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Purification and biochemical characterization of an atypical β-glucosidase from *Stachybotrys microspora*

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ABSTRACT

Stachybotrys microspora is a filamentous fungus secreting various β-glucosidases. The current work undertakes purification and biochemical characterization of the most particular one, named bglG, which is the only one to be highly produced on glucose and fairly on cellulose-based medium. Although produced on glucose, bglG activity continues to be highly inhibited by this sugar. After two chromatographic steps, bglG was purified to homogeneity and shown to be a monomeric protein with the molecular mass of 225 kDa. The highest bglG activity was obtained at pH 5 and a temperature range of 50–60 °C. This enzyme was shown to act through a retaining-enzyme mechanism. The N-terminal sequence analysis did not reveal any homology with all available sequences in the database. BglG is somehow atypical for multiple reasons: (1) BglG is insensitive to the conventional Coomassie staining protocol and $CuCl_2$ method was applied to reveal the protein; (2) the bglG activity is strongly enhanced by ferrous ion (Fe^{2+}) to 161% at 5 and 10 mM of Fe^{2+}. Flame spectrometry analysis showed that iron was stoechiometrically and strongly bound to bglG; (3) besides cellobiose, BglG is active on sucrose (114%); a rarely described property among β-glucosidases and (4) bglG is significantly stimulated by xylose. BglG could be considered as very original, since all known β-glucosidases, did not share these properties.

1. Introduction

Cellulose is the major component of plant cell wall and the most abundant renewable biological resource in the biosphere [1]. The biological depolymerisation of cellulose requires a multi enzymatic system composed of three enzymes functioning in synergy [2,3]: endoglucanases (EC3.2.1.9), cellobiohydrolases (EC3.2.1.4) and β-glucosidases (EC3.2.1.21) [4,5]. Indeed, endoglucanases attack the amorphous regions of cellulose fibres, liberating long oligosaccharides, which are readily taken by the cellobiohydrolases, processive enzymes which sequentially split cellobiose units from their extremities [3]. Finally, the β-glucosidases terminate the cellulolysis by hydrolysing cellobiose and other cellooligosaccharides into glucose monomers.

β-Glucosidase enzymes are important for cellulolytic fungi as by hydrolysing cellobiose, which is a strong cellobiohydrolase inhibitor, they minimize such end-product inhibition of cellulases [6]; they also convert cellobiose into sophorose, which is a very potent inducer of cellulolytic genes [7]. β-Glucosidases are also important for insects and animals as they cleave various glycosilated biomolecules into deglycosilated forms more or less actives [8,9]. Other studies proved that *Bacillus thuringiensis* δ-endotoxin exerts its bio-insecticide effect through β-glucosidase activity [10]. Industrially, β-glucosidases provide glucose stream for their use as carbon and energy sources such as in bio-conversion to ethanol [11–13] and other industrially important molecules by fermentative microorganisms [14]. Commercially, available cellulase preparations are often supplemented with β-glucosidase to boost the overall cellulolytic activity on biomass [15].

Among cellulases, only β-glucosidases possess the capacity not only to hydrolyse cellobiose and other cellooligosaccharides but also to synthesize oligosaccharides [8,16–18]. Thus, they contribute efficiently to the synthesis of valuables molecules in medical, pharmaceutical and industrial fields [19].

The catalytic mechanism mediated by β-glucosidase requires the presence of two acidic amino-acid residues in the catalytic site. The reaction occurs in one or two displacement steps depending on the space between the two acidic residues, 9.5 or 5.5 Å respectively, leading to an inverting or retaining mechanism according to the anomeric carbon configuration of the liberated glycone molecule [20]. β-Glucosidases are classified into three GH families: GH1, GH3 and GH9. GH1 and GH3 are families with a retaining mechanism while GH9 presented an inverting mechanism and mostly contains endoglucanases. Retaining enzymes often display transglycosylation abilities [21].

Glucose is the most known catabolic repressor of several genes, which are therefore controlled negatively by glucose at the transcriptional level. Being the end-product of the reaction

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catalyzed by the cellulolytic complex, glucose is also a very potent inhibitor of the cellulolytic enzymes and particularly the β-glucosidases. Nonetheless, exceptionally, some β-glucosidase genes are expressed/induced in presence of glucose even though they continue to be inhibited by the same substrate [8,22].

Stachybotrys microspora secretes a large number of β-glucosidases. The production of the last ones was directed differentially by the use of various carbon sources [23], two of these β-glucosidases were already purified and characterized [8,22]. In the current work, we undertake the purification and biochemical characterization of the most particular one, named (bglG). This β-glucosidase is not only produced in presence of glucose as unique carbon source, but also characterized by some peculiar properties such as its activation by xylose and ferrous ion or its capacity to efficiently hydrolyze sucrose, in addition to its insensitivity to ordinary protein staining methods. BglG thermo-activity, thermo-stability and refolding recovery were improved by wheat dehydrin, a somehow chaperone-like molecule, as we have already proven in our previous work [24].

2. Materials and methods

2.1. Biological strain

The biological stain used in this work is a filamentous fungus A19 that was isolated in our laboratory and belongs to *S. microspora* [22].

2.2. Production and purification of bglG

A19 strain was grown on potato dextrose agar medium at 30 °C for 4 days. The spores were harvested in 0.1% Tween 80 solution and used to inoculate Mandels medium [25] that was modified as follows, per litre: 2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 1 g yeast extract, 0.69 g urea, 0.3 g $CaCl_2{\cdot}2H_2O$, 0.3 g $MgSO_4{\cdot}7H_2O$, 1 mL Tween 80 and 1 mL trace element solution composed of 1.6 g/L $MnSO_4$, 2 g/L $ZnSO_4$, 0.5 g/L $CuSO_4$, 0.5 g/L $CoSO_4$. Glucose at the concentration of 1% was used as a unique carbon source, during the pre-culture and the culture of the fungus. 10 mL of pre-culture inoculated 100 mL of modified Mandels medium in 500 mL erlenmmeyer.

S. microspora was grown at 30 °C for 5 days and the supernatant was used for the purification and characterization of the bglG. The culture medium was centrifuged for 15 min at 4000 × *g*. The supernatant was concentrated using twice volumes of cold acetone, incubated for 2 h at −20 °C and centrifuged for 30 min at 10,000 × *g* at 4 °C. The pellet was resuspended in buffer A (20 mM Tris–HCl buffer pH 8), dialysed against the same buffer and applied to an anionic exchange column (Q sepharose big beads, 1.5 cm × 20 cm) equilibrated with the same buffer. Proteins were eluted with a linear gradient of NaCl from 0 to 1 M in buffer A (Buffer B). The active fractions were pooled, concentrated with cold acetone as described previously, resuspended in 50 mM sodium acetate buffer pH 5.6 and applied onto a Sephacryl S-200 gel filtration column (1 cm × 90 cm) pre-equilibrated in the same acetate buffer. The active fractions were pooled and analyzed by gel filtration-HPLC system (Schodex 300 mm × 8 mm) and SDS-PAGE analysis in order to judge the protein purity and to determine its molecular mass.

2.3. Enzyme assays

The β-glucosidase activity was monitored using para-nitrophenyl-β-D-glucopuranoside (pNPG) as substrate. 0.2 mL of 1 mM pNPG (in 0.1 M sodium acetate buffer pH 5) was incubated with bglG at the appropriate dilution at 50 °C for 15 min. The reaction was stopped by adding 0.6 mL of 0.4 M glycine–NaOH buffer pH 10.8; the amount of the liberated *p*-nitrophenol (pNP) was determined by measuring the optical density at 400 nm. The molecular extinction coefficient of the pNP is 18,000 L/mol cm. One unit of enzymatic activity was determined as the amount of enzyme required to release 1 μmol of pNP per min under the assay conditions.

Various substrates were used to measure the hydrolytic capacity and specificity of bglG. The substrates used were chromogenic substrates like pNPG, oNPG (ortho-nitro-phenyl-β-D-glucopuranoside), pNPX (para-nitro-phenyl-β-D-xyloside), pNPA (para-nitro-phenyl-β-D-arabinofuranoside) and others such as salicine, amygdaline, arbutine, esculine, cellobiose, lactose, sucrose, avicel cellulose, CM-cellulose, starch, xylan, pectin and casein. For the chromogenic substrates, we used the same assay as with pNPG. For the remaining substrates, the activity was assessed by determining the liberated reducing sugars using the method of 3,5-dinitrosalicilic acid (DNS) [26]. Alternatively, the liberated glucose was also determined via the glucose oxidase/peroxidase kit (GOD kit was purchased from Biomaghreb company) as follows: 10 μL of the sample was added to 1 mL of GOD-kit, incubated at 37 °C for 10 min and the optical density was determined at 505 nm against standard curve prepared with glucose solution.

2.4. Protein assay, electrophoresis and staining

The protein content of the samples was determined using the Biorad-Bradford assay with optical density reading at 595 nm and BSA used as standard [27]. After electrophoresis, proteins were stained using conventional Coomassie blue staining solution.

Alternatively, $CuCl_2$ staining method was applied. Such method is 2–3 times more sensitive than Coomassie blue staining, much more easier and faster and the gels can be stored at 4 °C for several months without fading [28–30]. Practically, the gel was immersed in $CuCl_2$ solution at the concentration of 0.2 M for 5 min, washed with water and observed in dark phase. Using this method, the gel can be subsequently used in zymogram analysis, just after washing the gel with water, which eliminates the copper ion. This staining method depends partly on the tyrosin and tryptophan content [31].

The protein band was excised from the gel and transferred onto PVDF membrane as described in [31,32]. The purified enzyme was then subjected to N-terminal sequence analysis using an automated protein sequencer (Procise 492 cLc, Applied Biosystem).

2.5. Zymogram analysis

Proteins were mixed with the same loading buffer as in SDS-PAGE but they were not heated before loading on SDS-gel [33]. After electrophoresis, the gel was incubated during 2 h in 20 mM Tris–HCl pH 8 to get rid of SDS, allowing the renaturation of proteins. After 15 min of equilibration in 0.1 M sodium acetate buffer pH 5, the gel was superposed against 1% agar gel containing 100 μL of 4-methylumbelliferyl-β-D-glucoside (MUG) at 25 μg/mL. Following a suitable period of incubation, the system was observed under UV light, with excitation at 366 nm and emission at 445 nm. The zone of activity was indicated by the fluorescence emitted by methylumbelliferol (MU) released during enzyme action. The zymogram analysis can also be conducted using pNPG as substrate. Indeed, after the renaturation step, the gel was immersed in sodium acetate buffer 0.1 M pH 5 containing 10 mM pNPG and incubated for up to 15 min at 50 °C. The gel was briefly washed in distilled water and then immersed in 0.4 M glycine–NaOH buffer pH 10.8. The yellow color characteristic of pNP develops thereafter at the site of enzyme activity.

2.6. Determination of the molecular mass of proteins

The purified enzyme was applied onto a Sephacryl S-200 (93 cm × 1.5 cm) gel filtration chromatography and eluted with