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Purification and some properties of a β-glucanase from a strain, *Trichoderma reesei* GXC*

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Abstract: β-glucanase was purified from a solid-state culture of *Trichoderma reesei* on wheat bran in three steps which comprised ammonium sulfate precipitation, Sephadex G-100 chromatography, and DEAE-Sephadex A-50 chromatography. The molecular mass was determined to be 35.21 kilodaltons by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis. The β-glucanase at low pHs was more stable than that at high pHs, and optimum pH was 5.0. The optimum temperature was 60 ℃, and β-glucanase was relatively stable at below 40 ℃ for 60 min. The K_m of the enzyme on β-glucan was 10.86 mg/ml, and the V_{max} on β-glucan was 14286 μmol of glucose equivalents per mg of the pure enzyme per min. The β-glucanase activity was significantly inhibited by Fe^{3+} ions, and was reduced in the presence of Cu^{2+} ions, Mn^{2+} ions and Mg^{2+} ions at 5 mmol/L and 10 mmol/L, respectively. The β-glucanase activity was stimulated by Co^{2+} ions, Ca^{2+} ions, Zn^{2+} ions, and Fe^{2+} ions at 1 mmol/L and 5 mmol/L, respectively.

Key words: *Trichoderma reesei*, β-glucanase, purification and characterization, stability
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INTRODUCTION

Hemicelluloses, which include β-glucan, mannans, and xylans, are major constituents of plant cell walls. The utilization of starch from plant material requires the degradation of hemicellulose material that coats starch grains, and endosperm cell walls of cereals such as barley are particularly rich in β-glucans and arabinoxylans (Fincher et al., 1986). β-glucans account for up to 70% of the cell wall in the barley endosperm (Buliga et al., 1986). β-glucans consist of glucose units joined by β-1,4 and β-1,3 linkages and include lichenin, or barley β-glucan yielding solutions of high viscosity which can lead to problems such as reduced rate of wort and beer filtration in the brewing industry, and can also lead to haziness, precipitation, and gel formation in stored beer. These polymers also have antinutritional properties, particularly in chicken diets where their "gumminess" and indigestibility severely affect food intake. β-glucanase (1, 3-1, 4-β-D-glucan 4-glucanohydrolase; lichenase) (EC 3.2.1.73) cleaves β-1,4 linkages adjacent to β-1,3 bonds in glucans, yielding chiefly cellobiosyltriose and cellotriosyltetraose (Anderson et al., 1975; Fleming et al., 1977). There is considerable interest in β-glucanases in the brewing industry. For example, the application of β-glucanase can reduce the wort or beer filtration time and prevent haziness in the finished products. In the feed industry β-glucanase can be used as feed additive to decompose the β-glucan, and is a valuable tool as a natural way to improve feed utilization and control pollution through reducing animal wastes (Brenes et al., 1993; Broz et al., 1994; Jensen et al., 1996; Partrige et al., 1995; Viveros et al., 1994). β-glucanases had been found in several *Bacillus* species (Murphy et al., 1984; Hofemeister et al., 1986; Borriss et al., 1990; Lloberas et al., 1991; Gosalbes et al., 1991).

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A β-glucanase from the anaerobic Fungus *Orpinomyces* strain PC-2 had been reported (Chen et al., 1997). *Trichoderma reesei* is one of the most potent producers of cellulase and hemicellulase system. The present work reports the purification and characterization of a β-glucanase from *Trichoderma reesei* strain GXC.

MATERIALS AND METHODS

Material

β-glucan was obtained from Sigma Chemical (St. Louis, MO). Sephadex G-25, Sephadex G-100, DEAE Sephadex A-50 and high molecular weight range kit (14.3 – 220.0 KD) were purchased from Pharmacia (Amersham Pharmacia Biotech in China). All other chemicals used were of reagent grade obtained from standard sources.

Organism and culture conditions

The organism used was *Trichoderma reesei* GXC supplied by Feed Science Institute of Animal Science College of Zhejiang University. It was kept as a spore at −20 ℃.

Solid state fermentation (SSF) technology was used for the production of β-glucanase by *Trichoderma reesei* GXC. The culture medium used was wheat bran with moisture level of 50%, and was sterilized for 30 min at 121 ℃. The organism was cultivated for 2 days in a 500 ml flask containing 50 g medium at 32 ℃. All inoculations were performed with spores at a final concentration of 10^6 spores/ml of culture medium.

Purification of enzymes

All operations were performed at 4 ℃ unless otherwise mentioned. The fermented about 200 g medium was suspended in 2000 ml of 50 mmol/L sodium acetate buffer, pH 5.3. After 2 h at 20 ℃ on a shaker, the supernatant used in the ammonium sulfate precipitation step was obtained by centrifuging (5000 g, 15 min). The crude extract of enzyme was precipitated with ammonium sulfate (30% saturation) followed by centrifuging (5000 g, 15 min); the supernatant was precipitated again with ammonium sulfate (60% saturation) followed by centrifuging (10 000 g, 15 min). The ammonium sulfate precipitate was dissolved in 5.0 ml of 50 mmol/L sodium acetate buffer, pH 5.3, and de-salted on Sephadex G-25 column (2 cm × 50 cm) and eluted with sodium acetate buffer (50 mmol/L, pH 5.3). Fractions with large molecular size proteins were collected. The fractions containing β-glucanase were pooled, concentrated. The enzyme containing sample was applied on Sephadex G-100 column (2 cm × 50 cm) and eluted with the same buffer system. The enzyme containing fractions were pooled, concentrated and reapplied to a DEAE-Sephadex A-50 column (1.2 cm × 20 cm) under a new set of conditions. β-glucanase was eluted with a buffer system consisting of 20 mmol/L NaCl in 50 mmol/L sodium acetate, pH 5.3 and 300 mmol/L NaCl in 50 mmol/L sodium acetate, pH 5.3. After elution with starting buffer (20 mmol/L NaCl in 50 mmol/L sodium acetate, pH 5.3), a linear gradient of NaCl (20 to 300 mmol/L) was applied. The flow rate was 12 ml/h, and fractions (3 ml each) were collected and analyzed for β-glucanase activity and protein concentration. Active fractions were pooled and concentrated.

Polyacrylamide gel electrophoresis

The molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel was done as described by Laemmli (1970). High molecular weight protein (code RPN 756) standards were used for molecular mass determination. The markers used were lysozyme (14.3KD), trypsin inhibitor (21.5KD), carbonic anhydrase (30.0KD), ovalbumin (46.0KD), bovine serum albumin (66.0KD), phosphorylase b (97.4KD) and myosin (220.0KD). Proteins were stained with Coomassie brilliant blue R-250.

pH optimum and stability

The effect of pH on β-glucanase activity was measured over a range of 3.0 to 8.0 by using a Na_2HPO_4-citric acid buffer system under standard enzyme assay conditions.

The effect of pH on β-glucanase stability was determined by using the Na_2HPO_4-citric acid buffer system over a pH range of 3.0 to 7.0. After incubation of the enzyme in various buffers at 30 ℃ for 1 h, the pH was adjusted to 5.3, and β-glucanase activities were determined un-

der standard enzyme assay conditions.

Temperature optimum and stability

The temperature optimum was measured by using the standard assay within the temperature range of 25 to 85 ℃.

Thermal stability was determined by assaying for residual β-glucanase activity after incubation of β-glucanase in sodium actetate buffer (50 mmol/L, pH 5.3) at various temperatures for 60 min. One ml portions were removed at 15 min intervals, the enzyme was cooled and residual activity was determined under standard enzyme assay conditions.

Effects of various compounds on β-glucanase activity

β-glucanase samples were incubated with various compounds at 1.0 mmol/L, 5.0 mmol/L and 10.0 mmol/L, respectively; the β-glucanase activity was determined under standard enzyme assay conditions. The salts used were $CaCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $MnSO_4$, $FeCl_3$, $FeSO_4$ and $CoCl_2$.

Michaelis-Menten kinetics

β-glucanase assays were performed by using a range of 0 to 10 mg/ml β-glucan in sodium acetate (50 mmol/L, pH 5.3). Assays were performed at 50 ℃ by using the same enzyme concentration of the assay mixture. Michaelis-Menten constants were determined by using a Lineweaver-Burk plot.

Determination of β-glucanase activity

β-glucan solution concentration used in the β-glucanase assay was 1% in 100 mmol/L sodium acetate buffer (pH 4.8). One g of β-glucan was suspended in 6 ml of ethanol. About 80 ml of sodium acetate buffer was added and heated to boiling point while stirring until the sodium acetate dissolved, then followed by cooling to room temperature by continued stirring with a magnetic stirrer. Then the volume was made up to 100 ml with sodium acetate buffer. The substrate may be stored for a maximum of one week at 4 ℃. For the determination of β-glucanase, two tubes with 1.8 ml of β-glucan solution were equilibrated at 50 ℃ for 5 min, then 0.2 ml of diluted enzyme solution was added to one of the tubes and mixed well. The reaction mixture was incubated at 50 ℃ for 10 min, and stopped by addition of 3.0 ml of DNS reagent. To the other tube without enzyme was added 3.0 ml DNS reagent too, then 0.2 ml of enzyme solution was added and mixed well. Both tubes was boiled for exactly 5 min and cooled to room temperature. The reducing sugar released against the enzyme blank was determined by the dinitrosalicylic acid method with glucose as the standard (Miller, 1959).

One unit of β-glucanase activity was defined as the amount of the enzyme that catalyzed the formation of 1 nmol of glucose from β-glucan in one second under the conditions described above.

Measurement of protein content

Protein concentrations were estimated by the dye-binding assay method of Bradford (1976), and bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

β-glucanase purification

After two day of cultivation, the fermented medium was suspended in 50 mmol/L sodium acetate buffer (pH 5.3), and centrifuged to remove insoluble material, the supernant was used for enzyme purification. The Sephadex G-100 chromatograph of the ammonium sulfate precipitated enzyme (60% saturation) is shown in Fig. 1 Fractions 44-55 which showed high activity on β-glucan, were collected and concentrated by ammonium sulfate precipitation, desalted by gel filtration with Sephadex G-25 column equilibrated with 50 mmol/L sodium acetate buffer (pH 5.3), and eluted with the same buffer.

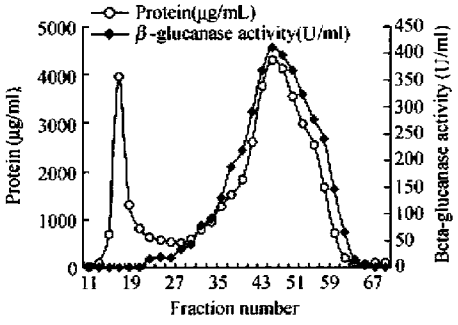

Fig.1 Chromatograph of β-glucanase on a Sephadex G-100 column

The enzyme containing sample was applied on a DEAE-Sephadex A-50 column equilibrated with 50 mmol/L sodium acetate buffer containing 20 mmol/L NaCl, and enzyme protein was eluted with a linear gradient composed of 300 ml 20 mmol/L NaCl in 50 mmol/L sodium acetate buffer (pH 5.3) and 300 ml 300 mmol/L NaCl in the same buffer. The DEAE-Sephadex A-50 column chromatograph is shown in Fig. 2 Several protein peaks were eluted, peak I showed β-glucanase activity. Fractions 24-28, which showed high β-glucanase activity were pooled and concentrated by 60% saturation of ammonium sulfate and desalted as described above, and then reapplied to the DEAE-Sephadex A-50 column with the same buffer described as above. The β-glucanase was eluted as a single peak. The purification steps are summarized in Table 1. The purification scheme resulted in a 14.60-fold purification of β-glucanase relative to the crude enzyme extract and a substantial increase in the specific activity, from 1469 to 21361 U/mg.

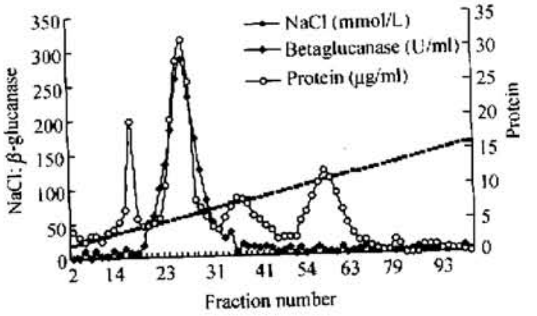

Fig.2 Elution pattern of β-glucanase on a DEAE-Sephadex A-50 column

Table 1 Summary of purification of a β-glucanase from culture medium extract

Steps	Total protein (mg)	Total activity (Units)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	1 228.57	1 804 724	1 469	1	100
Ammonium sulfate	273.38	634 765	2 322	1.58	35.17
Sephadex G-100	28.00	379 160	13 541	9.22	20.01
DEAE-Sephadex A-50	5.59	119 406	21 361	14.60	6.62

Molecular weight of β-glucanase

The molecular weight of β-glucanase was 35.21 kilodaltons as determined by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (Fig. 3). β-glucanases from different sources showed variation in their molecular weight. The molecular mass of the mature enzyme, from *Rhodothermus marinus* expressed in Escherichia coli was estimated to be 29.7 kDa (Spilliaert et al., 1994), from *Bacillus brevis* cloned in Escherichia coli was estimated to be about 29 kDa by sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (Louw et al., 1993), from *Clostridium thermocellum* was about 35 kDa (Schimming et al, 1991), from *Bacteroides succinogenes* cloned in *Escherichia coli* was estimated to be 35,200 by electrophoresis (Erfle et al., 1988), from *Orpinomyces sp.* strain PC-2 constructed in *Escherichia coli* had a molecular mass of 27 kDa on sodium dodecyl sulfate-polyacrylamide gels (Chen et al., 1997).

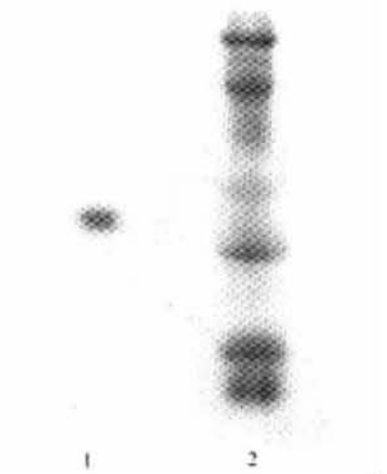

Fig.3 SDS-Polyacrylamide gel electrophoresis of purified β-glucanase. Lane 1, purified β-glucanase; Lane 2, protein standards (from the bottom): lysozyme (14.3KD), trypsin inhibitor (21.5KD), carbonic anhydrase (30.0KD), ovalbumin (46.0KD), bovine serum albumin (66.0KD), phosphorylase b (97.4KD) and myosin (220.0KD).

Effect of pH on β-glucanase activity and stability

The Na_2HPO_4-citric acid buffer system (3.0-8.0) was used to study the effect of pH on β-glucanase activity. The buffer used to study the effect of pH on β-glucanase stability was Na_2HPO_4-citric acid (pH 3.0 - 7.0). Fig. 4 shows the β-glucanase activity curve with respect to pH. The β-glucanase showed high activity in a pH range of 3.0 – 5.0. The optimum pH was 5.0. The application of β-glucanase in the feed industry to digest the hemicellulose and decrease the digesta viscosity demands good enzyme activity and stability under acidic conditions. The β-glucanase activity was 76.0% of the optimum pH at pH 3.0. The stability of the (β-glucanase at pH 3.0 – 7.0 was determined. As seen in Fig. 5, the β-glucanase activity was relatively more stable at low pHs than at high pHs. The optimum was at pH 5 when β-glucanase activity was 94.87% . The optimum activity appearing in acidic condition was found to be the same as that of fungal β-glucanases from *Trichoderma longibrachiatum* (Sharma et al., 1987). It was found to be different from that of other enzyme sources. The relative activity of β-glucanase from *Bacillus subtilis* was in the region of 6.5 to 6.6 (Ezio et al., 1961). The enzyme from *Rhodothermus marinus* had a pH optimum of 7.0 (Spilliaert et al., 1994). The lichenase activity of BgaA from an alkalophilic *Bacillus* strain (N137) is stable from pH 6 to pH 12. A thermoactive beta-1,3-1,4-glucanase from *Clostridium thermocellum* is active pH 5 to pH 12 (Schimming et al., 1991). The pH-optimum for enzymatic activity of *B. amyloliquefaciens* β-glucanase was found to be pH 6 to 7 and for *B. macerans* at pH 6.0 to 7.5, but their hybrid enzyme had maximum activity from pH 5.6 to pH 6.6 (Borriss et al., 1989). A 1,3-1,4-beta-D-glucanase from *Bacteroides succinogenes* cloned in *Escherichia coli* had a broad pH optimum with maximum activity at approx. pH 6.0 (Erfle et al., 1988).

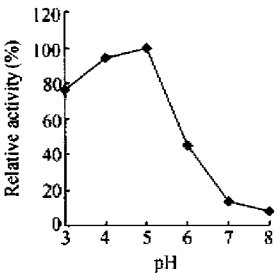

Fig.4 Effect of pH on the activity of β-glucanase

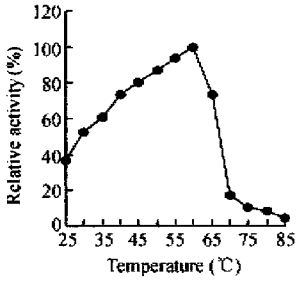

Fig.5 The stability of the β-glucanase at pH 3.0 – 7.0 for 1 h at 30 ℃

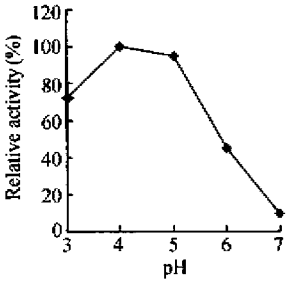

Fig.6 Effect of temperature on the β-glucanase activity

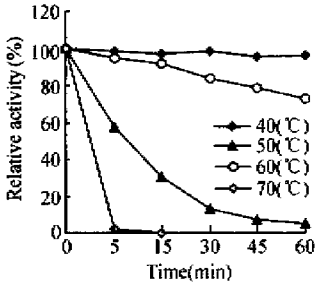

Fig.7 The thermostability of β-glucanase

Effect of temperature on β-glucanase activity and stability

The temperature optimum of purified β-glucanase was measured by using the standard assay in the temperature range of 25 ℃ to 85 ℃. Fig. 6 shows that the β-glucanase activity increased with rise of temperature, maximized at 60 ℃;

then decreased rapidly with the temperature, and exhibited about 17% of the maximal activity at the temperature of 70 ℃; and was about 87% of the maximal activity at 50 ℃ used for standard activity measurements.

For determining the thermostability, the enzyme solution in 50 mmol/L sodium actetate buffer (pH 5.3) at various temperatures for different times, the enzyme was cooled and residual activity was determined under standard enzyme assay conditions. Fig. 7 shows that the β-glucanase was stable during 60 min incubations at temperature of ≤40 ℃; that loss of enzyme activity was rapid above 50 ℃; and that the loss rate of activity increased with rise of temperature.

The optimum temperature may differ significantly depending on different sources. For some fungal β-glucanases, the optimum temperature was around 50 ℃(Sharma et al., 1987). However, the optimum temperatures of some enzymes, such as those produced by bacteria are higher than those of fungal enzymes. For example, the enzyme from *Rhodothermus marinus* expressed in *Escherichia coli* had a temperature optimum of 85 ℃, and was shown to retain full activity after incubation for 16 h at 80 ℃ and had a half life of 3 h at 85 ℃ (Spilliaert et al., 1994). Enzyme activity of an alkalophilic *Bacillus sp.* strain N137 expressed in *Escherichia coli* showed maximal activity at temperature of 60 ℃ to 70 ℃, and retained 65% of its activity after incubation at 70 ℃ for 1 h (Tabernero et al., 1994). For an enzyme from a *Bacillus brevis* cloned in *Escherichia coli*, the optimum temperature was 65 to 70 ℃; when incubated at 75 ℃ for 1 h, 75% residual activity was measured (Louw et al., 1993). A thermoactive enzyme from *Clostridium thermocellum* had a temperature optimum of about 80 ℃ (Schimming et al., 1991). However, enzyme from *Bacteroides succinogenes* cloned in *Escherichia coli* had a lower temperature optimum of 50 ℃ (Erfle et al., 1988).

Michaelis-Menten kinetics

For determining the Michaelis-Menten constants, purified enzyme was incubated with different substrate concentrations (0-10 mg/ml β-glucan) under standard assay conditions. K_m and V_{max} were 10.86 mg/ml and 14286 μmol/mg, respectively, determined from Lineweaver-Burk plots (Fig.8).

Effect of various compounds on β-glucanase activity

β-glucanase standard reaction mixtures were incubated with various compounds at 1 mmol/L, 5 mmol/L and 10 mmol/L, respectively. The enzyme activity was determined under standard enzyme assay conditions.

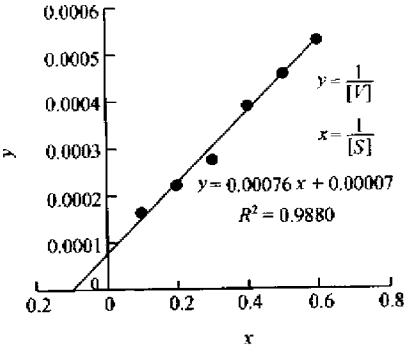

Fig.8 K_m and V_{max} from Lineweaver-Burk plots

Table 2 Effect of compounds on β-glucanase activity

Compound	Relative activity (%)		
	1 mmol/L	5 mmol/L	10 mmol/L
Control	100	100	100
$CaCl_2$	106.36	114.71	63.95
$MgSO_4$	101.49	92.05	86.51
$Cu\ SO_4$	93.18	70.11	54.65
$ZnSO_4$	125.00	108.45	103.84
$MnSO_4$	98.86	90.92	84.88
$FeCl_3$	71.59	47.70	31.40
$FeSO_4$	124.55	114.94	89.53
$CoCl_2$	121.59	137.24	130.81

As seen in Table 2, Cu^{2+}, Mn^{2+} and Fe^{3+} had inhibitory effect on the β-glucanase activity, Fe^{3+} ions were the most inhibitory at 10 mmol/L concentration, inhibiting β-glucanase activity by 68%. Cu^{2+} had the same negative effects on β-glucanase activity at the different concentrations. The β-glucanase retained more enzyme activity in the presence of Fe^{2+} than in the presence of Fe^{3+} at the same concentration. Fe^{2+} and Ca^{2+} had negative effect on enzyme activity at 10 mmol/L, but had positive effect at 1mmol/L and 5 mmol/L. Mg^{2+} exhibited inhibitory effect on

enzyme activity at 5 mmol/L and 10 mmol/L. β-glucanase activity was also stimulated by Zn^{2+} ions at 1 mmol/L and 5 mmol/L, and Co^{2+} ions at concentration of 1 – 10 mmol/L.

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