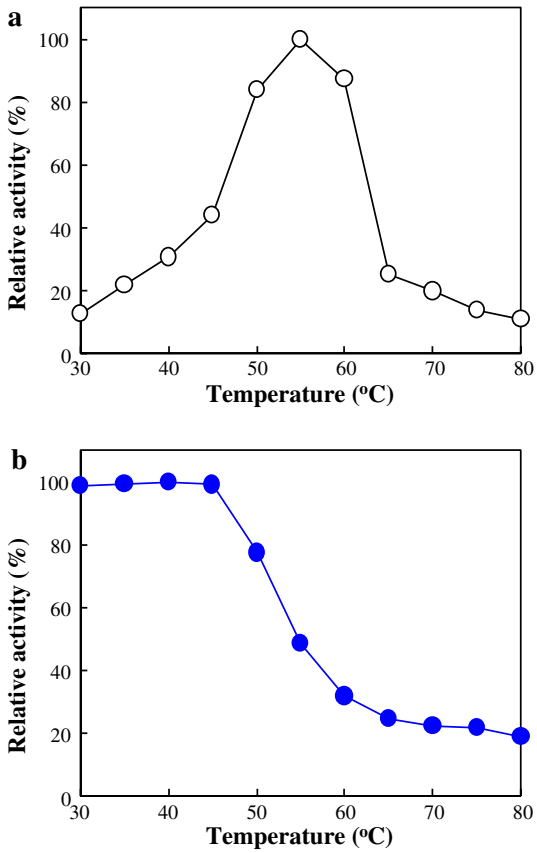


**Fig. 3** Optimal temperature (**a**) and thermal stability (**b**) of the purified McLic1 from *M. cinnamomea* S168. The optimal temperature was determined at different temperatures (30–80 °C) in 50 mM glycine–NaOH buffer (pH 10.0). To determine the thermostability, the residual activities were measured at 55 °C after the enzyme was incubated in 50 mM glycine–NaOH buffer (pH 10.0) at different temperatures (30–80 °C) for 30 min

## Characterization of the purified McLic1

McLic1 displayed optimal activity at pH 10.0 in glycine–NaOH buffer (Fig. 2a). It retained more than 90 % of its activities after being treated in different buffers with a pH range of 4.5–10.0 for 30 min (Fig. 2b). The optimal temperature of McLic1 was determined to be 55 °C in glycine–NaOH buffer (pH 10.0) (Fig. 3a), and it was stable up 45 °C (Fig. 3b).

McLic1 activity was strongly inhibited by $Fe^{3+}$ (19.8 %) and $Mn^{2+}$ (56.4 %), while significantly inhibited by $Hg^{2+}$ (87.3 %), $Cr^{3+}$ (84 %) and $Ag^{+}$ (71.4 %). However, its activity was enhanced in the presence of $Cu^{2+}$ (120 %), $Ca^{2+}$ (116.1 %), $Ni^{2+}$ (115.3 %), $Mg^{2+}$ (120 %) and $Co^{2+}$ (147.2 %) (Table 3). EDTA, SDS and β-mercaptoethanol

slightly promoted McLic1's activity (Table 3), suggesting that no metal ions or disulfide bonds were needed for the enzymatic reaction.

### Substrate specificity and kinetic parameters

The enzyme exhibited the highest specific activity toward barley β-glucan (52.7 U $mg^{-1}$) followed by oat β-glucan (43.2 U $mg^{-1}$) and lichenan (23.0 U $mg^{-1}$) (Table 4). No activity was detected toward other tested substrates. Thus, McLic1 should be a strict β-1,3-1,4-glucanase based on its substrate specificity.

The kinetic parameters, $K_m$ and $V_{max}$ of McLic1 for barley β-glucan, oat β-glucan and lichenan were determined to be 0.69 mg $mL^{-1}$ and 21.9 μmol $min^{-1}$ $mg^{-1}$, 1.11 mg $mL^{-1}$ and 29.3 μmol $min^{-1}$ $mg^{-1}$, and 0.63 mg $mL^{-1}$ and 9.35 μmol $min^{-1}$ $mg^{-1}$ (Table 5).

**Table 3** Effect of metal ions and reagents on the activity of McLic1

| Metal ions and agents | Specific activity[a] (U $mg^{-1}$) | Relative activity (%) |
|---|---|---|
| Control | 52.7 ± 0.2 | 100 |
| $Cu^{2+}$ | 63.3 ± 1.1 | 120.0 |
| $Ca^{2+}$ | 61.3 ± 1.0 | 116.1 |
| $Ni^{2+}$ | 60.9 ± 0.4 | 115.3 |
| $Mg^{2+}$ | 49.1 ± 3.0 | 93.0 |
| $Zn^{2+}$ | 65.9 ± 0.5 | 124.8 |
| $Mn^{2+}$ | 29.8 ± 0.4 | 56.4 |
| $Co^{2+}$ | 77.7 ± 4.1 | 147.2 |
| $Hg^{2+}$ | 46.1 ± 0.7 | 87.3 |
| $Fe^{3+}$ | 10.5 ± 1.2 | 19.8 |
| $Cr^{3+}$ | 44.4 ± 2.5 | 84.0 |
| $Fe^{2+}$ | 49.5 ± 1.7 | 93.7 |
| $Ag^{+}$ | 37.7 ± 1.9 | 71.4 |
| $K^{+}$ | 56.7 ± 3.4 | 107.4 |
| SDS | 62.2 ± 4.0 | 117.9 |
| EDTA | 53.9 ± 1.1 | 102.1 |
| DTT | 59.5 ± 1.9 | 112.8 |
| β-mercaptoethanol | 62.5 ± 1.9 | 118.3 |

[a] Activity was measured in 50 mM glycine–NaOH buffer (pH 10.0) at 55 °C for 10 min, using barley β-D-glucan as the substrate

**Table 4** Substrate specificity of McLic1

| Substrate | Specific activity[a] (U $mg^{-1}$) | Relative activity (%) |
|---|---|---|
| Barley β-glucan | 52.7 ± 0.2 | 100 |
| Oat β-glucan | 43.2 ± 0.1 | 82.0 |
| Lichenan | 23.0 ± 1.7 | 43.6 |

[a] Activity was measured in 50 mM glycine–NaOH buffer (pH 10.0) at 55 °C for 10 min, using various polysaccharides as the substrate

### Hydrolysis property of McLic1

The products of barley β-glucan and lichenan hydrolyzed by the purified β-1,3-1,4-glucanase were analyzed. McLic1 efficiently hydrolyzed barley β-glucan to yield trisaccharides, tetrasaccharides and other oligosaccharides with higher degree of polymerization (DP), with trisaccharides and tetrasaccharides as the predominant products, while it hydrolyzed lichenan to yield mainly bisaccharides, trisaccharides and tetrasaccharides as well as a series of oligosaccharides with DP higher than 5 (Fig. 4).

### Compatibility of McLic1 in detergent

McLic1 exhibited an excellent stability in the presence of all the tested ingredients commonly used in detergent, including Tween 80, Triton ×100, SDS, sodium hypochlorite and $H_2O_2$, retaining more than 90 % of its original activity (Table 6). Furthermore, the enzyme also showed remarkable stability in the presence of some commercial liquid and solid detergents such as OMO (washing liquid), Blue moon (washing liquid), Fresh'n Hygiene (washing liquid), OMO (washing powder), Diao (washing powder), Tide (washing powder) and Super (natural soap powder), with 101, 97, 103, 106, 100, 90 and 93 % of enzyme activities were retained, respectively (Table 6).

## Discussion

Though a number of bacterial β-1,3-1,4-glucanases have been identified and characterized, there have been few reports on fungal β-1,3-1,4-glucanases [2, 5]. Here, we reported the purification and biochemical characterization of a novel alkaline β-1,3-1,4-glucanase from a thermophilic fungus, *Malbranchea cinnamomea*.

The highest β-1,3-1,4-glucanase production of 100.3 U $mL^{-1}$ was obtained in submerged culture. This value is comparable to that produced by *P. thermophila* (135.6 U $mL^{-1}$ [16]), but obviously higher than those produced by *P. occitanis* Pol6 (50 U $mL^{-1}$ [5]) and *Aspergillus niger* (2.2 U $mL^{-1}$ [23]) and much lower than that produced by *R. miehei* (6,230 U $mL^{-1}$ [18]). The purified McLic1 was a monomer with a molecular mass of approximately 44.7 kDa (Fig. 1). The value is higher than that of most bacterial β-1-3,1-4-glucanases with molecular masses in the range of 25–30 kDa [2]. It is also higher than those of β-1,3-1,4-glucanases from other fungi, including *P. occitanis* (20 kDa [5]), *Orpinomyces* sp. PC-2 (27 kDa [29]), *A. niger* (32 kDa [23]), *Rhizopus microspores* var. *microsporus* (33.7 kDa [22]), *Thermoascus aurantiacus* (37 kDa [30]) and *Talaromyces emersonii* (40.7 kDa [1]), but lower than that of a β-1,3-1,4-glucanase from an

**Table 5** Kinetic parameters of McLic1

| Substrate | $V_{max}$ (μmol $min^{-1}$ $mg^{-1}$) | $K_m$ (mg $mL^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ mL $mg^{-1}$) |
|---|---|---|---|---|
| Barley β-glucan | 21.9 ± 1.13 | 0.69 ± 0.05 | 0.016 | 0.023 |
| Oat β-glucan | 29.3 ± 1.37 | 1.11 ± 0.10 | 0.022 | 0.019 |
| Lichenan | 9.35 ± 0.26 | 0.63 ± 0.04 | 0.007 | 0.011 |

Activity was measured in 50 mM glycine–NaOH buffer (pH 10.0) at 55 °C for 5 min, using various polysaccharides as the substrates

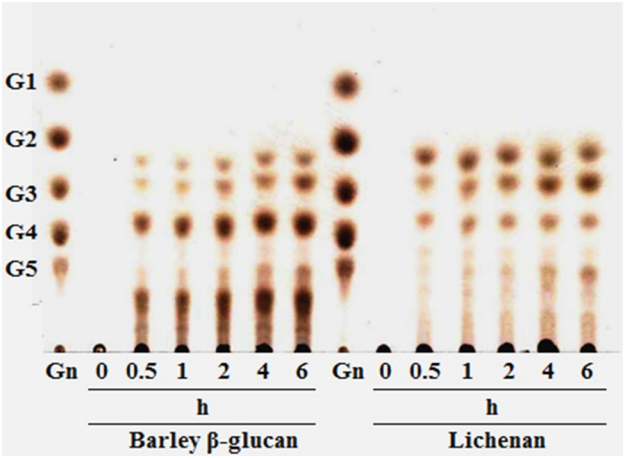


**Fig. 4** TLC analysis of barley β-glucan and lichenan hydrolyzed by the purified McLic1 from *M. cinnamomea* S168. The hydrolysis reactions were performed at 50 °C in 50 mM glycine–NaOH buffer (pH 10.0) for 6 h. Substrates and incubation times were indicated in the figures. Glucose ($G_1$), cellobiose ($G_2$), cellotriose ($G_3$), and cellotetraose ($G_4$) were used as the standards (M)

alkalothermophilic actinomycete, *Thermomonospora* sp. (64.5 kDa [31]).

Most of fungal β-1,3-1,4-glucanases are optimally active at acidic or neutral pH values, such as the enzymes from *P. occitanis* (pH 3.0 [5]), *Talaromyces emersonii* (pH 4.8 [1]), *A. niger* (pH 5.0 [23]), *Rhizopus microspores* var. *microsporus* (pH 5.0 [22]), *R. miehei* (pH 5.5 [18]), *T. aurantiacus* (pH 6.0 [30]), *Orpinomyces* sp. PC-2 (pH 6.0 [29]) and *P. thermophila* (pH 7.0 [16]). However, McLic1 in the present study was most active at an alkaline pH of 10.0, which is much higher than those of other fungal β-1,3-1,4-glucanases. In addition, McLic1 also exhibited a wide range of pH stability (pH 4.0–10.0), the property of which is similar to that of the β-1,3-1,4-glucanase from *P. occitanis* [5]. Similar to fungal β-1,3-1,4-glucanases, most reported bacterial β-1,3-1,4-glucanases also exhibited pH optima around neutrality (pH 6.0–7.0) except for the enzymes from *B. brevis* (pH 9.0) and alkalophilic *B.* sp. N137 (pH 7.0–12.0) [2]. To the best of our knowledge, this is the first report on an alkaline β-1,3-1,4-glucanase

**Table 6** Stability of the purified McLic1 in the presence of various surfactants, bleaches and commercial detergents

| Surfactant/oxidizing/detergent[a] | Substrate specificity (U $mg^{-1}$) | Relative activity (%) |
|---|---|---|
| Control | 52.7 ± 0.2 | 100 |
| Tween-80 (1 %) | 50.5 ± 0.5 | 96 |
| Tween-80 (5 %) | 50.6 ± 0.3 | 96 |
| Triton ×100 (1 %) | 51.8 ± 0.8 | 98 |
| Triton ×100 (5 %) | 51.1 ± 0.7 | 97 |
| SDS (0.1 %) | 52.9 ± 0.3 | 100 |
| SDS (0.5 %) | 62.4 ± 0.9 | 118 |
| $H_2O_2$ (0.5 %) | 52.4 ± 0.2 | 99.4 |
| $H_2O_2$ (1 %) | 47.0 ± 0.4 | 89 |
| NaOCl (0.2 %) | 50.1 ± 0.4 | 95 |
| NaOCl (1 %) | 51.8 ± 0.7 | 98 |
| OMO (washing liquid) | 53.2 ± 0.6 | 101 |
| Blue moon (washing liquid) | 51.2 ± 0.7 | 97 |
| Fresh'n Hygiene (washing liquid) | 54.2 ± 0.9 | 103 |
| OMO (washing powder) | 56.0 ± 1.0 | 106 |
| Diao (washing powder) | 52.7 ± 0.2 | 100 |
| Tide (washing powder) | 47.3 ± 0.3 | 90 |
| Super (natural soap powder) | 49.0 ± 0.6 | 93 |

[a] The purified enzyme (1 mg $mL^{-1}$) was incubated in the presence of various surfactants, oxidants and commercial detergents at 40 °C for 1 h, respectively, and then the retained activities were measured in 50 mM glycine–NaOH buffer (pH 10.0) at 55 °C by the standard enzyme assay

from fungal species. The unique property may possess the enzyme special potential in several industries that should be carried out in alkaline conditions, such as detergent, paper and pulp industries. The optimal temperature of McLic1 (55 °C) is comparable to those of the β-1,3-1,4-glucanases from *R. microspores* var. *microsporus* (50 °C [22]), *A. niger* (60 °C [23]), *T. aurantiacus* (60 °C [30]) and *P. occitanis* (60 °C [5]), obviously higher than those of the β-1,3-1,4-glucanases from *T. emersonii* (40 °C [1]) and *Orpinomyces* sp. PC-2 (40 °C [29]), and lower than that of the β-1,3-1,4-glucanases from *T. aurantiacus* (70 °C [30]).

McLic1 exhibited strict substrate specificity for barley β-glucan, oat β-glucan and lichenan, which consist of mixed β-1,3 and β-1,4 glycosidic linkages, whereas displayed no activity on other tested substrates containing only α-1,4; α-1,6; β-1,3, β-1,4 and other linkages (Table 4), suggesting that the present enzyme is a strict β-1,3-1,4-glucanase (lichenase). Most of other reported fungal β-1,3-1,4-glucanases also showed strict substrate specificity [5, 16, 18, 23], but there are still some differences among them. For example, the β-1,3-1,4-glucanases from *M. cinnamomea* (Table 4, in the present study), *P. thermophila* [16] and *A. niger* [23] prefer to hydrolyze barley β-glucan, with the specific activities for barley β-glucan more than two times higher than that for lichenan, while the β-1,3-1,4-glucanases from *R. miehei* [18] and *P. occitanis* (60 °C [5]) both showed almost the same specificity for barley β-glucan and lichenan. Besides, the β-1,3-1,4-glucanase from *R. miehei* exhibited trace activity on laminarin [18], while the others did not [5, 16, 23]. The $K_m$ value of McLic1 for barley β-glucan (0.69 mg $mL^{-1}$) is similar to that of the β-1,3-1,4-glucanases from *Orpinomyces* sp. PC-2 (0.75 mg $mL^{-1}$ [29]) and *A. niger* (0.62 mg $mL^{-1}$ [23]). However, it is lower than that of the β-1,3-1,4-glucanases from most other fungi displaying $K_m$ values in the range of 1.98–13.38 mg $mL^{-1}$ for barley β-glucan [1, 5, 16, 18, 22]. It is reported that endo-β-1,3-1,4-glucanases (lichenases) specifically catalyze the cleavage of β-1,4 glycosidic linkages adjacent to β-1,3 bonds to yield chiefly trisaccharides and tetrasaccharides [12, 32, 33]. The β-1,3-1,4-glucanase in the present study hydrolyzed barley β-glucan and lichanan, releasing bisaccharide, trisaccharide and tetrasaccharide as the predominant products (Fig. 4). A trisaccharide and a tetrasaccharide from both β-D-glucan and lichenan migrating ahead of cellotriose and cellotetraose are possibly laminaritriose and laminaritetraose, respectively, which is similar to the results of other reports [16, 29, 34].

Enzymes being active at extreme alkaline pH and stable over a wide range of pH are desired for several industrial applications, especially those in alkaline conditions, such as detergent, paper and pulp industries [35]. Hence, the potential of McLic1 in laundry detergent was further evaluated. McLic1 exhibited excellent stability in the presence of several commonly used ingredients in bleach-based detergent formulations, such as Tween 80, Triton ×100, $H_2O_2$, NaOCl and SDS (Table 6). Furthermore, it also exhibited remarkable stability in the presence of several tested commercial washing liquids and washing powders (Table 6). The property is similar to the bacterial lichenases from *B. licheniformis* UEB CF [9] and *Bacillus* sp. UES-S [7]. However, the enzyme in the present study displayed higher stability in high concentration of $H_2O_2$ (1 %, v/v) when compared with the lichenase from *B. licheniformis* UEB CF, whose enzyme activity was completely inhibited [9]. The excellent properties indicated that McLic1 can be used in both liquid and solid detergent formulations.

In conclusion, a novel β-1,3-1,4-glucanase (McLic1) from a thermophilic fungus, *Malbranchea cinnamomea*, was purified and functionally characterized. McLic1 was purified to apparent homogeneity with a molecular mass of 44.7 kDa on SDS-PAGE. It was an alkaline enzyme having an extreme alkaline optimal pH of 10.0, and was stable in a wide range of pH (4.0–10.0). The enzyme was most active at 55 °C and was stable at temperatures below 45 °C. McLic1 showed strict substrate specificity for barley β-glucan, oat β-glucan and lichenan. Besides, the enzyme exhibited excellent stability in non ionic surfactants, oxidizing agents and solid detergents. The unique properties may make the enzyme a good candidate in several industrial applications.

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