African Journal of Biotechnology Vol. 10(84), pp. 19607-19614, 26 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2144
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Purification and characterization of β-1,4-glucosidase from *Aspergillus glaucus*

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Accepted 9 November, 2011

A β-1,4-glucosidase (BG) was purified from fermentation liquor of *Aspergillus glaucus* by the following procedures: Ammonium sulfate precipitation, gel filtration on Sephadex G-100, and hydrophobic chromatography on Phenyl Sepharose Fast Flow. The specific activity on salicin was determined as 1747.4 U/mg. The molecular weight of this enzyme was determined as 23.5 and 92.5 kDa with SDS-PAGE and gel filtration on Sephadex G-100, respectively indicating that BG is a tetramer. The enzyme was stable at the pH ranging from 2.2 to 7.5, and its maximum activity was obtained at pH 3.6. The enzymatic activity gradually increased in the range from 40 to 60°C, but a sharp decrease occurred at 65°C. Enzymatic kinetics indicated that Michaelis-Menten contant (K_m) of the hydrolysis for salicin by BG was 2.58 mmol/L (pH 3.6, 60°C). Na^+, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, NO_3^- and SO_4^{2-} had no effects on BG activity. The enzyme activity was activated by Mn^{2+} and Fe^{2+}, while it was strongly inhibited by Cu^{2+}, Pb^{2+}, Cd^{2+}, SDS and EDTA, and slightly inhibited by Zn^{2+}.

Key words: *Aspergillus glaucus*, β-1,4-glucosidase, purification, enzymatic properties.

INTRODUCTION

Cellulose, which constitutes the highest proportion of municipal and plant wastes, represents a major source of renewable energy and raw materials (Gruno et al., 2004; Jatinder et al., 2007). It is a linear polymer of D-glucose units linked by 1, 4-β-D-glucosidic bonds. Cellulolytic enzymes hydrolyze cellulose to glucose, which may be further converted to other chemical products. Cellulolytic enzymes can be divided into three types: endoglucanase (endo-1,4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase (exo-1,4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1, 4-β-D-glucosidase, BG, EC 3.2.1.21) (Hong et al., 2001; Li et al., 2006). Endo- and exoglucanases act on the cellulose chain, catalyzing the random cleavage of internal bonds and the release of cellobiose from reducing and nonreducing ends, respectively whereas BG act on cello-oligosaccharides and cellobiose, releasing glucose monomers (Beguin and Aubert, 1994; Kumar et al., 2008). Thus, it does not only produce glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently (Saha et al., 1994), because both endoglucanase and exoglucanase activities are usually

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Abbreviations: BG, β–Glucosidase; **K_m,** Michaelis-Menten constant; **PAGE,** polyacrylamide gel electrophoresis; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **DNS,** 3,5-dinitrosalicylic; **HAc-NaAc buffer,** sodium acetate buffer; **EDTA,** ethylene diamine tetraacetic acid.

#These authors contributed equally to this work.

inhibited by cellobiose and short cello-oligosaccharides. Product inhibition and thermal inactivation of BG constitute two major barriers to the development of enzymatic hydrolysis of cellulose as a commercial process (Woodward et al., 1982).

BG exist widely in nature. Until now, BGs have been isolated from bacteria, fungi, plants and animals (Joo et al., 2010; Han and Chen, 2008; Pontoh et al., 2002). Many fungal strains secrete higher amounts of cellulases than bacterial ones, with *Trichoderma* as the leading one. Although, the cellulolytic enzymes of *Trichoderma reesei* have been investigated thoroughly (Saloheimo et al., 1997; Arja et al., 2004), the quantity of BG secreted by *T. reesei* is insufficient for effective conversion of cellulose to glucose (Workman and Day, 1982).

In addition, there is an increasing demand for the production of BG in the conversion of cellulose to glucose for the subsequent production of fuel ethanol (Saha et al., 1994). *Trichoderma* species are major agents of decompositions and decay, and thus possess the capability to produce a brand range of enzymes. BG production has been described for many *Trichoderma* species (Claudia et al., 2000).

We have previously reported a purified endoglucanase (EG) from *A. glaucus* grown on rice straw (Tao et al., 2010). But the extracellular BG from this strain has never been reported. Thus, the purpose of this present work is to isolate and purify the enzyme and to study its physical-chemical and kinetic parameters. It will provide some additional data to understand the new strain.

MATERIALS AND METHODS

Reagents

Salicin, bovine insulin, trypsin, albumin egg, bovine serum albumin (BSA) and bovine intestine alkaline phosphatase (BIALP) were products of Sigma (St. Louis, MO, USA). Standard marker proteins for SDS-PAGE were from Amersham. Sephadex G-100 and Phenyl Sepharose Fast Flow were purchased from Pharmacia Biotech (Piscataway, NJ, USA). Acryl amide (Acr) and Bis-acryl amide (Bis) were products of Fluka (Buchs SG, Switzerland). The water used was a double-distilled water and ion free. All other reagents were local products of analytical grade.

Organism and culture conditions

In our previous work, a cellulase producing fungus was isolated from mildew maize cob and was identified by 18s rRNA gene sequence analyses and morphological characteristics as *A. glaucus* (Xu et al., 2006). The fungus was cultured in a modified medium of Mandels and Sternburg (MS medium) (Mandels and Sternburg, 1976), containing per liter: 3 g sugarcane bagasse, 1.4 g $(NH_4)_2SO_4$, 0.3 g urea, 2.0 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.75 g tryptone, 5 mg $FeSO_4{\cdot}7H_2O$, 1.4 mg $ZnSO_4$, 1.6 mg $MnSO_4{\cdot}H_2O$ and 2 ml Tween 80.

The culture medium (200 ml) in 500 ml shake flasks was inoculated with 2% (v: v) overnight grown culture and the cultivation was performed at 30°C with rotatory shaking at 180 rpm. Finally, the culture was centrifuged at 9000 rpm for 20 min and the supernatant was collected as crude enzyme.

Assay of BG activity and protein determination

BG activity was measured by Baruch and swiain assay using salicin (Sigma) as substrate. The total assay mixture (1 ml), consisting of 500 µl of salicin (10 mmol/L) ,100 µl of enzyme and 400 µl of 0.2 mol/L HAc-NaAc buffer (pH 3.6) was incubated at 60°C for 45 min. The amount of reducing sugar produced was measured by the 3, 5-dinitrosalicylic (DNS) reagent method (Miller, 1959). One unit (U) of the enzyme activity was defined as the amount of enzyme required to liberate 1 µg of glucose per min at the pH and temperature of maximum activity (pH 3.6 and 60°C).

Protein was determined according to the Coomassie blue method described by Bradford using bovine serum albumin as a standard (Bradford, 1976).

Purification of BG

Unless otherwise noted, all procedures were performed at 4°C, and 50 mmol/L NaAc buffer (pH 5.0). Protein in the column effluents was monitored by measuring the absorbance at 280 nm. BG was purified by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G-100 and Phenyl-Sepharose™ 6 Fast Flow column.

Solid ammonium sulfate was added to the crude enzyme preparation to achieve 90% saturation. After standing overnight at 4°C, the precipitate formed was collected by centrifugation at 7000 rpm for 30 min at 4°C and dissolved in a small volume of 50 mmol/L HAc-NaAc buffer (pH 5.0), and then dialyzed against the same buffer until no ammonium sulfate was detected by $BaCl_2$. Then, the dissolved enzyme solution (about 20 ml) was sealed in a dialytic bag covered with polyethylene glycol 20 000 (PEG-20 000). PEG-20 000 absorbed water and concentrated the solution within the dialysis bag. The concentrated enzyme solution (about 2 ml) was applied onto a Sephadex G-100 column (1.5 × 65 cm) and equilibrated in a 50 mmol/L HAc-NaAc buffer (pH 5.0) to remove excess salt. Elution was undertaken with the same buffer at a flow rate of 1 ml/min. Fractions showing BG activity were pooled and applied onto a Phenyl-Sepharose™ 6 Fast Flow column (1.6 × 20 cm) equilibrated in 50 mmol/L Tris-HCl buffer (pH 7.8) and eluted using the same buffer at a flow rate of 0.5 ml/min, with a sodium chloride linear gradient from 0.4 to 0 mol/L.

Fractions with higher BG activities were pooled and stored at 4°C for further analysis.

Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Davis (Davis, 1964) in 7% acryl amide rod gels. Sodium dodecyl sulfate (SDS)-PAGE was carried out in slab gels (stacking and separating gels containing 5 and 10% acryl amide, respectively), according to the procedure proposed by Laemmli (1970). Protein bands were revealed with Coomassie blue.

Molecular weight estimation

The whole molecular weight of BG was determined by using the method of gel filtration on Sephadex G-100. Insulin (12.8 kDa), trypsin (25.0 kDa), albumin egg (45.0 kDa), bovine serum albumin

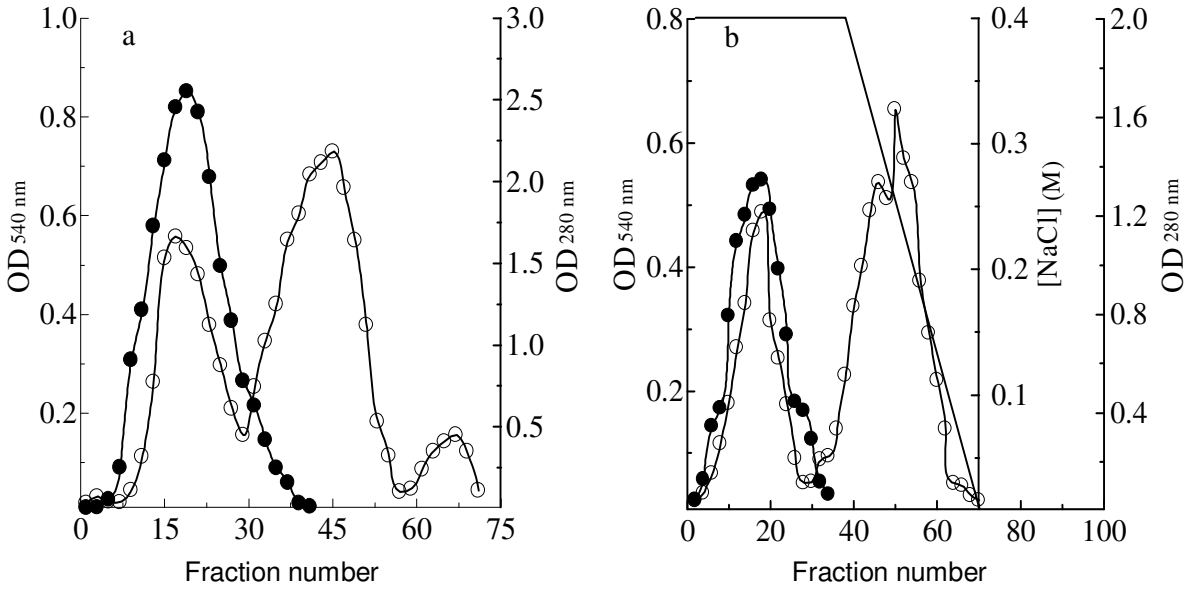

Figure 1. Chromatography of BG from *Aspergillus glaucus*. (a) Sephadex G-100, (b) Phenyl Sepharose Fast Flow. —•—•— U/mg, enzyme activity; —○—○—$OD_{280\ nm}$, protein concentration; —NaCl, concentration of NaCl.

(68.0 kDa) and bovine intestine alkaline phosphatase (115 kDa) were used as standard proteins. The molecular weight of the subunit was determined by SDS-PAGE.

Determination of kinetic parameters

The values of the Michaelis constant (K_m) and the maximum velocity (V_m) were determined for BG by incubating it in 0.2 mol/L HAc-NaAc buffer, pH 3.6 at 60°C with salicin at concentrations ranging from 0 to 10 mmol/L. The kinetic constants (K_m and V_m) were determined by the Lineweaver-Burk plot.

Optimal temperature and thermal stability

The optimum temperature for BG activity was determined in the range from 40 to 80°C in 0.2 mol/L HAc-NaAc buffer, pH 3.6, using salicin (10 mmol/L) as substrate. The thermal stability of BG was monitored by incubating BG at various temperatures (4 to 80°C) for 1 h. Then, 50 µl of treated enzyme was assayed at the optimum pH and temperature.

Optimal pH and pH stability

The optimum pH was determined by measuring the enzyme activities as earlier described, at pH ranging from 2.0 to 10.0 at 60°C. The pH stability of the enzyme was monitored by incubating the enzyme in buffers with pH ranging from 2.0 to 10.0 for 3 h at 4°C. 50 µl of the mixture was taken for activity assay at the optimum pH. The following buffers were used: Gly-HCl buffer (pH 2.0 to 4.0), HAc-NaAc buffer (pH 4.5 to 5.5), Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0 to 8.0), and Gly-NaOH buffer (pH 8.5 to 10.0).

Effects of metal ions and other reagents

The effects of the different components on the activity of the purified BG were tested by incubation with 10 mmol/L salicin in the presence of different chemicals for 45 min at 60°C in 0.2 mol/L HAc-NaAc buffer, pH 3.6. The degree of inhibition of enzyme activity was expressed as a percentage of the enzyme activity in the control sample.

RESULTS

Purification of BG

An extracellular BG from *A. glaucus* was purified using Sephadex G-100 (Figure 1a) and Phenyl-Sepharose™ 6 Fast Flow (Figure 1b) columns. During Sephadex G-100 column chromatography, the major BG peak was eluted and pooled; this peak also contained contaminating exoglucanase activities. Finally, on Phenyl-Sepharose™ 6 Fast Flow column, the BG peak was separated from exoglucanase. The results of purification procedure are summarized in Table 1.

The purified enzyme preparation exhibited a salicin specific activity of 1747.4 U/mg, while the purification fold and yield were 8.8 and 38.3%, respectively. The final preparation was determined to be homogeneous by native PAGE (Figure 2b), showing a single protein band corresponding to 92.5 kDa.

Determination of the molecular weight

The apparent molecular mass of the purified BG estimated by gel filtration on a Sephadex G-100 column was 92.5 kDa, whereas Figure 2a indicates that the single

Table 1. Purification steps and folds of BG produced by *A. glaucus*.

Step	Total protein (mg)	Total activity (U)	SA [a] (U/mg)	Recovery (%)
Filtrate	315	62460	198.3	100.0
$(NH_4)_2SO_4$	114	53460	468.9	85.5
Sephadex G-100	42	45000	1071.4	72.0
Phenyl-Sepharose™ 6 FF	13.7	23940	1747.4	38.3

[a] SA: Specific activity.

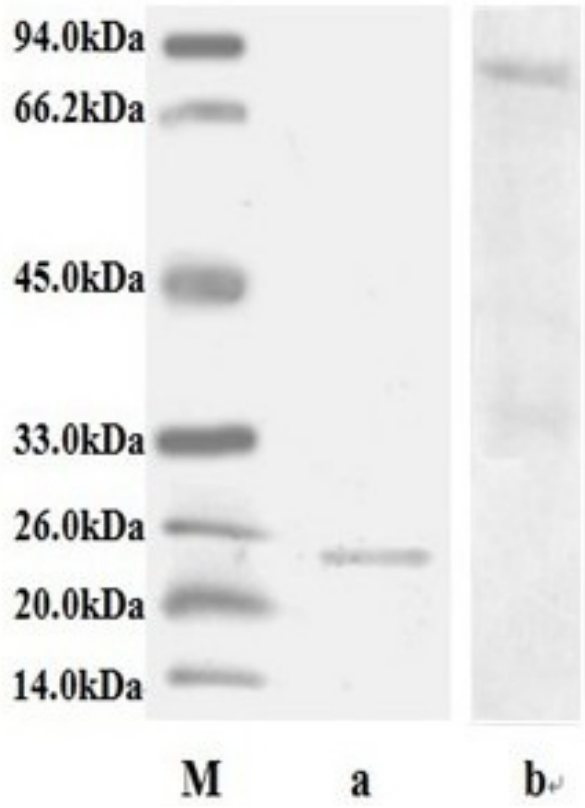

Figure 2. Native PAGE and SDS-PAGE of BG produced by *A. glaucus*. (Lane M: Protein markers; lane a: SDS-PAGE of BG; lane b: Native-PAGE of BG).

protein band revealed in SDS-PAGE analysis corresponded to 23.5 kDa. Hence, it was assumed that the native enzyme is a tetramer.

Kinetic parameters

BG from *A. glaucus* hydrolyzed salicin is shown in the Michealis-Menten equation. Kinetic constants for salicin hydrolysis by the enzyme were determined using Lineweaver-Burk double-reciprocal graph as shown in Figure 3. K_m and V_m of BG were calculated as 2.58 mmol/L and 48 mol/L/min, respectively.

Optimum temperature and thermal stability

The enzyme activities were measured at various temperatures to assess the effect of temperature on the enzyme activity. These results are shown in Figure 4. The enzymatic activity gradually increased in the range from 40 to 60°C, but a sharp decrease occurred at 65°C, with a residual activity of only 60% of the maximal value and 15% at 70°C. So we concluded that the optimum temperature was 60°C at pH 3.6 (curve a in Figure 4). The stability of purified BG was studied at various temperatures from 4 to 80°C and the enzyme was stable below 65°C (curve b in Figure 4). The enzyme retained

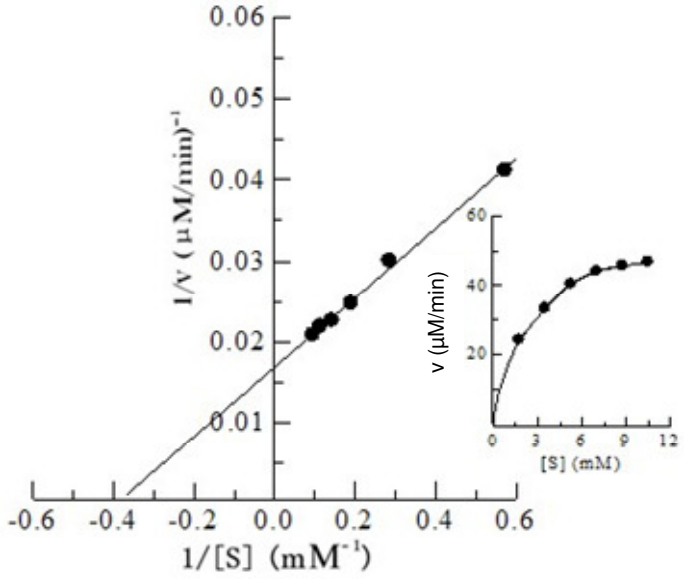

Figure 3. Lineweaver-Burk plot for the determination of K_m and V_m for BG from *A. glaucus* on the hydrolysis of salicin. Conditions were 0.2 mol/L HAc-NaAc buffer (pH 3.6) with different concentrations of salicin at 60°C.

almost 100% activity at 60°C after 1 h.The enzyme became unstable when the temperature was above 65°C. At 70°C, only 20% activity was retained after incubation for 1 h.

Optimum pH and pH stability

The enzymatic activity was determined at 60°C. These results of optimum pH and pH stability are shown in Figure 5. It revealed that the optimum pH of the enzyme was at 3.6 (curve a in Figure 5) and the enzyme was stable in the pH range from 3.0 to 7.0 (curve b in Figure 5). It showed that BG was fairly stable and highly active over a broad acid pH range. About 90 and 70% of activity remained after incubation at pH 2.2 and 8.0, respectively.

Effects of metal ions and other reagents

Several reagents were assayed for their effects on enzyme activity. These results are shown in Table 2. The activity of the purified BG was severely depleted in the presence of 4 mmol/L Cu^{2+}, Pb^{2+}, Cd^{2+}, with residual activities of 59.9, 81.3 and 75.9%, respectively. Zn^{2+} also inhibited the enzyme with a residual activity of 92.7% at 4 mmol/L concentration, while at 8 mmol/L concentration 83.8%. In contrast, Na^+, K^+, Ba^{2+}, Ca^{2+} and Mg^{2+} have no effects on BG activity. The acid radicals SO_4^{2-}, Cl^- and NO_3^- do not influence enzyme activity either. Fe^{2+} and Mn^{2+} activated the enzyme. Ethylene diamine tetraacetic acid (EDTA) abolished nearly half of the activity at both 10 and 20 mmol/L concentration. The activity was almost totally inhibited in the presence of SDS with a 2 mmol/L concentration. There was little effect on the BG when urea concentration was 1 mol/L.

DISCUSSION

The purified BG from *A. glaucus* has a similar mass of molecular weight with that from *Humicola insolens* (Souza et al., 2010) and *Fomitopsis pinicola* (Joo et al., 2009). In contrast, it is smaller than that from *Humicola lanuginosea* (Anand and Vithayathil, 1989), *Cellulomonas biazotea* (Andy and Lau, 2001), *Paecilomyces thermophila* (Yang et al., 2008) and *Thermomyces lanuginosus* (Lin et al., 1999), and could more easily combine with substrate, so we can primarily focus on the gene of this enzyme to build engineering strains.

Depending on the optimal temperature, enzymes can be classified as mesophilic (40 to 60°C), thermophilic (50 to 80°C) and hyperthermophilic (> 80°C) (Chaabouni et al., 2005). The maximum activity for the enzyme was observed at 60°C, which is similar to BG purified from *Thermomyces lanuginosus* (Lin et al., 1999), *Trichoderma reesei* (Chen et al., 1992), *Aspergillus japonicus* (Claudia et al., 2000), *Penicillium pinophilum* (Joo et al., 2010), and *Humicola insolens* (Souza et al., 2010). In contrast, BGs from various thermophilic fungi show optimum tempera-

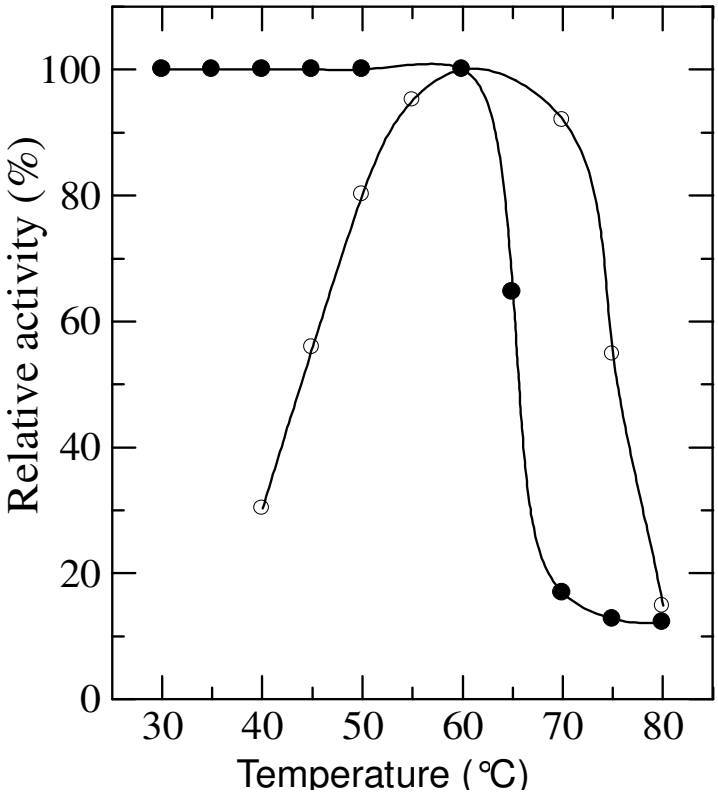

Figure 4. Effects of temperature on the BG activity (curve a), the enzyme activity was measured at various temperatures in 0.2 mol/L HAc-NaAc buffer (pH 3.6). Thermal stability of the BG (curve b), the enzyme was incubated at different temperature for 1 h in 0.2 mol/L HAc-NaAc buffer (pH 3.6), then 50 µl of the mixture was taken for activity assay at the optimum temperature.

ture ranging from 55 to 75°C and are usually more thermostable than those from mesophilic fungi (Jatinder et al., 2007; Yang et al., 2008; Venturi et al., 2002; Mashewari et al., 2000).

The optimum pH of BG from *A. glaucus* was lower than that from *Trichoderma reesei* (pH 4.5) (Chen et al., 1992), *Aspergillus* Species (Claudia et al., 2000) and *Aspergillus niger* (pH 5.0) (Qi et al., 2009). It was still active in the extreme acid pH 2.0, while for others reported, no activity was observed below pH 3.0 (Claudia et al., 2000; Souza et al., 2010 ;Yoon et al., 2008).

The enzymatic properties showed that this enzyme owns high thermal stability and acid tolerance. These advantages indicate striking prospect for application of this enzyme.

The effect of EDTA indicated that BG is a metallo-enzyme, and divalent cations are required for enzyme activation (Riou et al., 1998), whereas the effect of urea on BG proved that BG has resistance to denaturant. Strong inhibition by Cu^{2+}, Pb^{2+}, Cd^{2+} on enzymatic activity were reported for several BG; nevertheless, sensitivity to Zn^{2+} appears to be a similar characteristic of the enzyme from *A. glaucus* (Karnchanatat et al., 2007; Bhiri et al., 2008; Zanoelo et al., 2004 ; Peralta et al., 1997).

Although, the stability of BG is good enough, its activity cannot be of requirement for application. In order to improve the stability and activity of BG, the gene encoding the enzyme should be cloned; over expression and modification of this kind of BG would facilitate to better understand the structure of enzyme and could result in the production of large amounts of efficient BG biocatalyst, which are undergoing in our further studies.

ACKNOWLEDGEMENTS

This present investigation was supported by the grant of the National Key Basic Research Program (NKBRP) (No:2010CB732201), the grant 2009J01196 of the Natural Science Foundation of Fujian Province and the grant from the National Foundation for fostering talents of basic science (J1030626).

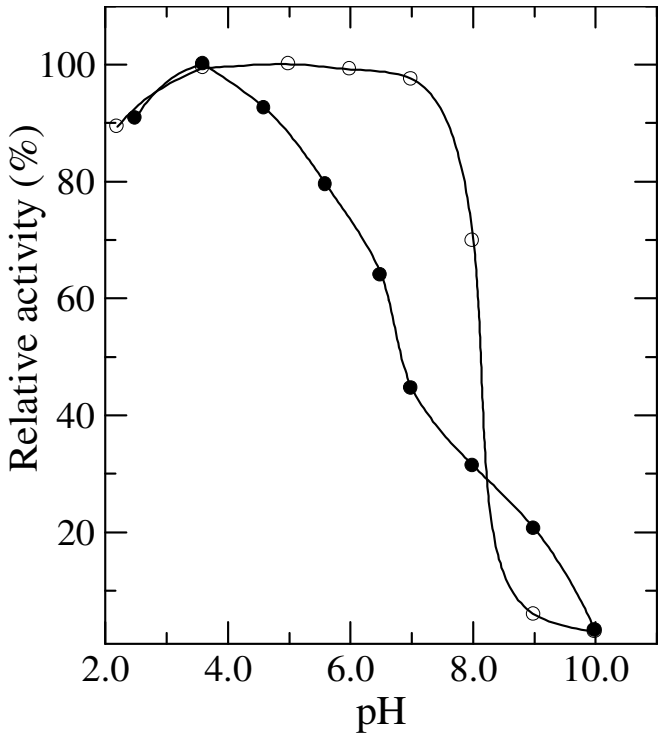

Figure 5. Effects of pH on BG activity (curve a), the enzyme activity was assayed at 60℃. The pH stability of the BG (curve b), the enzyme was incubated at different pH buffers for 3 h at 4℃, then 50 µl of the mixture was taken for activity assay at the optimum pH. The following buffers were used: Gly-HCl buffer (pH 2.0 to 4.0), HAc-NaAc buffer (pH 4.5 to 5.5), Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0 to 8.0), and Gly-NaOH buffer (pH 8.5 to 10.0).

Table 2. Effects of metal ions and other reagents on BG activity.

Compound	Concentration (mmol/L)	RA [a] (%)	Compound	Concentration (mmol/L)	RA [a] (%)
Control	-	100.0	$CuCl_2$	4	59.9
NaCl	4	97.2	$CdCl_2$	4	75.9
$NaNO_3$	4	98.5	$ZnCl_2$	4	92.7
Na_2SO_4	4	97.9	$ZnCl_2$	8	83.8
KCl	4	97.2	SDS	1	55.8
$CaCl_2$	4	97.0	SDS	2	5.2
$MnCl_2$	4	120.9	EDTA	10	59.8
$BaCl_2$	4	100.3	EDTA	20	50.7
$MgCl_2$	4	97.5	Urea	1000	95.2
$FeCl_2$	4	111.5	Urea	2000	86.7
$Pb(NO_3)_2$	4	81.3			

[a] RA: Relative activity. The final concentration of each compound was as reported. Enzyme activities were determined in 0.2 mol/L HAc-NaAc buffer (pH 3.6) at 60℃ for 45 min.

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