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Purification and Characterization of a Thermostable β-1,3-1,4-Glucanase from *Laetiporus sulphureus* var. *miniatus*

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A β-1,3-1,4-glucanase from the fungus *Laetiporus sulphureus* var. *miniatus* was purified as a single 26 kDa band by ammonium sulfate precipitation, HiTrap Q HP, and UNO Q ion-exchange chromatography, with a specific activity of 29 U/mg. The molecular mass of the native enzyme was 52 kDa as a dimer by gel filtration. β-1,3-1,4-Glucanase showed optimum activity at pH 4.0 and 75°C. The half-lives of the enzyme at 70°C and 75°C were 152 h and 22 h, respectively. The enzyme showed the highest activity for barley β-glucan as β-1,3-1,4-glucan among the tested polysaccharides and *p*-nitrophenyl-β-D-glycosides with a K_m of 0.67 mg/ml, a k_{cat} of 13.5 s^{-1} and a k_{cat}/K_m of 20 mg/ml/s.

Keywords: Characterization, β-1,3-1,4-glucanase, *Laetiporus sulphureus* var. *miniatus*, purification, thermostable enzyme

β-1,3-1,4-D-Glucans are polysaccharide components of the cell wall in the endosperm of cereals such as barley, rye, sorghum, rice, and wheat [14]. β-1,3-1,4-Glucanase has been used in brewing and feedstuff industries because β-1,3-1,4-glucan can be dissolved in water and often has high viscosity in the solution owing to its unique structure [4]. The addition of β-1,3-1,4-glucanase reduces viscosity during beer filtration and improves the digestibility of feedstuffs [1, 10, 21]. However, the thermo-instability of the enzyme reduces its activity in many of these industrial processes.

Many bacterial β-1,3-1,4-glucanases have been identified, purified, and characterized [3, 6, 12, 15, 16], and several fungal β-1,3-1,4-glucanases including *Achlya ambisexualis* [11], *Aspergillus japonicus* [7], *Cochliobolus carbonum* [5], *Melanocarpus* sp. [9], *Orpinomyces joyonii* [19], *Paecilomyces thermophila* [18], *Rhizopus microsporus* var. *microsporus* [2], and *Talaromyces emersonii* [13] have been characterized to date. The enzymes, except for the enzyme produced by *T. emersonii,* showed little or no activity for the β-1,3-glucan laminarin. To achieve more efficient hydrolysis of β-1,3-1,4-glucans, it will be necessary to discover different types of enzymes with different properties.

The basidiomycete *Laetiporus sulphureus* var. *miniatus* has been used in the isolation of new benzofuran glycoside and C10 acetylenic acid [20] and in the production of extracellular polysaccharides [8]. However, there have been no reports on β-1,3-1,4-glucanase from this fungus. In this study, we found that *L. sulphureus* var. *miniatus* produces a thermostable β-1,3-1,4-glucanase. The purification, molecular size, optimum pH and temperature, and substrate specificity of the enzyme were investigated.

Materials and Methods

Microorganism and Culture Conditions

The basidiomycete *L. sulphureus* var. *miniatus* KACC 50117 was cultivated in a 250-ml Erlenmeyer flask containing 50 ml of potato dextrose broth (PDB; Difco) at 25°C with agitation at 150 rpm for 5 days. The broth of the seed culture (15 ml) was transferred to a 1-l flask containing 200 ml of production medium, which consisted of 20 g/l barley β-glucan, 10 g/l corn steep powder, 5 g/l KH_2PO_4, 5 g/l K_2HPO_4, 3 g/l $MgSO_4 \cdot 7H_2O$, and 5 mg/l thiamine, and the culture was performed at 25°C with agitation at 150 rpm for 5 days.

Enzyme Purification

The culture solution was filtered through a 0.45 μm filter. The filtrate was precipitated by ammonium sulfate, collecting the fraction up to 80% saturation. After standing overnight at 4°C, the precipitate formed was collected by centrifugation at 10,000 ×*g* for 20 min at 4°C. The crude protein was applied to a HiTrap Q HP column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM sodium acetate buffer (pH 5.0). The column was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer at 2 ml/min.

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Table 1. Purification of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture broth	56	101	2	100	1
Ammonium sulfate	6.5	49	8	49	4
HiTrap Q HP	1.2	20	17	19	9
UNO Q	0.2	5	29	5	16

The culture broth was purified as a single band enzyme by 80% ammonium sulfate precipitation, HiTrap Q HP and UNO Q chromatography.

Highly active fractions were pooled and then loaded onto a UNO Q column (Bio-Rad, Hercules, CA, U.S.A.). The sodium acetate buffer of 20 mM containing 0.5 M NaCl was used to elute the protein and pooled enzyme fractions. The resulting solution was used as a purified enzyme.

Determination of Molecular Mass

The subunit molecular mass of β-1,3-1,4-glucanase was examined by SDS–PAGE under denaturing conditions, using the proteins of a prestained ladder (MBI Fermentas, Hanover, MD, U.S.A.) as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of the native enzyme was estimated using a Sephacryl S-300 preparative-grade column HR 16/60 gel filtration chromatography (Amersham Biosciences). The enzyme solution was applied to the column and eluted with 50 mM sodium acetate buffer (pH 4.0) containing 150 mM NaCl at a flow rate of 1 ml/min. The column was calibrated with aldolase (158 kDa), albumin (67 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa) as reference proteins (Amersham Biosciences), and the molecular mass of the native enzyme was calculated by comparing with the migration length of reference proteins.

Enzyme Assay

Unless otherwise stated, all reactions by the enzyme were performed in 50 mM sodium acetate buffer (pH 4.0) containing 0.6 U/ml of enzyme (or 0.06 U/mg substrate) and 1.0% (w/v) barley β-glucan as β-1,3-1,4-glucan, other 1.0% (w/v) polysaccharide, or 1 mM *p*-nitrophenyl-β-D-glycoside at 75°C for 10 min. Total released reducing sugar was determined by the 3,5-dinitrosalicylic acid (DNS) reagent method. One unit of activity was defined as the amount of the enzyme required to produce 1 μmol reducing sugar per minute at 75°C and pH 4.0.

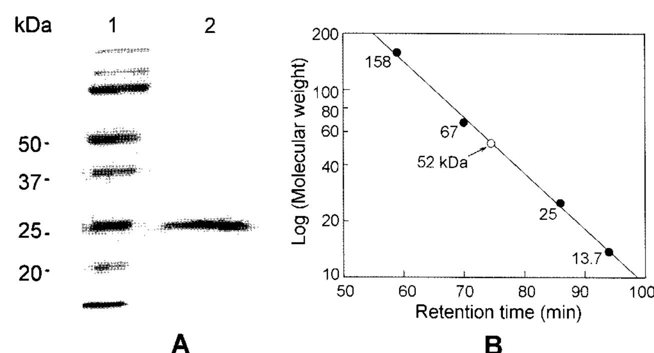

Fig. 1. Determination of molecular mass of purified β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.
A. Subunit molecular mass of the enzyme by SDS–PAGE with Coomassie blue. Lane 1, molecular mass markers; lane 2, purified enzyme. **B.** Molecular mass of the native enzyme by gel-filtration chromatography.

Effects of pH, Temperature, and Metal Ions

To determine the maximum activity of the enzyme, the pH and temperature were varied from 3.0 to 5.5 using 50 mM citrate buffer and from 45 to 90°C, respectively. The relative activity was defined as the relative value to the maximum activity.

To investigate thermostability, the enzymes were incubated at temperatures ranging from 65 to 90°C for varying periods of time. A sample was withdrawn at each time interval and the relative activity was determined after the reaction described above. The experimental data for thermal deactivation of enzyme were fitted to

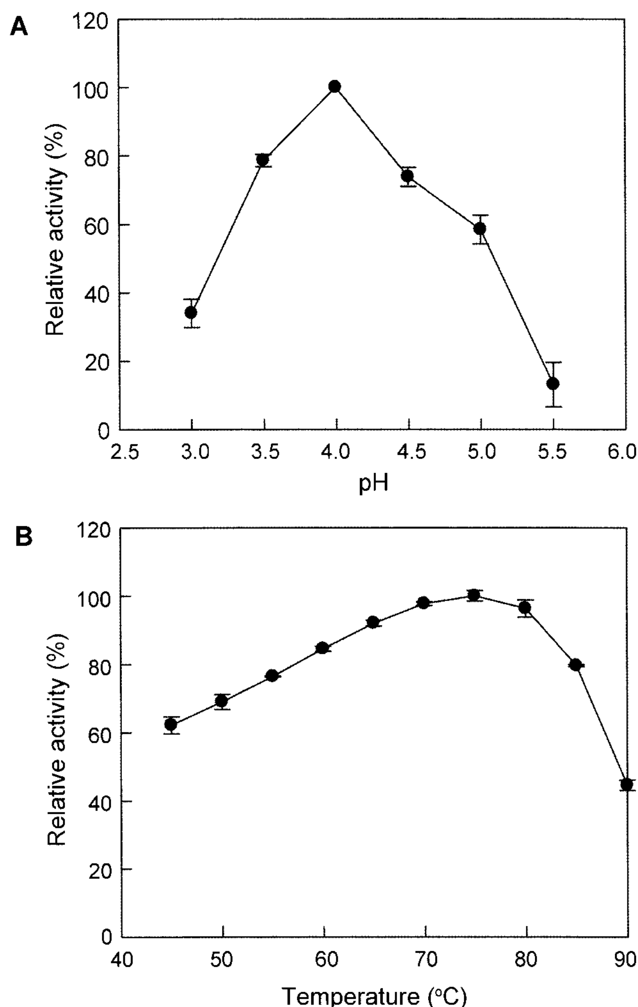

Fig. 2. Effects of pH and temperature on the activity of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.
Effects of pH (**A**) and temperature (**B**). Data represent the means of three experiments and error bars represent standard deviation.

a first-order curve and the half-lives of the enzyme were calculated using SigmaPlot 9.0 software (Systat Software, San Jose, CA, U.S.A.).

The enzyme was treated with 1 mM ethylenediaminetetraacetic acid (EDTA) at room temperature for 1 h. The EDTA-treated enzyme solution was dialyzed against 20 mM sodium acetate buffer (pH 4.0). Various divalent ions at a concentration of 1 mM were added to the dialyzed enzyme, which was then assayed.

Determination of Kinetic Parameters

The reaction was carried out in 50 mM sodium acetate buffer (pH 4.0) containing various concentrations of barley β-glucan (from 0.2 to 0.8 mg/ml) and carboxymethyl cellulose (CMC) (from 0.6 to 8 mg/ml) at 75°C for 5 min. The K_m (mg/ml) and k_{cat} (s^{-1}) were calculated by standard methods.

Results and Discussion

Enzyme Purification and Molecular Mass

The basidiomycete *L. sulphureus* var. *miniatus* was grown on the production medium containing corn steep powder because the nitrogen source was the most effective for the production of β-1,3-1,4-glucan-hydrolyzing enzyme among various nitrogen sources (data not shown). The culture broth obtained from harvested cells as a soluble protein was purified by 80% ammonium sulfate precipitation, HiTrap Q HP, and UNO Q ion-exchange chromatography (Table 1). The enzyme was purified with a final purification of 16-fold, a yield of 5%, and a specific activity of 29 U/mg against β-1,3-1,4-glucan as barley β-glucan.

The purified enzyme showed a single band in SDS-PAGE with a molecular mass of approximately 26 kDa (Fig. 1A). The molecular mass of the native enzyme was estimated to be 52 kDa using Sephacryl S-300 HR gel filtration chromatography based on the masses of reference proteins (Fig. 1B). This result indicates that the native form of the enzyme is a dimer.

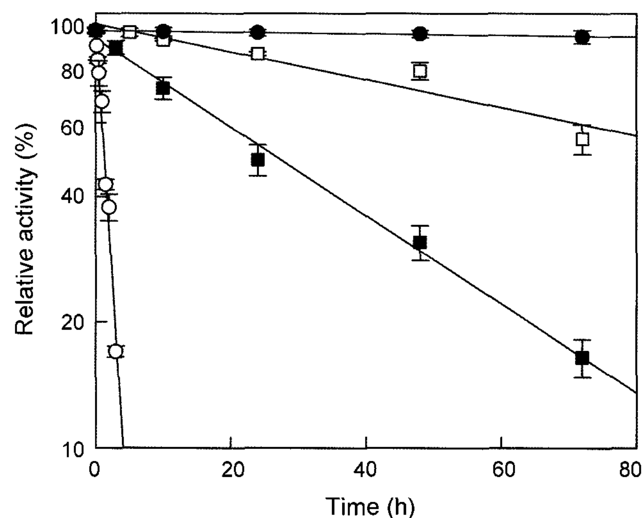

Fig. 3. Thermal inactivation of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus* at temperatures of 65°C (●), 70°C (□), 75°C (■), and 80°C (○).

Effects of pH and Temperature on β-1,3-1,4-Glucanase Activity

The activity of β-1,3-1,4-glucanase to hydrolyze barley β-glucan as β-1,3-1,4-glucan was examined at pH values ranging from 3.0 to 5.5 at 75°C (Fig. 2A). When the reactions were performed without the enzyme under the experimental conditions, reducing sugar was not formed. The maximum activity was observed at pH 4.0. At pH 3.5 and 4.5, the activities were 79% and 73% of the maximum, respectively. The temperature was also varied from 45 to 90°C, and maximum enzyme activity was observed at 75°C (Fig. 2B). At temperatures of 60 and 85°C, the activities were 85% and 80% of the maximum, respectively.

Generally, the activities of fungal β-1,3-1,4-glucanases, such as *Achlya ambisexualis* [11], *Aspergillus japonicus* [7], *C. carbonum* [5], *O. joyonii* [19], *R. microsporus* var. *microsporus* [2], and *T. emersonii* [13] were optimal at the range of 37–50°C. However, the optimum temperature of β-1,3-1,4-glucanase from the thermophilic fungus *P. thermophila* was 70°C [18]. The optimal activity of β-1,3-1,4-glucanases from *L. sulphureus* var. *miniatus* was determined at 75°C. The optimum temperature for the fungal growth was 28°C and the fungus did not grow above 42°C. It was interesting that the mesophilic fungus produced a thermophilic enzyme.

Thermostability of β-1,3-1,4-Glucanase

Thermostability of the enzyme was evaluated by measuring the enzyme activity over time (Fig. 3). Thermal inactivation followed first-order kinetics with half-lives of 1,650, 125, 22, 1.0, 0.08, and 0.02 h at 65, 70, 75, 80, 85, and 90°C, respectively, indicating that the enzyme is relatively unstable above 80°C.

Table 2. Effects of metal ions on the activity of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.

Metal ions	Relative activity (%)
None	100±2.1
EDTA	88±1.6
$MnCl_2$	33±0.1
$MgSO_4$	92±1.9
$CaCl_2$	102±2.2
$BaCl_2$	92±1.9
$CuCl_2$	86±5.3
$FeSO_4$	103±1.8
$ZnSO_4$	92±2.4
$NiSO_4$	119±0.6
$CoSO_4$	109±3.4

Data are means±SD for three separate experiments. The relative activity of 100% is 16.6 U/mg.

The half-life of β-1,3-1,4-glucanase from *P. thermophila* was 2.9 h at 70°C and 0.8 h at 75°C. In terms of thermal inactivation, the β-1,3-1,4-glucanase of *L. sulphureus* var. *miniatus* showed a higher thermal stability than that of *P. thermophila*. The high temperatures employed in malting processes (50–70°C) or feed pelleting processes (65–90°C) using β-1,3-1,4-glucanase can cause a severe inactivation of the enzyme [17]. Thus, the thermostable β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus* is a desirable enzyme in industrial applications.

Effects of Metal Ions on β-1,3-1,4-Glucanase Activity

The enzyme activity was assayed in the presence of various metal ions after treatment with EDTA (Table 2). The addition of Ni^{2+}, Co^{2+}, Fe^{2+}, or Ca^{2+} ions enhanced the activity of *L. sulphureus* var. *miniatus* β-1,3-1,4-glucanase, whereas Mn^{2+} and Cu^{2+} ions inhibited the activity. The addition of Mn^{2+} strongly inhibited the enzyme, resulting in 33% relative activity. Mn^{2+} inhibition was also found for the β-1,3-1,4-glucanases from *Aspergillus japonicus* [7], yielding relative activities of 75%.

Substrate Specificity of β-1,3-1,4-Glucanase

The substrate specificity of the enzyme was examined for polysaccharides and *p*-nitrophenyl-β-D-glycosides (Table 3). The enzyme activities for β-1,3-1,4-glucan, such as barley β-glucan and lichenan, were 5.6- and 4.8-fold higher than for β-1,4-glucan (CMC), respectively. The enzyme activity showed moderate activity for β-1,3-glucan (laminarin) showing relative activity of 86% in comparison with CMC, but low activity towards crystalline β-glucans, such as cellulose, rice straw, and Avicel (microcrystalline cellulose partially hydrolyzed with acid). The β-1,3-1,4-glucanases from *L. sulphureus* var. *miniatus* and *T. emersonii* exhibited activity for the β-1,3-glucan laminarin, giving relative activities of 15% and 25–27% [13], respectively, in comparison with barley β-glucan, whereas the other fungal β-1,3-1,4-glucanases showed little or no activity for the β-1,3-glucan laminarin [2, 5, 7, 9, 11, 18, 19]. These results indicate that two enzymes have different substrate specificities when compared with the other fungal β-1,3-1,4-glucanases, the β-1,3-1,4-glucanases showed little or no activity towards *p*-nitrophenyl-β-D-glycosides, suggesting that the enzyme is not a β-glycosidase.

Table 3. Substrate specificity for β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.

Substrate	Relative activity (%)
Barley β-glucan (1% w/v)	100±1.64
Lichenan (1% w/v)	85±1.14
Laminarin (1% w/v)	15±0.35
CMC (1% w/v)	18±0.34
Cellulose (1% w/v)	7±0.04
Avicel (1% w/v)	4±0.02
Rice straw (1% w/v)	4±0.05
Xylan (1% w/v)	4±0.03
p-Nitrophenyl-β-D-glucopyranoside (1 mM)	<0.1
p-Nitrophenyl-β-D-galactopyranoside (1 mM)	ND
p-Nitrophenyl-β-D-xyloside (1 mM)	ND
p-Nitrophenyl-β-D-cellobioside (1 mM)	ND

ND, not detected. Data are means±SD for three separate experiments. The relative activity of 100% is 16.6 U/mg.

Table 4. Kinetics parameters of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus*.

Substrate	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (mg/ml/s)
Barley β-glucan	0.67±0.01	13.5±0.18	20.1±0.40
CMC	3.7±0.08	5.3±0.09	1.45±0.04

Data are means±SD for three separate experiments.

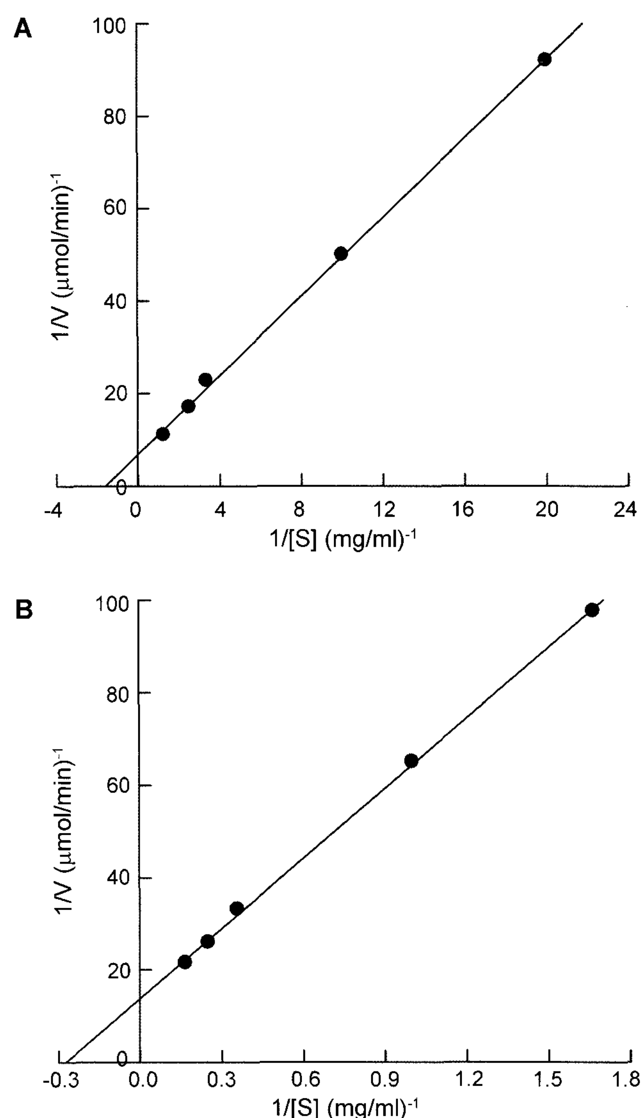

Fig. 4. Lineweaver–Burk plots of β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus* for (**A**) barley β-glucan and (**B**) CMC.

The Michaelis constant (K_m), turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) of the enzyme from *L. sulphureus* var. *miniatus* for barley β-glucan and CMC are shown in Table 4 and Fig. 4. The enzyme showed lower K_m, higher k_{cat}, and higher k_{cat}/K_m values for barley β-glucan than for CMC. The k_{cat}/K_m values of β-1,3-1,4-glucanases V–VII from *T. emersonii* for barley β-glucan and CMC were 7.7–12.6 mg/ml/s and 2.3–8.2 mg/ml/s, respectively [13]. The β-1,3-1,4-glucanase from *L. sulphureus* var. *miniatus* was more active for β-1,3-1,4-glucan and β-1,3-glucan, and less active for β-1,4-glucan, than that from *T. emersonii*. Barley β-glucan and lichenan, which are mixed glucans containing β-1,3- and β-1,4-linkages, were preferred substrates to CMC, which is a β-1,4-linked cellulose with carboxymethyl substitutions as a model substrate for detecting β-1,4-glucanases. The substrate specificity indicates that the enzyme is a β-1,3-1,4-glucanase.

In conclusion, we have purified and characterized an enzyme from *L. sulphureus* var. *miniatus*. This enzyme was determined to be a thermostable β-1,3-1,4-glucanase, in accordance with its thermal property and substrate specificity. This new thermostable fungal β-1,3-1,4-glucanase has potential industrial applications for β-1,3-1,4-glucan hydrolysis.

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