Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm

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**Abstract**

An extracellular β-glucosidase was purified from culture filtrates of the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm grown on 1.0% (w/v) carboxymethyl-cellulose using ammonium sulfate precipitation, ion-exchange, hydrophobic interaction and gel filtration chromatography. The enzyme is monomeric with a molecular weight of 64.2 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and has a pI of 8.55. The enzyme catalyzes the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate, with a *K*ₘ of 1.52 mM, and *V*ₘₐₓ of 3.21 U·min⁻¹·mg⁻¹ protein. Glucose competitively inhibited β-glucosidase with a *K*ᵢ value of 0.79 mM. Optimal activity with PNPG as the substrate was at pH 5.0 and 50 °C. The enzyme was stable at pH 5.0 at temperatures up to 50 °C. The purified β-glucosidase was active against PNPG, cellobiose, sophorose, laminaribiose and gentiobiose, but did not hydrolyze lactose, sucrose, Avicel or *o*-nitrophenyl-β-D-galactopyranoside. The activity of β-glucosidase was stimulated by Ca²⁺, Co²⁺, Mg²⁺, Mn²⁺, glycerol, dimethyl sulfoxide (DMSO), dithiothreitol and EDTA, and strongly inhibited by Hg²⁺. The internal amino acid sequences of *D. eschscholzii* β-glucosidase have similarity to the sequences of the family 3 β-glucosyl hydrolase.

**Introduction**

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-glycosidic linkages, such as those in alkyl- or aryl-β-glucosides as well as diglucosides and oligosaccharides. They represent an important group of enzymes because of their potential use in various biotechnological processes, including biomass degradation (Coughlan, 1985), production of fuel ethanol from cellulosic agricultural residues (Bothast & Saha, 1997), release of aromatic compounds in the flavour industry (Gueguen et al., 1996) and synthesis of useful β-glucosides (Makropoulou et al., 1998). A complete set of cellulolytic enzymes is minimally composed of the following components: endoglucanases (endo-β-1,4-glucanase, EC 3.2.1.4), cellobiohydrolases (exo-β-1,4-glucanase, EC 3.2.1.91) and β-glucosidases (Ryu & Mandels, 1980). The role of the β-glucosidase in the saccharification of cellulose is to degrade cellobiose, an inhibitor of the depolymerizing enzyme, and cellulooligosaccharides to glucose. However, β-glucosidase is frequently a rate-limiting factor during enzymatic hydrolysis of cellulose and is very sensitive to glucose inhibition, which limits its activity (Howell & Stuck, 1975).

The *Xylariaceae* are wood-decaying fungi found in terrestrial habitats, are particularly diverse in tropical regions and are able to degrade lignocellulose. Many of the *Xylariaceae* are saprotrophs on decaying plant material such as logs and stumps, dead twigs and branches of trees, dead leaves and stems of herbaceous plants (Whalley, 1996). It is also clear that many species can degrade lignin (Sutherland & Crawford, 1981) and that others exhibit impressive production of cellulolytic enzymes (Wei et al., 1992). *Daldinia* (*Xylariaceae*) is a genus of wood-inhabiting pyrenomycetes with perithecia embedded in large stromata that are internally concentrically zoned and produce enzymes that digest the...
cell wall components for nutrition and energy (Johannesson et al., 2000). This paper reports on the purification and detailed biochemical characteristics of an extracellular β-glucosidase from the wood-decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm. It is noted that the activities of D. eschscholzii have not as yet been reported.

Materials and methods

Organism and culture conditions

The specimen was collected from a dead mango tree in the Royal Forest Department Park, Bangkok, during June 2003. The fruiting structure was identified as Daldinia eschscholzii based on morphological characteristics and then confirmed using molecular techniques. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB284189. The stock culture of D. eschscholzii was maintained on potato dextrose agar medium. Unless otherwise stated, actively growing fungal mycelium from a 7-day-old culture was transferred to a 250-mL Erlenmeyer flask containing 100 mL of Mandels medium (Mandels & Weber, 1969) composed of urea 0.3 g L\(^{-1}\), \((NH_4)_2SO_4\) 1.4 g L\(^{-1}\), KH\(_2\)PO\(_4\) 2.0 g L\(^{-1}\), CaCl\(_2\) \(\cdot\) 2H\(_2\)O 0.4 g L\(^{-1}\), MgSO\(_4\) \(\cdot\) 4H\(_2\)O 0.3 g L\(^{-1}\), peptone 1.0 g L\(^{-1}\), FeSO\(_4\) \(\cdot\) 7H\(_2\)O 5.0 mg L\(^{-1}\), MnSO\(_4\) \(\cdot\) 4H\(_2\)O 1.6 mg L\(^{-1}\), ZnSO\(_4\) \(\cdot\) 7H\(_2\)O 1.4 mg L\(^{-1}\), CoCl\(_2\) \(\cdot\) 6H\(_2\)O 2.0 mg L\(^{-1}\) and Tween 80.20 mL L\(^{-1}\) pH 5.5 to which 1% (w/v) of various carbon sources such as Avicel® PH-101 (Fluka), Carboxymethyl-cellulose (Fluka), Filter paper (Whatman No.1), and glucose (Fluka) were added. The medium was sterilized by autoclaving at 121 °C for 20 min. Inoculated flasks were incubated on a rotary shaker (New Brunswick Scientific) at 150 r.p.m. at 25 °C for 14 days under natural light conditions. The culture fluid was filtered through filter paper (Whatman No.1) and the supernatant fluid was used as the crude enzyme preparation.

Assay of β-glucosidase

β-Glucosidase activity was determined by measuring the hydrolysis of \(p\)-nitrophenyl β-\(D\)-glucopyranoside (PNPG) as described previously (Cai et al., 1998). The reaction mixture (1 mL) contained 5 mM PNPG (Sigma) in 0.1 M sodium acetate buffer (pH 5.0) and an appropriately diluted enzyme solution. After incubation at 50 °C for 30 min, the reaction was stopped by adding 1.0 mL ice-cold 0.25 M Na\(_2\)CO\(_3\) and the colour formed was measured at 410 nm (Tecan Sunrise). One unit of β-glucosidase activity was defined as the amount of enzyme liberating 1 \(\mu\)mole of \(p\)-nitrophenol per minute under the assay conditions. Specific activity is defined as the number of units per milligram of protein.

Purification of β-glucosidase

\((NH_4)_2SO_4\) precipitation

To 5 L of culture supernatant, \((NH_4)_2SO_4\) was added to give 80% saturation. After standing overnight, the precipitate formed was collected by centrifugation at 10 000 g for 20 min (Beckman Coulter), dissolved in 20 mM sodium acetate buffer, pH 5.0. The dissolved sample was dialyzed against the same buffer and concentrated by lyophilization (Labconco).

Cation exchange chromatography

The sample solution was applied on a column (1.6 × 10 cm) of an SP Sepharose Fast Flow (Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0. Elution was undertaken with the same buffer at a flow rate of 1.0 mL min\(^{-1}\). A linear gradient of 0–1.0 M NaCl in the same buffer was then applied. Fractions of 10.0 mL each were collected and assayed for β-glucosidase activity. The fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

Hydrophobic interaction chromatography

To the active fraction from the SP Sepharose Fast Flow, \((NH_4)_2SO_4\) was added to a concentration of 30%. The mixture was applied to a column (1.6 × 10 cm) of Phenyl Sepharose Fast Flow (Amersham Biosciences) equilibrated with 30% \((NH_4)_2SO_4\) in 20 mM sodium acetate buffer, pH 5.0. The column was then eluted with a gradient of 30%–0% \((NH_4)_2SO_4\) in 20 mM sodium acetate buffer, pH 5.0, at a flow rate of 1.0 mL min\(^{-1}\). Fractions of 5.0 mL were collected and assayed for β-glucosidase activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

Gel filtration chromatography

The active fraction from Phenyl Sepharose Fast Flow was applied to a column (1.6 × 60 cm) of Superdex 200 HR (Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 100 mM NaCl at a flow rate of 1.0 mL min\(^{-1}\). Fractions of 5.0 mL were collected and assayed for β-glucosidase activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further analysis.
Protein determination

Protein concentrations in the enzyme preparations were determined by the method of Bradford (1976) with reference to a standard calibration curve for bovine serum albumin (BSA). During the column chromatographic separations, the elution profiles of proteins were determined by measuring absorbance at 280 nm.

Molecular weight and isoelectric point determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli (1970). Samples to be analyzed were treated with sample buffer and boiled for 5 min before application to the gel. Electrophoresis was run from the cathode to anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Amersham Biosciences). High and low molecular weight standards (Sigma) were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie Blue R-250 (Laemmli, 1970).

Isoelectric-focusing PAGE

IEF-PAGE was performed on the Phast-System of Pharmacia LKB using a precast gel, PhastGel IEF 3–9. Isoelectric points were determined using standard pH markers (BioRad).

Effect of temperature on β-glucosidase activity and stability

The optimum temperature for enzyme activity was determined by monitoring each activity at pH 5.0 at various temperatures from 30 to 80°C. Stability was measured by incubating the enzyme in 0.1 M acetate buffer pH 5.0 for 30 min at temperatures from 30 to 80°C. Following incubation, the enzyme solution was cooled, and the remaining activity was determined under standard enzyme assay conditions (Cai et al., 1998).

Effect of pH on β-glucosidase activity and stability

The optimum pH of activity was determined by monitoring each activity at 50°C at various pH values ranging between 3.0 and 9.0. The following buffers were used: 0.1 M sodium acetate buffer (pH 3.0–6.0), 0.1 M phosphate buffer (pH 6.0–7.0) and Tris-HCl buffer (pH 7.0–9.0). The β-glucosidase stability was examined at the pH values 3.0–9.0. Enzyme samples were preincubated in the above-cited buffers at 30°C for 1 h before adding the substrate. After adjustment of the pH, the residual activity was determined under standard enzyme assay conditions (Cai et al., 1998).

Effect of metals and reagents

The effects of various metal ions and reagents at 1 mM on β-glucosidase activity were determined by preincubating the enzyme with the individual reagents in 0.1 M sodium acetate buffer pH 5.0 at 30°C for 30 min. Activities were then measured at 50°C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

Substrate specificity

Several α- and β-glucosides (20 mM), saccharides (1%, w/v) and arylglycosides (5 mM) were tested as substrates for the purified enzyme. The p-nitrophenol released was determined under standard enzyme assay conditions (Cai et al., 1998). The total amount of reducing sugars (expressed as equivalent glucose) in 1.0 mL supernatant was determined by the modified dinitrosalicicylic acid (DNS) method (Miller, 1959).

Determination of kinetic parameters

The values of the Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) were determined for β-glucosidase by incubating in 0.1 M sodium acetate buffer pH 5.0 at 50°C with PNPG at concentrations ranging from 0.5 to 25 mM. Values for $K_m$ and $V_{max}$ were determined from Lineweaver–Burk plots.

Determination of inhibition constants

Inhibition of β-glucosidase by glucose was determined in the presence of PNPG as the substrate. Inhibition constants ($K_i$) were determined from corresponding Lineweaver–Burk plots using standard linear regression techniques.

Internal amino acid sequence of β-glucosidase by LC-MS/MS

The internal amino acid sequence of β-glucosidase was performed by in-gel digestion of the protein and sequencing of the different peptides by mass spectrometry. The ion spectra were analyzed and the sequence determined. The analysis was performed at the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand.
Sequence comparison of internal peptides of β-glucosidase with those of the β-glucosidase family was performed using individual peptides. Peptide matching from sample mass spectra was based on an accuracy of ± 1 Da. Peptide amino acids were matched to the SWISSPROT data.

Results and discussion

Culture conditions and production of β-glucosidase activity

Carboxy-methylcellulose (CMC) was the most effective inducer of β-glucosidase activity of the carbon sources tested. Microcrystalline cellulose (Avicel® PH-101) and filter paper were also fairly good inducers, but with glucose as a carbon source, enzyme production was repressed. The highest level of total β-glucosidase activity (8.102 U mL⁻¹) was produced in CMC after 10 days growth.

Purification of β-glucosidase

β-Glucosidase was successfully purified through ammonium sulfate precipitation, SP Sepharose, Phenyl Sepharose and Superdex-200 column chromatography (Table 1). β-Glucosidase was purified 50.23-fold with 6.28% retention of total extracellular activity and 0.13% retention of total protein. The specific activity of the purified enzyme was 77.86 U mg⁻¹ of protein. The specific activities of purified β-glucosidases from various microorganisms examined by other researchers varied from 5 to 979 U mg⁻¹ of protein (Freer, 1985; Bronnenmeier & Staudenbauer, 1988; Li & Calza, 1991; Kwon et al., 1992; Watanabe et al., 1992).

Molecular weight and isoelectric point determination

SDS-PAGE analysis of the purified β-glucosidase showed the presence of a single band when stained with Coomassie Blue

Table 1. Purification table of β-glucosidase from Daldinia eschscholzii

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>695.47</td>
<td>1078.96</td>
<td>1.55</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>41.63</td>
<td>343.42</td>
<td>8.25</td>
<td>31.83</td>
<td>5.32</td>
</tr>
<tr>
<td>SP Sepharose (ion exchange)</td>
<td>3.75</td>
<td>175.61</td>
<td>46.83</td>
<td>16.28</td>
<td>30.21</td>
</tr>
<tr>
<td>Phenyl Sepharose (hydrophobic interaction)</td>
<td>1.52</td>
<td>98.53</td>
<td>64.72</td>
<td>9.13</td>
<td>41.75</td>
</tr>
<tr>
<td>Superdex-200 (gel filtration)</td>
<td>0.87</td>
<td>67.74</td>
<td>77.86</td>
<td>6.28</td>
<td>50.23</td>
</tr>
</tbody>
</table>

Fig. 1. (a) SDS-PAGE of purified β-glucosidase from Daldinia eschscholzii: lane 1, molecular weight marker of protein standard, i.e. myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa); lane 2 purified β-glucosidase (10 μg of protein). (b) IEF-PAGE of purified β-glucosidase from D. eschscholzii: lane 1, pH marker i.e. amyloglucosidase from Aspergillus niger (3.6), trypsin inhibitor (4.6), β-lactoglobulin A (5.1), carboxic anhydrase II (5.9), carboxic anhydrase I (6.6), myoglobin (6.8, 7.2), lentil lectin (8.2, 8.6, 8.8), trypsinogen from bovine pancreas (9.3); lane 2, purified β-glucosidase (5 μg of protein).
R-250 and its apparent molecular mass was about 64.2 kDa (Fig. 1a), indicating that the enzyme is a monomer. The molecular mass of \(\beta\)-glucosidases from aerobic fungi range from 40 to 480 kDa (Dekker, 1981; Wood & McCrae, 1982). Analytical IEF data demonstrated that \(\beta\)-glucosidase from \textit{D. eschscholzii} is an alkaline protein; it was isoelectric at pH 8.55 (Fig. 1b). \(\beta\)-Glucosidases from aerobic fungi generally have acidic pIs (Coughlan, 1985). However, \(\beta\)-glucosidases from fungi with basic pIs have also been reported, e.g. \textit{Trichoderma reesei} (Schmid & Wandrey, 1987; Hofer et al., 1989).

**Effect of temperature and pH on \(\beta\)-glucosidase activity and stability**

\(\beta\)-Glucosidase from \textit{D. eschscholzii} displayed maximal activity at 50°C (Fig. 2a). Similar temperature optima of \(\beta\)-glucosidases ranging from 50 to 65°C have been reported from several fungi, such as \textit{Aspergillus niger} (Yan & Lin, 1997), \textit{Fusarium oxysporum} (Christakopoulos et al., 1994) and \textit{Neurospora crassa} (Yazdi et al., 2003). Thermostability of the enzyme at different temperatures was monitored and the enzyme was found to be fairly stable at temperatures up to 60°C for 30 min. It was completely inactivated on incubation at 70°C for 30 min (Fig. 2a). The pH optimum for \(\beta\)-glucosidase activity was 5.0 (Fig. 2b), which is similar to that reported for the \(\beta\)-glucosidase from \textit{Aspergillus} species (Yan & Lin, 1997; Riou et al., 1998). The enzyme was fairly stable over the pH range of 5.0–8.0, retaining over 85% activity. The enzyme, however, was shown to be sensitive to pH below 4.0 since it lost its activity at pH 3.0.

Conversely, it was found to be very stable under neutral and alkaline pH since it retained up to 75% of its activity at pH 9.0 (Fig. 2b). Most fungal \(\beta\)-glucosidases exhibit pH optima ranging from 5.0 to 6.5 (Bhatia et al., 2002).

**Effect of metals and reagents**

The enzyme activity was strongly inhibited by the sulfhydryl oxidant, which has been generally reported to be a strong inhibitor for \(\beta\)-glucosidases, but it was observed that some metal ions activate the enzyme (Table 2). This result suggested that the thiol group was essential for \(\beta\)-glucosidase activity (Inglin et al., 1980). This was further confirmed by the observation that Hg\(^{2+}\) ions completely inactivated the enzyme. The chelating agent EDTA did not affect \(\beta\)-glucosidase activity indicating that \(\beta\)-glucosidase is not a metalloprotein. Furthermore, dithiothreitol is not an inhibitor suggesting that disulfide bonds are not essential for enzyme activity. Activation by Ca\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), glycerol and dimethyl sulfoxide (DMSO) may be explained by stabilization of the enzyme structure.

**Substrate specificity**

The purified \(\beta\)-glucosidase from \textit{D. eschscholzii} is a broad-specificity type since it can hydrolyze a range of (\(\beta\)-1, 2), (\(\beta\)-1, 3), (\(\beta\)-1, 4) and (\(\beta\)-1, 6) diglycosides, as well as saccharides and aryl-\(\beta\)-glycosides. The purified enzyme had none or very little (\(\leq 8.5\%\)) activity on lactose, maltose, sucrose, Avicel and carboxymethyl cellulose. It had very little (\(\leq 7.0\%\)) activity on \(p\)-nitrophenyl-\(\beta\)-D-xylopyranoside and
o-nitrophenyl-β-D-galactopyranoside. o-Nitrophenyl-β-D-glucopyranoside was hydrolyzed at 15.5% of the level of hydrolysis of PNPG (Table 3). β-Glucosidases with very broad specificity have been isolated from many fungi (Pitson et al., 1993; Gueguen et al., 1995).

Determination of kinetic parameters and inhibition constants

Reaction kinetics of the purified β-glucosidase were determined from Lineweaver–Burk plots with PNPG as substrate under defined assay conditions. The enzyme had $K_m$ values of 1.52 mM, and $V_{max}$ values of 3.21 U min mg$^{-1}$ of protein (Fig. 3a). In the case of β-glucosidase from D. eschscholzii, the $K_m$ values for PNPG are similar to those of other fungal β-glucosidases such as from Aspergillus fumigatus (Kitpreechavanch et al., 1986), Aspergillus wentii (Srivastava et al., 1984) and Sclerotium rolfsii (Sadana et al., 1988) with $K_m$ values of 1.4, 1.6 and 1.38 mM, respectively. Glucose was found to be a competitive inhibitor of the enzyme as shown by a Lineweaver–Burk plot in the presence of various concentrations of glucose. The $K_i$ was found to be 0.79 mM for glucose when PNPG was used as the substrate (Fig. 3b). Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases although there are exceptions like β-glucosidases produced by several Aspergillus species (Yan & Lin, 1997; Riou et al., 1998; Decker et al., 2001).

Table 2. Effect of some cations and other reagents on purified β-glucosidase activity from Daldinia eschscholzii

<table>
<thead>
<tr>
<th>Reagent*</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control1</td>
<td>100.0</td>
</tr>
<tr>
<td>CaCl2</td>
<td>107.3</td>
</tr>
<tr>
<td>CoCl2</td>
<td>101.6</td>
</tr>
<tr>
<td>FeCl2</td>
<td>78.7</td>
</tr>
<tr>
<td>HgCl2</td>
<td>6.8</td>
</tr>
<tr>
<td>CuSO4</td>
<td>85.2</td>
</tr>
<tr>
<td>MgSO4</td>
<td>122.3</td>
</tr>
<tr>
<td>MnCl2</td>
<td>118.5</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>92.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>104.6</td>
</tr>
<tr>
<td>DMSO</td>
<td>102.8</td>
</tr>
<tr>
<td>DTT</td>
<td>107.5</td>
</tr>
<tr>
<td>EDTA</td>
<td>108.3</td>
</tr>
</tbody>
</table>

*The assays were carried out in the presence of 1 mM concentrations of each of the metal ions and reagents.

1The relative activity was determined by measuring β-glucosidase at 50 °C in 0.1 M sodium acetate buffer pH 5.0 after preincubation at 30 °C for 30 min with individual cations or reagents. Results are shown as the average p-nitrophenol released from a representative assay performed in triplicate.

2The activity assayed in the absence of cations or reagents was taken as 100%.

Most microbial β-glucosidases have glucose inhibition constants ($K_i$) ranging from 0.35 mM to no more than 100 mM (Riou et al., 1998). The glucose inhibition constants for β-glucosidases are the smallest reported for fungal β-glucosidases (Christakopoulos et al., 1994; Gueguen et al., 1995).

Internal amino acid sequence of β-glucosidase by LC-MS/MS

The internal sequence analysis of the purified β-glucosidase was obtained by digestion with trypsin and sequence analysis with LC-MS/MS and was found to be TDGSNGDVASDVYHR. Comparisons were then made to all protein sequences in the swissprot database using the search protocol BLAST. A high degree of internal amino acid sequence identity between D. eschscholzii β-glucosidase and other β-glucosidases of family 3 suggested that this enzyme could be a member of glycoside hydrolase family 3 (Fig. 4). In addition, the active site of this enzyme contains several conserved residues, including a nucleophile (Asp) and an acid/base catalyst (Glu). Sequence alignment of the region containing the proposed catalytic nucleophile of over 15 β-glucosidases of family 3 shows the presence of Asp as a fully conserved catalytic nucleophile (Dan et al., 2000). The data presented here showed that the β-glucosidase from Daldinia eschscholzii...
D. eschscholzii follows a retention mechanism and most probably belongs to family 3 of the glycoside hydrolase, as well as possessing an Asp or a Glu residue as a catalytic nucleophile.

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