Characteristics and potential use of β-glucosidases from Zygomycetes

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β-Glucosidases (β-D-glucoside glucohydrolases) play important role in nature, including the degradation of cellulosic biomass by fungi and bacteria, breakdown of glycolipids in mammalian lysosomes, and the cleavage of glycosylated flavonoids in plants. These enzymes have broad substrate specificity, and are used in a range of biotechnological processes. The most intensively studied area of their application is the saccharification of cellulosic biomass for fuel ethanol production. Their synthetic activity in the production of oligosaccharides and aryl-glycosides is also subject of intensive research. β-Glucosidases from Zygomycetes can be produced in large amounts on cheap substrates using solid state fermentation which makes them promising candidates for biotechnological applications in the future.

Keywords Zygomycetes; β-glucosidases, transglycosylase activity, liberation of aglycons, fuel ethanol production

1. Introduction

β-Glucosidases (β-D-glucoside glucohydrolases; EC3.2.1.21) catalyze the hydrolysis of alkyl and aryl β-glycosides as well as disaccharides and short chain oligosaccharides. Many of them show also synthetic activity via reverse hydrolysis or transglycosylation [1]. β-Glucosidases have a great potential to be used in various biotechnological processes from liberating flavours, aromas and isoflavone aglycons to the synthesis of oligosaccharides and alkyl-glycosides.

β-Glucosidases are ubiquitous and can be found in bacteria, fungi, plants and animals. Fungal β-glucosidases are parts of the cellulose degrading enzyme system working synergistically with endoglucanases and cellobiohydrolases. They split cellobiose into two molecules of glucose, protecting the above mentioned enzymes from the product inhibition effect of cellobiose. Their application in the conversion of high-cellulose-content biomass to fermentable sugars for the production of fuel ethanol is an intensively studied area [2]. Good β-glucosidase-producer fungi, usable in various biotechnological processes, synthesize enzymes with high hydrolyzing activity, heat and glucose tolerance, acid resistance, and possible transglycosylase activity [3].

Several fungi belonging to the Zygomycetes group are well-known producers of extracellular enzymes, mainly proteases and lipases [4, 5]. These enzymes are used, e.g., in manufacturing of washing powders or in the food industry for cheese making. Some β-glucosidases from different Zygomycetes have been purified and characterised [6-9]. In our study, β-glucosidase was isolated from 94 strains representing 24 species of Zygomycetes, and 10 of them showed characteristics corresponding to the requirements described above [10]. The good enzyme production of these fungi under solid state fermentation conditions could be the bases of the cheap enzyme production. The acid- and thermotolerant characteristics of some of these enzymes make them promising candidates in various special applications.

2. Occurrence, function and cellular localization of β-glucosidases

β-glucosidases are ubiquitous in the nature and can be found in bacteria, fungi, plants and animals. Their activity is fundamental in many biological pathways, such as degradation of structural and storage polysaccharides, host-pathogen interactions, cellular signalling, and oncogenesis [1]. In plants, they release pathogen-defending compounds from their glycosidic bonds and activate phytohormons. β-glucosidase deficiency in humans causes Gaucher's disease.

Fungal β-glucosidases in wine-making yeasts contribute to the liberation of aromatic compounds. In moulds and white-rot fungi, they are parts of the cellulose degrading enzyme system together with various endo- and exoglucanases.

It seems that bacterial and yeast β-glucosidases are mainly intracellular. Moulds secrete their enzymes extracellularly [1]. However, there are some exceptions: a novel, intracellular β-glucosidase (BGLII) was purified from *Trichoderma reesei* [11]. It is suggested that cultivation conditions can influence the secretion of these enzymes in moulds: they are cell-wall-bound in submerged cultures but soluble under solid state fermentations [12]. We hypothesise that β-glucosidases in moulds are, as part of the cellulase complex, inducible and might be released from the loose bonds of the cell wall and secreted into the environment in the presence of natural cellulose substrates. This theory is supported by the findings that extracellular and for the most part cell-wall-bound activities are related to the same enzyme [6, 11].

3. Classification of β-glucosidases

Classification of β-glucosidases among hydrolytic enzymes is based on their substrate activity or their nucleotide sequence identity [1, 13].

3.1. Classification on the basis of substrate specificity

On the basis of substrate specificity, three classes have been defined: aryl β-glucosidases (class 1), true cellobiases (class 2) and broad substrate specificity enzymes (class 3). Most β-glucosidases belong to class 3 with various abilities for the cleavage of β 1,4; β 1,6; β 1,2 and α 1,3; α 1,4; α 1,6 glycosidic bonds [1, 14].

3.2. Classification on the basis of nucleotide sequence identity (NSI)

According to the classification of Henrissat and Bairoch [15], β-glucosidases are mainly in family 1 and 3 from the 88 glycosyl hydrolase families. In family 1, β-glucosidases from archeabacteria, plants and mammals can be found. They usually exhibit also β-galactosidase activity. Family 3 includes β-glucosidases from bacteria, fungi and plants with a characteristic two-domain globular structure [16].

4. Mode of action of β-glucosidases

β-glucosidases are retaining enzymes since their products retain the same anomeric configuration as the substrate. Their reaction follows a double-displacement mechanism [17].

4.1. Hydrolysis

β-glucosidases normally catalyze the hydrolysis of β 1,4-glucosidic bonds in aryl- and alkyl β-D-glucosides from the non-reducing termini. In the first step the enzyme's nucleophile in the active centre attacks the substrate and an α-glycosyl enzyme intermediate is formed. In the second step the intermediate is hydrolyzed by H_2O and β-glucose is released as the product [1]. The nucleophile residue is in many cases Asp or Glu. In our study [18] the analysis of the *Rhizomucor miehei* β-glucosidase identified, in line with known fungal β-glucosidases, the residue Asp254 as the catalytic nucleophile, situated in a conserved motif SDW. For the release of the aglycon, another amino acid residue in the catalytic domain acts as a H^+ donor to the glycosidic oxygen, resulting in the departure of the aglycon in R-OH form. The potential H^+ donor can be His. In case of *R. miehei* β-glucosidase, the residue His177 was proposed as H^+ donor in the motif KHY [18].

4.2. Reverse hydrolysis or transglycosylation

In the synthetic reactions, the reactive molecule in the second step is an R'-OH instead of H_2O, yielding oligosaccharides or other glycosides. In reverse hydrolysis, the substrate is a sugar, mainly glucose, yielding a disaccharide product. In transglycosylation process, formation of the product is the result of competition between water and the acceptor molecule [1]. In many cases, lowering the water activity would shift hydrolysis to transglycosylation.

5. Characteristics of β-glucosidases from Zygomycetes

There are only sporadic publications on the purification and characterization of β-glucosidases from Zygomycetes [6-9]. We studied the extracellular β-glucosidase activity of 94 strains, representing 24 species of the genera *Gilbertella, Mucor*, *Rhizomucor*, and *Rhizopus* [10].

5.1. Substrate specificity

Table 1 Substrate specificity of some β-glucosidases purified from Zygomycetes

Substrate	Linkage of glycosyl group	Source of enzyme [reference]			
Saccharides		*M. racemosus* [6]	*M. circinelloides* [7]	*Rh. oryzae* [8]	*R. miehei* [9]
Cellobiose	β (1,4) Glc	+	n.d.	+	+
Gentiobiose	β (1,6) Glc	n.d	n.d	+	n.d.
Maltose	α (1,4) Glc	n.d	n.d	-	n.d
Aryl-glucosides					
p-nitrophenyl –β-D-glucoside	β Glc	+	+	+	+
p-nitrophenyl –α-D-glucoside	α Glc	n.d	n.d	-	n.d
Cyanogen glycosids					
Amygdalin	β (1,6) Glc	n.d	+	+	n.d

n.d. = not detected

β-glucosidases from Zygomycetes show, similarly to the enzymes isolated from other fungal groups, a broad substrate specificity (Table 1). The aryl-glucoside p-nitrophenyl-β-D-glucopyranoside is the standard substrate in β-glucosidase enzyme activity measurements. All known enzymes are able to hydrolyse this substrate with Km values from 0.31 mM to 1.87 mM [6]. Cellobiose was hydrolyzed by the enzyme of "*Mucor" miehei* (now *Rhizomucor miehei*) [9], *Mucor racemosus* [6], *Rhizopus oryzae* MIBA348 [8], and strains of *Gilbertella, Mucor*, *Rhizomucor*, and *Rhizopus* [5]. β-glucosidase from *Rh. oryzae* was active on salicine, gentiobiose and amygdaline, but was inactive on Avicel, carboxymethylcellulose, maltose and p-nitrophenyl-α-D-glucopyranoside [8]. Linamarase (β-glucosidase) of *M. circinelloides* LU M40 had a wide activity against plant cyanogen glycosides: prunasin, amygdalin, linamarin, sambunigrin, tetraphyllin B, taxiphyllin, lucumin, gynocardin with Km values from 0.36 to 2.93 mM [7].

5.2. Influence of pH on the enzyme activity

Most microbial β-glucosidases have an acidic pH optimum but are active over a broad pH range. The pH optimum for the enzyme purified from *R. oryzae* was 5.0, [8] and for the linamarase of *M. circinelloides,* 5.5 [7]. Surprisingly, an optimum of pH 8.0 was reported for the *M. (Rhizomucor) miehei* enzyme [9]. β-glucosidases from *Mucor racemosus f. chibinensis, Rhizomucor miehei* (NRRL5901 and ETHM 4908) and *Rhizopus microsporus v. oligosporus* were activated at pH 4 suggesting acidophilic character for these enzymes [10]. The pH stability of zygomycota β-glucosidases ranged from 2.5 to 10 (*R. miehei*); from 3.0 to 7.1 (*M. circinelloides*) and from 3.8 to 7.1 (*R. oryzae*) [7-9].

5.3. Influence of temperature on the enzyme activity

Heat tolerance or resistance is a very important feature of enzymes in biotechnological applications since some processes like cellulose saccharification take place at 60^{o}C or above.

In Table 2, optimum temperatures and temperature tolerances of β-glucosidases from various Zygomycetes are shown.

Table 2 Temperature optimums and ranges for zygomycota β-glucosidases

Source of enzyme	Temperature optimum (^{o}C)	Temperature range (^{o}C)	Max. temperature (^{o}C)
M. circinelloides [7]	40	-	60
Rh. oryzae [8]	55	48-62	-
R. miehei [9]	60	50-75	95

In our experiments, all investigated *R. miehei* strains retained 60% or more of their activity after heating to 75 ^{o}C for 5 min in the presence of 5 mM cellobiose [10]. The substrate plays a protective role during heating by maintaining the appropriate conformation of the enzyme. β-glucosidases generally possess a carbohydrate chain that may play a role in the heat tolerance; after cleavage of this carbohydrate chain, thermostability decreases [19].

5.4. Effect of inhibitors and activators on the enzyme activity

The enzyme of *R. miehei* was not sensitive to the metal ions Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} [9]. Linamarase from *M. circinelloides* was inhibited by Mn^{2+}, Cu^{2+}, Co^{2+} and Ag^{+}, while Zn^{2+} and Ca^{2+} showed no effect on the enzyme activity [7]. Also, EDTA had no effect on both enzymes; the activity of linemarase was even slightly increased [7, 9].

5.5. Influence of glucose on the enzyme activity

In most cases glucose, the main product of hydrolysis, has a strong inhibiting effect on β-glucosidases. This is a competitive mechanism in which substrate and product are in competition for the active site of the enzyme. In some biotechnological processes high glucose tolerance is desirable. Only few β-glucosidases were reported to be glucose tolerant, e.g. that from *Aspergillus oryzae* with K_i inhibition constant of 1.36 M [14]. The investigated *Gilbertella, Mucor*, *Rhizomucor* and *Rhizopus* species showed no or moderate tolerance, retaining 6.2-30.8% of activity in the presence of 10 mg/ml glucose. K_i for *Gilbertella persicaria* and *R. miehei* were 3.68 and 8 mM, respectively (Krisch, unpublished results).

5.6. Transglycosylation activity

Yoshioka and Hayashida [9] reported the formation of cellotriose and gentiobiose (β 1,6 linkages) during the hydrolysis of 5% cellobiose by *R. miehei*. In our study ethanol at 10% concentration increased the enzyme activity of *R. miehei* strains, suggesting transglycosylation effect [10]. It was supposed that ethanol was an acceptor for the liberated glucose leading to the formation of ethyl-glucoside.

6. Production of β-glucosidases from Zygomycetes

β-glucosidase production in submerged culture was detected in the presence of CMC [8], glucose [6, 7], xylose, sucrose and cellobiose [7]. Linamarase production was high with lactose and pectin in the medium [7]. *R. miehei* was cultivated on wheat bran for 4 days. Highest activity was achieved at 50°C [9]. Cultivation on cellobiose in submerged culture, or on wheat bran by solid state fermentation, resulted in significant differences in the β-glucosidase production of Zygomycetes investigated in our laboratory [10]. Extracellular enzyme production was higher during solid state fermentation, except for *M. amphibiorum, M. guillermondii* and *Rh. oryzae* enzymes. The best β-glucosidase producers were *G. persicaria* strains, yielding more than 350 U activities on 1 g wheat bran. The temperature optimum for enzyme production was 25 °C for *G. persicaria* strains and 30 °C for all other strains. Only *R. miehei* strains were able to produce the enzyme at 50 °C [10].

7. Application of β-glucosidases in biotechnology

Due to their hydrolyzing and synthetic activity, β-glucosidases are excellent candidates in a number of biotechnological processes. Production of acid-, thermo-, and glucose tolerant enzymes in large quantities on cheap substrates is an intensively studied area.

7.1. Applications based on hydrolysis

β-glucosidases as part of the cellulase enzyme complex hydrolyze cellobiose and cello-oligosaccharides to yield glucose which is fermentable by yeasts into fuel ethanol. The conversion of cellulosic biomass into fermentable sugars is a very attractive way of ethanol production. However, the most widely used cellulase from *Trichoderma viridae* has a poor β-glucosidase activity and the accumulation of cellobiose will lead to product inhibition [20]. Addition of thermo-tolerant β-glucosidases to commercial cellulase enzyme preparations resulted in synergistic effect and increased reducing sugar concentration [20, 21].

Using β-glucosidases as additives in cellulose-based feeds is beneficial for single-stomach animals, such as pigs and chickens, by enhancing the digestibility of the feed [22, 23].

In winemaking, β-glucosidases play a key role in the enzymatic release of aromatic compounds from glycosidic precursors present in fruit juices, musts and wines. The natural process by endogenous plant β-glucosidases is very time consuming. Supplementation with external enzymes may enhance aroma release [24]. In tea beverages treated with immobilized β-glucosidase, the essential oil content has been increased by 6.79 - 20.69% [25]. In citrus fruit juices the hydrolysis of naringenin to prunin reduced the bitterness of the juices [26].

Isoflavones found in soybean have phytoestrogenic properties, so they can relieve menopausal symptoms and can help prevent several chronic diseases and certain cancers. However, in soy-based foods the isoflavons are mainly in the inactive form of glycosides. Production of aglycons by the hydrolysis via β-glucosidases is highly desired [27, 28]. Enrichment of genistein, the most potent inhibitor of cancer cell growth, in soy protein concentrate was reported [28]. In

soy milk, the aglycon content was increased significantly either by treatment with β-glucosidase [29] or by fermentation with a β-glucosidase-producing *Lactobacillus* strain [30].

Detoxification of cassava (a staple food in tropical countries) was achieved by the addition of linamarase (β-glucosidase) of *M. circinelloides*. After 24 h of fermentation, all cyanogenic glycosides were hydrolyzed to remove the toxic -CN moiety [7].

Recently, solid state fermentation of cranberry and pineapple pomace with good β-glucosidase-producing fungal strains (*Lentinus edodes* and *Rh. oligosporus*) was carried out to enhance the amount of extractable free phenolics showing antioxidant activity [31, 32].

7.2. Applications based on transglycosylation

The synthetic activity of β-glucosidases is used for the biosynthesis of oligosaccharides and alkyl-glycosides. Oligosaccharides can be used as therapeutic agents, diagnostic tools, and growth promoting agents for probiotic bacteria. They have important functions in biological systems including fertilization, embryogenesis, and cell proliferation. Galacto-oligosaccharides, the transgalactosylation products from lactose, were found to be good growth factors for intestinal *Bifidobacteria.* Alkyl-glycosides are non-ionic surfactants with high biodegradability, and have good antimicrobial properties. Hence, they have potential application in pharmaceutical, chemical, cosmetic, food, and detergent industries [34]. Their enzymatic synthesis using the transglycosylation activity of glycoside hydrolases can be performed in one step, instead of the several protection-deprotection steps required in chemical synthesis [3, 33].

There are some examples for the use of the synthetic activity of β-glucosidases in the literature. Galacto-oligosaccharides and isobutyl-galactosides were synthesised from lactose in water-organic media via *Aspergillus oryzae* β-glucosidase [34]. Arbutin-β-glycosides were synthesized via the transglycosylation reaction of *Thermotoga neapolitana* β-glucosidase to develop a new skin whitening agent, and the products were evaluated for their melanogenesis inhibitory activities [35].

8. Concluding remarks

β-glucosidases are ubiquitous in nature, being produced by bacteria, fungi, plants and animals. Their main function is hydrolysis of glycosidic bonds in a variety of substrates, releasing simple sugars and aglycons. Most of the purified enzymes have also a synthetic activity via reverse hydrolysis or transglycosylation. Due to their dual activity and broad substrate specificity, these enzymes may have a wide field of application in biotechnological processes, including the liberation of aromatic compounds and isoflavone aglycons, or the biosynthesis of oligosaccharides and aryl-glycosides.
There have been only a few examples for the purification and characterization of β-glucosidases from Zygomycetes although the enzyme production of some strains under solid state fermentation is excellent. β-Glucosidases from the thermo-tolerant *R. miehei* strains might have a potential application in biomass conversion to fermentable sugar and fuel ethanol. Enzymes of the good producer *G. persicaria* strains might be cloned into wine-making yeasts to enhance aroma liberation under mild conditions. β-glucosidases of Zygomycetes deserve more attention in the future.

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