PH.D. THESIS

ANALYSIS OF BETA-GLUCOSIDASES FROM ZYGOMYCETES FUNGI: PURIFICATION AND CHARACTERIZATION OF THE ENZYME, MOLECULAR AND FUNCTIONAL ANALYSIS OF THE CODING GENES

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INTRODUCTION

β-Glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21) are ubiquitous and biologically important enzymes catalyzing the hydrolysis of alkyl- and aryl-β-glycosides as well as di- and oligosaccharides. They have an active role in many biological processes, such as degradation of structural and storage polysaccharides, host-pathogen interactions, cellular signaling and oncogenesis. Fungal β-glucosidases are parts of the cellulose degrading enzyme system, wherein they split the short chain oligosaccharides and cellobiose into glucose.

The hydrolyzing activity of the β-glucosidase is utilized in various applications, such as fuel ethanol production from cellulosic agro-industrial residues, or liberation of aroma compounds from plant-derived products. Under certain conditions, β-glucosidases also have a synthetic activity, wherein they are able to transfer glycosyl groups to saccharides and alcohols resulting in the formation of oligosaccharides, alkyl-glycosides, and different glycoconjugates, which can be used as therapeutic agents, diagnostic tools and growth promoting agents for probiotic bacteria. Enzymatic synthesis of these compounds by transglycosylation or reverse hydrolysis can be achieved in one step instead of the several protection-deprotection steps required in the chemical synthesis. Because of the increasing interest for the compounds synthesized by glucosidases in the pharmaceutical and food industry, characterization of the transglucosylation activity of fungal β-glucosidases is an intensively studied area.

Representatives of the Zygomycetes in the order Mucorales, are widely distributed in soil and plant debris, on dung and other moist organic matter contacting with soil. These fungi have practical significance from several medical and agricultural aspects; moreover, numerous species of the order are also used in different biotechnological and industrial applications

due to their effective extracellular enzyme production (e. g. aspartic protease and lipase).

Filamentous fungi are known to be good producers of β-glucosidases and a number of fungal enzymes have been isolated and analyzed, but zygomycetes are poorly characterized from this aspect. Only a few β-glucosidases have been purified and characterized in this fungal group so far. Additionally, synthetic activity of β-glucosidase from zygomycetes has not been identified until to date, and little information is available from the zygomycetes genes encoding β-glucosidase enzymes as well. Our project has been tended to investigate on the β-glucosidase enzymes produced by isolates, including several thermophilic strains, in the order Mucorales.

AIMS

The main objective of our study was the identification, detection and characterization of β-glucosidase activities in zygomycetes in order to find new producer strains, which are potentially useful in further basic studies and biotechnological applications. Moreover, we planned the purification and biochemical characterization of selected β-glucosidases with high extracellular enzyme activity, as well as the isolation, cloning and molecular analysis of the encoding genes.

For this purpose, the following specific objectives have been formulated:

1. Screening for the extracellular β-glucosidase activity in submerged culture and solid-state fermentation of fungal strains belonging to the genera *Mucor, Rhizomucor, Rhizopus* and *Gilbertella*, identification of new producer strains as far as possible.
2. Analysis of different biotechnologically important factors influencing the enzyme activity in the crude extracts of the good β-glucosidase producer strains.

3. Purification of the β-glucosidase produced by isolates with high extracellular enzyme activity.
4. Biochemical characterization of the hydrolytic activity of the purified β-glucosidases, optimization of the hydrolysis conditions.
5. Analysis of the oligosaccharide synthesis by the purified β-glucosidases, optimization of the reaction conditions on cellobiose with respect to the initial substrate concentration, pH and temperature. Analysis of the synthetic activity using other donor and acceptor compounds.
6. Analysis of the β-glucosidase catalyzed liberation of plant derived phenolic antioxidants.
7. Cloning, molecular and functional characterization of the β-glucosidase genes and their adjacent elements from isolates with high extracellular enzyme activity.

METHODS

Screening methods for enzyme activity
Analytical methods:

- Spectrofotometry (UV, VIS)
- Fluorometry
- High Performance Liquid Chromatography (HPLC)
- Liquid Chromatography - Mass Spectrometry (LC-MS)
- Size-exclusion chromatography
- Ion exchange chromatography

Electrophoretic techniques:

- Agarose gel electrophoresis
- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
- Isoelectric focusing

DNA based techniques:

- Purification of genomic and plasmid DNA
- Polymerase Chain Reaction (PCR, Inverse PCR)
- Cloning
- DNA sequencing
- Preparation of plasmid constructions

- Bacterial transformation
- Southern blot

Nucleotide and amino acid sequence assays:

- Nucleotide sequence analysis (BLAST, FASTA)
- Nucleotide and amino acid sequence alignment
- Enzyme protein analysis (ProtParam, SignalP, Motif Scan-MyHits)
- Protein structure determination (STRAP)
- Phylogenetic assay

Genetic transformation methods:

- Protoplast preparation
- Polyethylene glycol (PEG)-mediated protoplast transformation
- Transformants selection

RESULTS

Screening of zygomycetes for extracellular β-glucosidase activity (Takó et al., 2010a)

Extracellular β-glucosidase activity of 95 strains representing the genera *Gilbertella*, *Mucor*, *Rhizomucor* and *Rhizopus* was screened in minimal media containing 1% cellobiose as sole carbon source. The enzyme activities of the tested strains were found to be highly variable. Based on the liquid culture fermentation, six *Mucor*, four *Rhizomucor*, three *Rhizopus* and three *Gilbertella* isolates were chosen for solid-state fermentation studies, in which wheat bran was used as substrate. Solid-state fermentation generally resulted in significantly higher enzyme activities than the liquid cultures. *Gilbertella persicaria* (58.6 - 70.9 U ml^{-1} and 351.6 - 425.4 U g^{-1} wheat bran) and *Mucor corticolus* (94.9 U ml^{-1} and 569.4 U g^{-1} wheat bran) isolates gave the highest volumetric activity and yield on wheat bran. Among the tested thermophilic *Rhizomucor miehei* isolates, the NRRL 5282 and ETH M4918 strains proved to be outstanding in their β-glucosidase producing ability, where 38.3 U ml^{-1} and 42 U ml^{-1} volumetric activity was detected, respectively. Crude extracts after the solid-state fermentation of the *Rhizopus* isolates showed approximately the same volumetric activity (15.1 - 18.4 U ml^{-1}). The effect of cultivation temperature on enzyme

production had also been analyzed, wherein significant differences could be observed among the tested strains.

Analysis of β-glucosidases in crude extracts (Takó et al., 2010a)

In reference to the preliminary tests, effect of temperature, ethanol, glucose and acidic pH on the enzyme activity were tested in the crude extracts of the good β-glucosidase producer *Rhizopus*, *Rhizomucor*, *Mucor* and *Gilbertella* strains. Addition of ethanol to 5 - 10% (v/v) increased the enzyme activity of the *R. miehei*, *Rhizopus microsporus* var. *oligosporus* and *Mucor fragilis* strains. Glucose at 10 mg ml^{-1} concentration was less inhibitory to the enzymes of *Mucor guillermondii*, *M. fragilis* and *M. corticolus* than to those of the other investigated strains. β-Glucosidases of the *Rhizopus* and *Rhizomucor* isolates showed excellent acid tolerance. Alcohol and acid tolerance is an important feature of enzymes used for aroma liberation in wine making. *R. miehei* isolate NRRL 5282 gave the highest residual activity in the heat tolerance test at 75 °C; furthermore, in the presence of a protective cellobiose substrate, the *Rhizomucor* enzymes proved to be thermotolerant. It is supposed that cellobiose, the substrate of the enzyme, was bound to the active site and protected the enzyme from heat inactivation by retaining the correct conformation.

Purification of the extracellular β-glucosidases

On the basis of the preliminary studies, purification and detailed characterization of β-glucosidases produced by *R. miehei* NRRL 5282 (Takó et al., 2010b; Krisch et al., 2011), *M. corticolus* SZMC 12031, *Rhizopus niveus* CBS 403.51 and *G. persicaria* ATCC 201107 isolates have been performed. For production and isolation of the extracellular β-glucosidases in high amount, strains were grown on wheat bran medium, and the enzymes were purified from the crude extract to electrophoretically

homogeneous state by ammonium sulphate precipitation and gel filtration followed by anion exchange and size-exclusion chromatographies. After the fourth step, the purified *R. miehei*, *M. corticolus*, *Rh. niveus* and *G. persicaria* enzymes had a specific activity of 62.2, 118.5, 70.6 and 121.1 U mg^{-1} proteins, respectively. The molecular weight of all purified enzymes was estimated by SDS-PAGE and was found to be about 75 - 80 kDa.

Biochemical characterization of the purified β-glucosidases

Biochemical characterization of the purified β-glucosidases was performed with respect to some parameters influencing the hydrolytic activity of the enzymes. The temperature optimum of the enzymes proved to be about 50 °C, except for the *R. miehei* β-glucosidase which was about 65 °C. The optimum pH for the action of the enzymes was between 5.0 and 5.5; however, the *Rhizomucor* enzyme retained 20% of its initial activity at pH 3.0, which was not observed in the case of the enzymes of the other isolates. The enzymes had a relatively narrow pH stability range (pH 4.0 - 6.0 for *R. miehei*, and pH 4.5 - 7.0 for the other isolates), but it was within the range of most fungal β-glucosidases.

The enzymes efficiently hydrolyzed oligosaccharides having β-(1→4) glycosidic bonds, but weak enzyme activity could also be observed on sophorose which have β-(1→2) glycosidic bonds. The β-glucosidase purified from the *R. miehei* strain presumably belongs to the broad-specificity type, since it can hydrolyze the *p*-nitrophenyl-β-D-galactopyranoside substrate, and it showed moderate α-glucosidase and N-acetylglucosaminidase activities as well. Kinetic parameters of the purified β-glucosidases were also determined from Lineweawer - Burk plots by using *p*-nitrophenyl-β-D-glucopyranoside as substrate.

Glucose substantially inhibited the hydrolytic activity of the purified enzymes. The β-glucosidase from *M. corticolus* proved to be more tolerant

to glucose preserving up to 31% of its initial activity in the presence of 20 mg ml^{-1} glucose. The effect of other mono- and disaccharides on *p*-nitrophenyl-β-D-glucopyranoside hydrolysis was also investigated. In these tests, sucrose significantly increased the activity of the *R. miehei* and *M. corticolus* enzymes. As sucrose is not hydrolysed by these enzymes, it is suggested that they have remarkable transglycosylation activity. Considerable inhibition was observed with 5% (w/v) lactose at the *Rh. niveus*, *G. persicaria* and *R. miehei* β-glucosidases; additionally, activity of these enzymes was slightly increased by 5% (w/v) fructose followed by a reduction at higher concentrations.

Various metal ions and potential inhibitors modified the activity of the purified enzymes. The results may indicate that thiol groups are required for the appropriate enzyme activity, and important function of tryptophan and aspartic acid in the catalysis could be expected as well. Analysis of the effect of different alcohols showed that the hydrolytic activity of the *R. miehei* β-glucosidase was increased with 30% by 10% (v/v) ethanol, which suggests an extensive synthetic activity of the enzyme. It is supposed that ethanol is an acceptor for glucose, decreasing the amount and hereby the inhibitory effect of the free glucose.

Enzymatic synthesis assays

In these assays, synthetic activity of the purified *R. miehei* and *M. corticolus* β-glucosidases was studied in detail. Transglucosylation capacity of the enzymes was tested using cellobiose as substrate. Analysis of the reaction mixtures revealed that cellotriose was the main product of the synthetic reaction on cellobiose that acts as glucose donor and acceptor. After a long incubation period, cellotetraose was also formed in these experiments. Some parameters influencing the transglucosylation of cellobiose were investigated in order to optimize the synthetic reaction (e.g.

initial substrate concentration, pH, temperature). In case of both enzymes, the yield of oligosaccharides increased linearly with the increase of the initial cellobiose concentration, and the maximum oligosaccharide formation was observed at high initial substrate concentration (350 - 400 mg ml^{-1}). The maximum cellotriose concentration was detected between pH 4.0 and pH 6.0, and 70 °C and 50 °C appeared to be the optimal temperatures for the synthetic activity of the *R. miehei* and *M. corticolus* enzymes, respectively.

Besides its synthetic activity on cellobiose, the *R. miehei* β-glucosidase is also able to synthesize oligosaccharides by using lactose as substrate (Krisch et al., 2011). It is supposed that the oligosaccharide produced is a trisaccharide. The transgalactosylation capacity of the *R. miehei* β-glucosidase was also identified; accordingly, the formed oligosaccharide may be a galacto-oligosaccharide as well, similar to that had been detected by the transgalactosidase activity of various β-galactosidases and β-galactosidase-like β-glucosidases. Additional product formation was observed on cellobiose or *p*NPG in the presence of ethanol, suggesting that the *R. miehei* β-glucosidase is able to synthesize alkyl-glucosides too. However, since we could not identify the synthetic compounds formed in the presence of lactose and ethanol accurately, calibration of the appropriate standards, and purification, mass spectrometry and NMR spectroscopy of the products are in progress.

Liberation of phenolic antioxidants by the β-glucosidase (Krisch et al., 2011)

The hydrolytic activity of the β-glucosidase can be used to liberate phenolic aglycons from their glycosidic bonds, which increase the amount, and allow the bioavailability of these phenolic antioxidants. To investigate the production of free phenolic antioxidants by *R. miehei* β-glucosidase,

sour cherry fruit, which has high antioxidant capacity and contains large quantities of phenolics was used. In these analyses, the crude extract of lyophilised sour cherry pomace was treated with the purified enzyme. The amount of the different phenolic antioxidants changed in different degrees; however, increasing concentration could be observed in the concentration of 4-hydroxybenzoic acid, vanillic acid and syringic acid during the incubation period.

The β-glucosidase-coding gene from *Rhizomucor miehei* NRRL 5282 (Vágvölgyi et al., 2007; Takó et al., 2010b)

Analysis of the β-glucosidase-coding genes and gene expression studies were also started. In the frame of this objective, the β-glucosidase gene (*bgl*) of the *R. miehei* NRRL 5282 isolate has been investigated. Isolation and cloning of the entire *R. miehei bgl* gene together with its flanking region was performed by using the IPCR method, and the coding sequence of the BGL protein was predicted by similarities to other known fungal β-glucosidase genes found in sequence databases. Comparison of the *R. miehei* BGL with database sequences showed it highly similar to fungal β-glucosidases classified as the subfamily 4 of the family 3 glycoside hydrolases. The putative N- and C-terminal catalytic domains of the predicted protein could be identified as well as the potential catalytic nucleophile and H^+ donor residues. Mass spectrometry analysis of the purified *R. miehei* β-glucosidase identified the isolated protein as the predicted BGL and confirmed that the *bgl* gene encodes the purified enzyme. Additionally, a number of typical nucleotide sequence motifs were identified in the promoter and terminator regions of the gene.

Heterologous expression studies with the isolated *Rhizomucor* gene were performed in *Mucor circinelloides*. The expression vector contained the entire *bgl* gene of *R. miehei* fused with the promoter and terminator

regions of the *M. circinelloides gpd1* (glyceraldehyde-3-phosphate dehydrogenase) gene. This plasmid was introduced into *M. circinelloides* by PEG-mediated protoplast transformation. After culturing on cellobiose and wheat bran, the transformant strains showed significantly higher β-glucosidase activity than the original *Mucor* strain. Moreover, denaturing gel electrophoresis of the proteins, which were partially purified from the crude extracts, revealed the presence of an extra band in the protein pattern of the transformants. The size of this band corresponded to that of the *Rhizomucor* BGL suggesting that the introduced gene had been expressed in the transformants.

Blast searches with the amino acid sequence of the *R. miehei* BGL revealed the existence of highly similar hypothetical proteins in the databases of other zygomycetes genomes. These sequences were involved in phylogenetic analyses carried out to address the evolution of fungal β-glucosidases of subfamily 4 within the family 3 glycoside hydrolases. The resulted consensus tree supports the monophyly of zygomycetes β-glucosidases.

SUMMARY

1. Several isolates of zygomycetes have been identified as good extracellular β-glucosidase producer.
2. Effect of temperature, ethanol, glucose and acidic pH on enzyme activity has been tested in the crude extracts of the good β-glucosidase producer strains. Enzymes with acido- and thermotolerant properties have been identified among the tested β-glucosidases.
3. The β-glucosidases produced on wheat bran by *R. miehei* NRRL 5282, *M. corticolus* SZMC 12031, *Rhizopus niveus* CBS 403.51 and *G. persicaria* ATCC 201107 isolates have been purified.

4. Biochemical characterization of the purified β-glucosidases has been performed with respect to some parameters influencing the hydrolytic activity of the enzymes.
5. Synthetic activity of the purified *R. miehei* and *M. corticolus* β-glucosidases has been studied in detail using various monosaccharide donor and acceptor compounds. Significant transgalactosidase activity has been determined at the *R. miehei* enzyme; as we know, this is the first study about a filamentous fungus β-glucosidase, which has good ability to catalyze both transglucosylation and transgalactosylation reactions.
6. Liberation of plant derived phenolic antioxidants from glycosides by the purified *R. miehei* β-glucosidase has been studied.
7. Isolation and characterization of a β-glucosidase-coding gene (*bgl*) from the *R. miehei* NRRL 5282 isolate have been performed. Heterologous expression studies have also been carried out. To our knowledge, the *R. miehei bgl* is the first β-glucosidase gene from a zygomycete fungus that has been isolated and characterized.
8. Phylogenetic analyses have been carried out to address the evolution of fungal β-glucosidases of subfamily 4 within the family 3 glycoside hydrolases.

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Book chapter:

Krisch, J., **Takó, M.**, Papp, T., Vágvölgyi, Cs. (2010) Characteristics and potential use of β-glucosidases from Zygomycetes. In: Vilas, A. M. (ed.) Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Formatex Research Center, pp. 891-896.

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Papp, T., **Takó, M**., Galgóczy, L., Vágvölgyi, Cs. (2004) Cloning and partial sequence analysis of the gene encoding a β-glucosidase in *Rhizomucor miehei.* MMT 2004 évi Nagygyűlése, Keszthely, Hungary.

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Takó, M., Bencsik, O., Krisch, J., Papp, T., Vágvölgyi, Cs. (2009) Transglycosylation activity of a beta-glucosidase from *Rhizomucor miehei*. *Acta Microbiol. Immunol. Hung.* 56, 249-250.

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Papp, T., Nyilasi, I., **Takó, M.**, Nagy, L., Vágvölgyi, Cs. (2011) Rhizomucor. In: Liu, D. (ed.) Molecular Detection of Human Fungal Pathogens. CRC Press Taylor & Francis Group, pp. 783-790.

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Cumulative impact factor: 20,332