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ORIGINAL ARTICLE

Production and secretion of a multifunctional ß-glucosidase by *Humicola grisea* var. *thermoidea*: effects of L-sorbose

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Abstract The effects of L-sorbose on growth, morphology and production of a multifunctional ß-glucosidase by the thermophilic fungus *Humicola grisea* var. *thermoidea* were investigated. Sorbose increased the lag phase period 3-fold and drastically altered the morphology of the fungal hyphae. Cellobiose and lactose were good inducers of the enzyme. The addition of 5 % sorbose to cultures containing 1 % cellobiose enhanced the extracellular levels of the ß-glucosidase 3.3-fold with constant cytosolic and cell-wall bound levels, demonstrating stimulation of both enzyme synthesis and secretion. The stimulation of enzyme production by sorbose was dependent on the presence of cellobiose as inducer, since 2- to 3-fold inhibition was observed in lactose and glucose. Production and secretion of phosphatases and endoglucanases was not stimulated by sorbose, which did not affect the subcellular distribution of the ß-glucosidase also. However, it reduced the uptake rates of glucose and cellobiose. Taken together, the results discarded increased non-specific enzyme secretion and/or increased release of the enzyme from the cell-wall as possible molecular mechanisms for the effects of sorbose on the production of the multifunctional ß-glucosidase by *H. grisea*. An alternative mechanism, based on a prolonged action of cellobiose as inducer associated with a decreased catabolic repression by glucose, was discussed.

Keywords L-sorbose · ß-glucosidase · Enzyme induction · Enzyme secretion · *Humicola grisea* var. *thermoidea*

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Introduction

It is well known that L-sorbose induces morphological changes in many filamentous fungi, causing cell length reduction, increased branching, and localized bulges in the hyphal walls, a phenomenon known as paramorphogenesis (Tatum et al. 1949; Barnett and Lilly 1951; El-Shafei 1997). The specific growth rate may also be slightly reduced in the presence of this ketohexose (Crocken and Tatum 1968; Trinci and Collinge 1973; El-Shafei 1997). Apparently, these alterations are associated with changes in the composition of the fungal cell walls, possibly resulting from the inactivation of polysaccharide synthases (De Terra and Tatum 1961; Mahadevan and Tatum 1965; Bisaria et al. 1986; El-Shafei 1997). In fact, when sorbose was present in the culture medium, the specific activity of the ß-1,3-glucan synthase was reduced in *Neurospora crassa* (Mishra and Tatum 1972), and the glucosamine/glucose ratio was increased in *Aspergillus fumigatus* (El-Shafei 1997) and *Trichoderma reesei* (Bisaria et al. 1986) cell walls.

On the other hand, L-sorbose substantially enhances the extracellular levels of cellulases in *T. reesei* cultures, both in the virtual absence of any other carbon source or in the presence of glucose, cellobiose, or cellulose (Sahoo et al. 1986; Nanda et al. 1986; Bisaria et al. 1989; Kubicek et al. 1993; Nogawa et al. 2001; Aro et al. 2005). This has previously been attributed to the cell wall alterations (Nanda et al. 1986; Bisaria et al. 1989; Singh and Hayashi 1995) and to a reduction in the uptake and/or intracellular degradation of

cellobiose (Bisaria et al. 1989). However, Nogawa et al. (2001) recently showed that, like cellobiose and sophorose, L-sorbose regulates the expression of cellulases in *T. reesei* at the transcriptional level.

The induction of cellulases by L-sorbose has also been described for *Penicillium decumbens* JU-A10 (Wei et al. 2011) and *Sclerotium rolfsii* (Sachslehner et al. 1998). In contrast, negligible induction occurred in *Acremonium cellulolyticus* (Fang et al. 2008) and *Volvariella volvacea* (Zhang et al. 2011), since the inducers and inducing mechanisms of cellulases synthesis may vary among fungi. In addition to cellulases, L-sorbose also induced xylanases and α-L-arabinofuranosidases in *T. reesei* (Xu et al. 1998, 2000; Nogawa et al. 1999) and mannanases in *S. rolfsii* (Sachslehner et al. 1998). Moreover, it increased the production of N-acetyl galactosaminoglycan deacetylase in *N. crassa* (Jorge et al. 1999) and NAD(P)ase in *Aspergillus nidulans* (Da-Silva and Jorge 1988).

The induction of ß-glucosidases (EC 3.2.1.21) by L-sorbose has been investigated less to date. In *V. volvacea*, sorbose was the best carbon source for the production of extracellular ß-glucosidases (Cai et al. 1998) and also efficiently induced an intracellular form of the enzyme at the transcriptional level (Ding et al. 2007). Moreover, the addition of L-sorbose to cellobiose or cellulose highly increased the extracellular levels of the enzyme in cultures of *Trichoderma pseudokoningii* (Kubicek 1983) and *T. reesei* (Bisaria et al. 1986; Chen and Wayman 1993). These effects have been attributed to the release of cell-wall associated ß-glucosidase to the culture medium and/or to a general increase in extracellular enzyme secretion (Kubicek 1983; Bisaria et al. 1986; Sahoo et al. 1986; Nanda et al. 1986; Bisaria et al. 1989; Messner et al. 1990). However, the true mechanisms by which L-sorbose increases the production of extracellular ß-glucosidases by fungi remain to be elucidated.

Humicola is a well known genus of thermophilic fungi from soil (Cooney and Emerson 1964), and some species of this genus were described as good producers of cellulolytic enzymes (Maheshwari et al. 2000; Lynd et al. 2002). Two major forms of thermostable ß-glucosidases produced by a strain of *Humicola grisea* var. *thermoidea* isolated in Brazil have been previously purified and characterized. An extracellular form hydrolyzed cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu) but not lactose or *o*-nitrophenyl-β-D-galactopyranoside (*o*NP-Gal) (Peralta et al. 1997). In contrast, an intracellular multifunctional ß-glucosidase hydrolyzed cellobiose and lactose, as well as *p*NP-Glu, *p*NP-Fuc (*p*-nitrophenyl-β-D-fucopyranoside), and *o*NP-Gal, at a common catalytic site (Peralta et al. 1990; Nascimento et al. 2010).

During the last decade, a growing interest in the production and secretion of ß-glucosidases by fungi has arisen, related to their diverse industrial applications. In this study, we investigated the effects of sorbose on the production and secretion of the multifuncional ß-glucosidase from this Brazilian *H. grisea* strain, assessed by its activity on both *p*NP-Glu and *o*NP-Gal.

Materials and methods

Organism and growth conditions

The *Humicola* strain was isolated from Brazilian soil and classified as *H. grisea* var. *thermoidea* on the basis of its morphological and physiological characteristics, according to Cooney and Emerson (1964). The fungus was maintained in the laboratory at 45 °C, on slants of solid medium containing 4 % oatmeal baby food (Quaker, Brazil).

Conidia were harvested from 7- to 10-day-old cultures and inoculated at a concentration of 10^7 conidia per mL into M5 medium (0.1 % peptone (Difco, Franklin Lakes, NJ, USA), 0.25 % gelatin (Oxoid, Basingstoke, UK), 0.8 % yeast extract (Difco), 0.1 % $CaCO_3$, and 0.5 % NaCl) with or without (deficient medium) a desired additional carbon source (glucose, cellobiose or lactose) at 1 % (w/v) concentration, in the presence or absence of sorbose. Initial pH was adjusted to 6.0 with HCl. Culture media were previously autoclaved for 20 min at 127 °C and 1.5 atm. A concentrated sorbose solution (25 %, w/v, in water) was autoclaved separately from the other components and adequate volumes were added to the culture media immediately before the inoculum. Cultures (25 mL) were incubated at 45 °C and 145 rpm in 125-mL Erlenmeyer flasks.

Enzymatic assays

β-Galactosidase activity was assayed at 50 °C in 50 mmol L^{-1} sodium phosphate buffer, pH 6.0, containing 8.0 mmol L^{-1} *o*NP-Gal (Sigma-Aldrich, St. Louis, MO, USA). The reactions were initiated by the addition of the crude cell extract or the culture filtrate suitably diluted. After adequate time intervals, the reactions were interrupted by the addition of two volumes of saturated sodium tetraborate solution. The hydrolysis rates were estimated by quantifying the liberation of the *p*-nitrophenolate ion ($\varepsilon_{410nm,\ pH\ 12}$=17,500 mol^{-1} L cm^{-1}). ß-glucosidase activity was estimated as above using 3.0 mmol L^{-1} *p*NP-Glu (Sigma-Aldrich) as substrate.

Endoglucanase activity was assayed at 50 °C in 50 mmol L^{-1} sodium acetate buffer, pH 5.0, using 0.1 % soluble starch or 1 % CMC (Sigma-Aldrich) as substrates, respectively. The reducing sugars released were estimated using the dinitrosalicylic acid (DNS) method, as described by Miller (1959).

Alkaline phosphatase activity was assayed at 50 °C in 200 mmol L^{-1} glycine buffer, pH 9.0, using 10 mmol L^{-1} *p*-

nitrophenylphosphate (*p*NPP; Sigma-Aldrich) as substrate. The reaction was initiated by the addition of the enzyme suitably diluted to the reaction medium and interrupted by the addition of one volume of sodium tetraborate saturated solution after convenient time intervals. The hydrolysis rates were estimated by quantifying the liberation of the *p*-nitrophenolate ion ($\varepsilon_{410nm,\ pH\ 12}$=17,500 mol^{-1} L cm^{-1}). Acid phosphatase activity was determined as described above, except that glycine buffer, pH 9.0, was substituted by sodium acetate buffer, pH 5.4.

The experimental conditions (reaction times, enzymatic units) employed in all experiments were adjusted to guarantee the estimation of initial velocities (hydrolysis of no more than 5 % initial substrate, linear response of product formation in respect to reaction time). One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min. Specific activities for intracellular and extracellular enzymes were expressed as U per mg protein.

Localization of the multifunctional ß-glucosidase

Cultures grown in media supplemented with 1 % glucose, 1 % cellobiose, or 1 % cellobiose plus 5 % L-sorbose (Sigma-Aldrich) were prepared in triplicate and filtered through synthetic bath sponge. The culture filtrates were used as sources of extracellular enzymes. The mycelial pads were rinsed twice with five volumes of chilled distilled water and stored at −20 °C until use. Cell extracts were prepared by grinding the mycelial pads with glass beads in chilled porcelain mortars. The slurries were suspended in known volumes of 50 mmol L^{-1} sodium phosphate buffer, pH 6.0, and the glass beads were separated by gravity. The resulting suspensions were centrifuged at 11,000*g* and 4 °C for 30 min and the supernatants were used as sources of intracellular enzymes. The pellets were extensively washed with phosphate buffer, pH 6.0, to remove cytoplasmic contaminants, suspended in a known volume of the same buffer and used as sources of cell wall-bound enzymes (Sahoo et al. 1986).

Analytical methods

L-sorbose concentrations were estimated according to the method of Dische and Devi (1960). Glucose concentrations were determined by the glucose-oxidase method (Bergmeyer and Bernt 1974). Cellobiose concentrations in the culture filtrates were determined by complete hydrolysis with an excess of ß-glucosidase followed by quantification of the glucose released using the glucose-oxidase method (Bergmeyer and Bernt 1974). Protein concentrations were estimated according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

Results

Effects of sorbose on *Humicola* growth

The effect of sorbose on *H. grisea* var. *thermoidea* growth in media supplemented with glucose, cellobiose, or lactose is shown in Fig. 1. Independent of the additional carbon source, the presence of sorbose retarded the onset of the growth phase, increasing almost 3-fold the lag growth period (Fig. 1). Negligible growth occurred up to 24 h in all media containing sorbose, compared to those containing glucose, cellobiose or lactose only. However, after the initial lag period, cultures in sorbose-containing media developed biomass. Moreover, cultures in the presence of glucose plus sorbose or cellobiose plus sorbose showed growth rates comparable to those observed in glucose or cellobiose alone, attaining final growth yields only slightly lower. In contrast, cultures in lactose plus sorbose exhibited lower growth rates than those carried out in lactose alone, and a final growth yield about 50 % lower.

Microscopic observation of sorbose-grown *H. grisea* mycelia showed swollen hyphae, some of them apparently burst at their tips (Fig. 2). These gross morphological alterations in mycelial structures were independent of the carbon source.

Effect of sorbose on the production of ß-glucosidase and ß-galactosidase activities by *H. grisea* var. *thermoidea*

The fungus was cultivated in deficient medium or in media supplemented with glucose, cellobiose or lactose at 1 % concentration, with or without the addition of 5 % sorbose (Table 1). Very poor growth was observed when only sorbose was added to the deficient medium, with growth yields (24 mg dry weight)

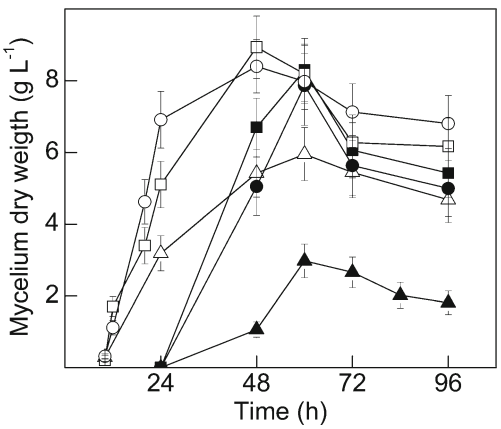

Fig. 1 Effect of sorbose on *H. grisea* var. *thermoidea* growth The fungus was cultivated at 45 °C and 145 rpm in medium supplemented with: (○) 1 % glucose; (●) 1 % glucose plus 5 % sorbose; (□) 1 % cellobiose; (■) 1 % cellobiose plus 5 % sorbose; (Δ) 1 % lactose; (▲) 1 % lactose plus 5 % sorbose. After convenient time intervals, the cultures were harvested for the determination of mycelial dry weight. The values shown represent means from triplicate experiments (n=3)

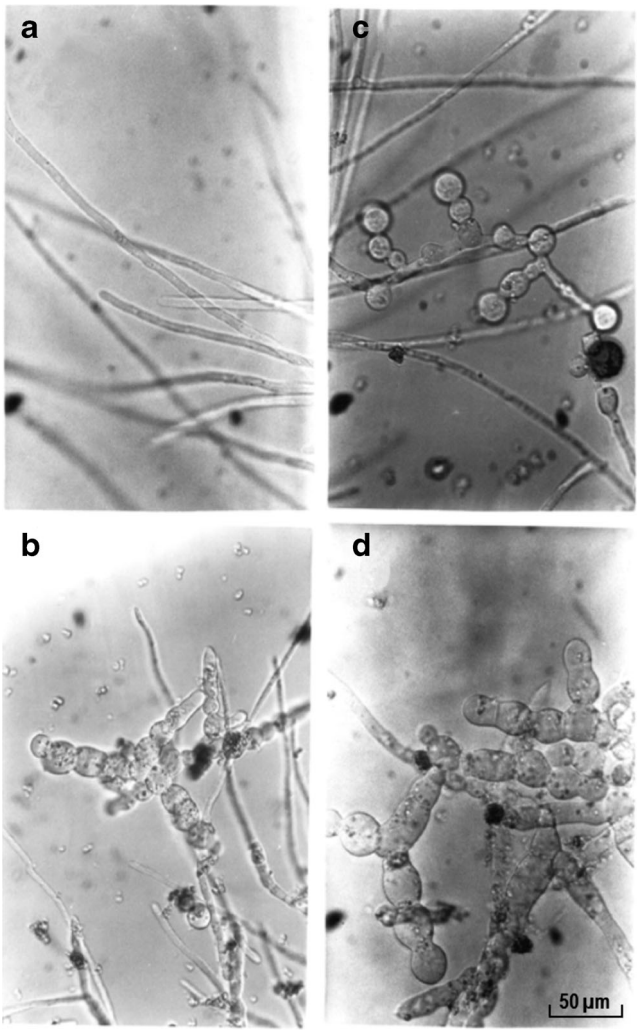

Fig. 2 Effect of sorbose on the morphology of *H. grisea* var. *thermoidea* The fungus was cultivated for 60 h at 45 °C and 145 rpm in M5 medium supplemented with: (**a**) 1 % glucose; (**b**) 1 % cellobiose; (**c**) 1 % glucose plus 5 % sorbose; (**d**) 1 % cellobiose plus 5 % sorbose

much lower than in its absence (59 mg dry weight). Moreover, cultures in deficient medium plus sorbose alone showed extracellular levels of ß-galactosidase (73 U) and ß-glucosidase (42 U) activities about 1.8- and 3.5-fold lower, respectively, compared to those obtained in cultures in deficient medium. In contrast, 2.9- and 1.6-fold higher intracellular levels of ß-galactosidase (131 U) and ß-glucosidase (362 U) activities, respectively, were estimated in the presence of sorbose. However, total ß-galactosidase and ß-glucosidase activities were not substantially affected by the presence of sorbose.

The addition of glucose to the deficient medium has not substantially increased the levels of total ß-galactosidase and ß-glucosidase activities in *H. grisea* cultures, in spite of an increase of about 3-fold in the mycelial dry weight. Moreover, the presence of glucose decreased the specific activity of the extracellular ß-glucosidase from about 2.46 to 0.68 U mg^{-1} protein (Table 1). Further, the addition of sorbose to the glucose-supplemented medium led to expressive reductions of the extracellular levels of ß-galactosidase and ß-glucosidase activities, which decreased respectively from 383 to 189 U and from 111 to 82 U, without appreciable variation in the dry cell mass (Table 1). The intracellular levels of the ß-galactosidase (37 U) and ß-glucosidase (53 U) activities produced in the presence of sorbose were also about 2- and 3-fold lower, respectively, than those estimated with glucose only, resulting in a reduction of 2-fold in the total production of both activities. Similarly, the addition of sorbose to media containing lactose negatively affected the intra- and extracellular levels of ß-galactosidase and ß-glucosidase activities (Table 1).

Cellobiose and lactose were good inducers of ß-galactosidase and ß-glucosidase activities in *H. grisea*. Compared to the deficient medium, total ß-galactosidase activity (cell extract plus culture filtrate) was enhanced about 16-fold by cellobiose, attaining 2,818 U, and 12-fold by lactose, attaining 2,114 U. Cellobiose and lactose also enhanced total ß-

Table 1 Effect of sorbose on the levels of glycosidase activities in *H. grisea* var. *thermoidea* cultures[a]

Carbon source (1%w/v)	Sorbose (5%w/v)	Dry weight (mg)	ß-Galactosidase activity				ß-Glucosidase activity			
			Extracellular		Intracellular		Extracellular		Intracellular	
			Total U	U mg^{-1}	Total U	U mg^{-1}	Total U	U mg^{-1}	Total U	U mg^{-1}
Deficient	−	59	130	2.11	45	6.74	147	2.46	231	33.55
	+	24	73	2.80	131	14.43	42	1.66	362	23.63
Glucose	−	173	383	2.17	73	12.63	111	0.68	159	28.41
	+	166	189	2.72	37	10.79	82	1.11	53	31.41
Cellobiose	−	201	955	5.46	1,863	194.18	453	2.34	994	103.52
	+	171	3,152	13.12	1,714	173.42	1,485	8.84	975	98.51
Lactose	−	129	920	6.93	1,194	129.78	955	7.12	1,011	109.88
	+	80	378	4.31	593	20.38	81	1.05	548	18.77

[a] The fungus was cultivated for 60 h at 45 °C and 145 rpm in M5 medium supplemented with the indicated carbon sources, in the presence and absence of 5 % (w/v) L-sorbose. The results are expressed as means of at least three cultures ($n \geq 3$).

glucosidase activity about 3.8- and 5.2-fold, reaching 1,447 and 1,966 U, respectively (Table 1). In parallel, the mycelial dry weight increased about 3.4- and 2.2-fold when the fungus was grown in the presence of cellobiose or lactose, respectively, compared to that obtained in deficient medium (59 mg). The higher production of the enzymatic activities in the presence of cellobiose or lactose was not directly related to the increase in cell growth, since the addition of glucose to the deficient medium increased the mycelial dry weight to similar values without a similar stimulation of enzyme production (Table 1).

When sorbose was added to *H. grisea* cultures in cellobiose-supplemented deficient medium the extracellular levels of ß-galactosidase and ß-glucosidase activities increased about 3.3-fold, from 955 to 3,152 U and from 453 to 1,485 U, respectively, without appreciable change in mycelial dry weight. Moreover, the intracellular levels remained practically unaltered, resulting in net increases of 1.7-fold in total activities (Table 1). The fact that sorbose increased both activities in a parallel fashion strongly suggested that it indeed affected the levels of the multifunctional ß-glucosidase from *H. grisea*, which shows good hydrolytic activity against both *p*NP-Glu and *o*NP-Gal.

Figure 3 depicts the effect of increasing concentrations of sorbose (up to 5 %, w/v) on the levels of extracellular ß-glucosidase and ß-galactosidase activities in *H. grisea* cultures in medium supplemented with 1 % cellobiose. Maximal levels of both activities occurred with 4–5 % sorbose, reaching values about 4-fold higher than those obtained in cellobiose-supplemented medium without the ketohexose.

The effect of sorbose on the time course of the production and secretion of *H. grisea* multifunctional ß-glucosidase in media supplemented with 1 % glucose or 1 % cellobiose is shown in Fig. 4. In media containing glucose, the presence of 5 % sorbose decreased the extracellular levels of ß-galactosidase (Fig. 4a) and ß-glucosidase (Fig. 4b) activities, in

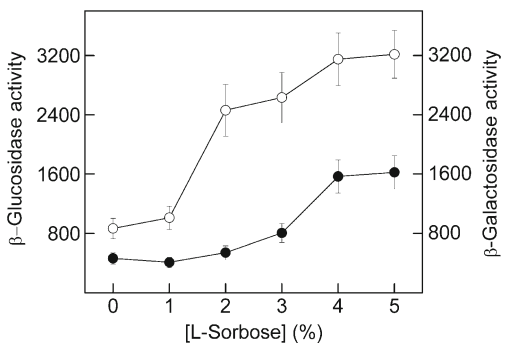

Fig. 3 Effect of increasing concentrations of sorbose on the levels of extracellular ß-glucosidase and ß-galactosidase activities in *H. grisea* var. *thermoidea* cultures The fungus was cultivated for 60 h at 45 °C and 145 rpm in medium supplemented with 1 % cellobiose and increasing concentrations of sorbose. Extracellular ß-glucosidase (●) and ß-galactosidase (○) activities were estimated in the culture filtrates. The values shown represent means from triplicate experiments ($n=3$)

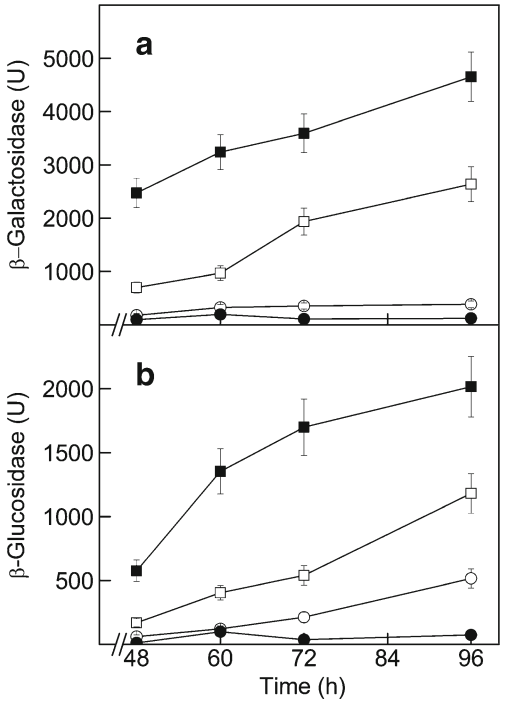

Fig. 4 Time-course of the extracellular levels of ß-galactosidase and ß-glucosidase activities in *H. grisea* var. *thermoidea* cultures The fungus was cultivated at 45 °C and 145 rpm for varying time intervals in medium supplemented with: (○) 1 % glucose; (●) 1 % glucose plus 5 % sorbose; (□) 1 % cellobiose; (■) 1 % cellobiose plus 5 % sorbose. ß-galactosidase (**a**) and ß-glucosidase (**b**) activities were assayed in the culture filtrates. The values shown represent means from triplicate experiments ($n=3$)

agreement with data from Table 1. However, the presence of sorbose in media containing cellobiose resulted in increases of about 1.8- to 3.5-fold in the extracellular levels of both ß-galactosidase and ß-glucosidase activities, relative to the absence of sorbose. The effect of sorbose was more pronounced from 48 to 60 h of culture.

Effect of sorbose on the production of different exoenzymes by *H. grisea* var. *thermoidea*

The effect of increasing sorbose concentrations (1 to 5 %) on the levels of extracellular endoglucanases, alkaline phosphatases and acid phosphatases in *H. grisea* cultures in medium supplemented with 1 % cellobiose was also evaluated (Fig. 5). The production of all enzymes was practically unaffected by the presence of sorbose. The intracellular levels of the enzymes were also not significantly affected (not shown).

Effect of sorbose on the localization of the multifunctional ß-glucosidase in *H. grisea*

The localization of the enzyme was investigated by following ß-glucosidase and ß-galactosidase activities in mycelia grown in media supplemented with glucose, cellobiose, or cellobiose plus sorbose (Table 2). In all culture conditions, a major

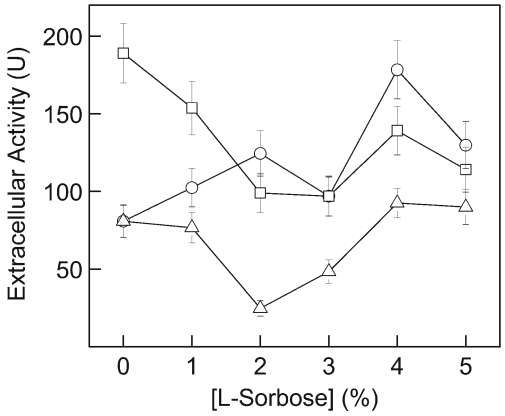

Fig. 5 Effect of increasing concentrations of sorbose on the levels of phosphatase and endoglucanase activities in *H. grisea* var. *thermoidea* cultures The fungus was cultivated for 60 h at 45 °C and 145 rpm in medium supplemented with 1 % cellobiose and increasing concentrations of sorbose. The levels of extracellular (○) endoglucanase, (□) acid phosphatase and (Δ) alkaline phosphatase activities were determined in the culture filtrates. The values shown represent means from triplicate experiments (n=3)

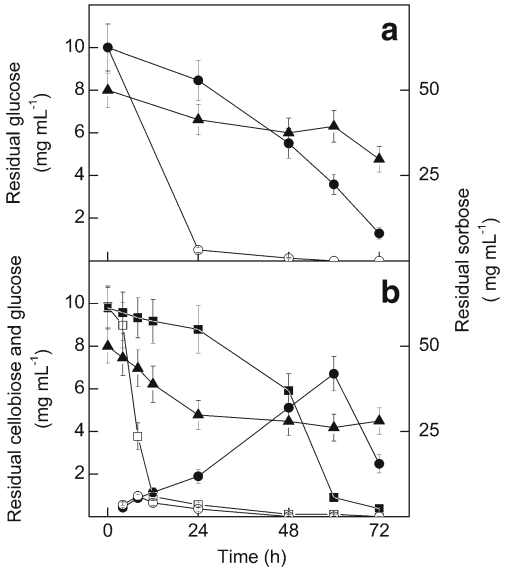

Fig. 6 Effect of sorbose on glucose (**a**) and cellobiose (**b**) mobilization by *H. grisea* var. *thermoidea* The fungus was cultivated at 45 °C and 145 rpm for different time intervals in medium supplemented with 1 % glucose (**a**) or 1 % cellobiose (**b**), alone (*open symbols*) or plus 5 % sorbose (*closed symbols*). Residual levels of glucose (*circles*), cellobiose (*squares*) and sorbose (*triangles*) were estimated in the culture filtrates. The values shown represent means from triplicate experiments ($n=3$)

fraction of *H. grisea* ß-glucosidase was partitioned in cytosolic and extracellular forms, with an insignificant cell wall-bound percentage. Sorbose increased about 3.3-fold the extracellular levels of both activities, but their intracellular and cell-wall bound levels remained practically unchanged. Moreover, the localization of the enzyme was unaffected by sorbose, in spite of the gross morphological changes induced by the ketohexose (see Fig. 2).

Effect of sorbose on glucose and cellobiose uptake by *H. grisea*

The residual concentrations of glucose, cellobiose, and sorbose were measured after different growth times of *H. grisea* in media containing 1 % glucose or 1 % cellobiose with or without 5 % sorbose (Fig. 6). Sorbose was slowly internalized/metabolized by the fungus: after 24 h, about 60 and 45 % of the initial concentration was still present in media containing glucose (Fig 6a) and cellobiose (Fig. 6b), respectively, decreasing to about 40 % after 72 h.

When *H. grisea* was grown in the absence of sorbose, glucose (Fig. 6a) and cellobiose (Fig. 6b) were practically fully consumed in 24 h. In contrast, about 90 % of the initial amount of both carbon sources remained in the medium after 24 h growth in the presence of sorbose, and about 60 and 72 h were necessary to nearly remove cellobiose and glucose, respectively, from the culture medium. Moreover, when the fungus was cultivated in the presence of cellobiose plus sorbose (Fig. 6b), the levels of glucose in the culture medium abruptly increased after 24 h, reaching maximum levels (about 6.7 mg mL^{-1}) after 60 h but decreasing to about 2.5 mg mL^{-1} at 72 h. In contrast, the residual levels of glucose were very low throughout the entire time interval in cultures containing only the disaccharide.

Table 2 Effect of sorbose on the distribution of glycosidase activities in subcellular fractions of *H. grisea* var. *thermoidea*[a]

Carbon source	ß-Galactosidase activity (total U)				ß-Glucosidase activity (total U)			
	Filtrate	Crude extract	Intracellular	Cell wall-bound	Filtrate	Crude extract	Intracellular	Cell wall-bound
1 % Glucose	389	93 (100)	75 (83)	6.5 (7)	115	198 (100)	165 (83)	19 (10)
1 % Cellobiose	998	2,167 (100)	1,777 (82)	195 (9)	415	1,092 (100)	897 (82)	120 (11)
1 % Cellobiose +5 % Sorbose	3,254	2,006 (100)	1,685 (84)	140 (7)	1420	1,029 (100)	885 (86)	94 (9)

[a] The fungus was cultivated for 60 h at 45 °C and 145 rpm in 25 mL of M5 medium supplemented with the indicated carbon sources. The results are expressed as means of at least three cultures ($n \geq 3$). The values in parenthesis represent percent activities relative to total activities.

Discussion

The effects of sorbose on the growth, morphology, and metabolism of filamentous fungi have been the subject of some studies, after the publication of the first investigation on this topic by Tatum et al. (1949). However, the biochemical mechanisms underlying the stimulation of the production and secretion of ß-glucosidases in fungal systems in response to L-sorbose are still virtually unknown.

The slow uptake of glucose and cellobiose by *H. grisea* may justify the negligible growth occurred up to 24 h and the increased lag growth period in media containing sorbose. Similarly, sorbose increased the lag growth period of *N. crassa* in glucose (Crocken and Tatum 1968) and *T. reesei* in cellobiose (Bisaria et al. 1986). In contrast to our results, however, the addition of sorbose to glucose-containing media lowered the specific growth rates and yields of *N. crassa* (Crocken and Tatum 1968; Trinci and Collinge 1973), *T. reesei* C5 (Sahoo et al. 1986) and *A. fumigatus* (El-Shafei 1997), but not *T. reesei* QM 9414 (Bisaria et al. 1986). The lower growth yields attained by *H. grisea* in media containing lactose may be tentatively attributed to the generation of galactose as hydrolysis product, since this fungus usually grows poorly in this monosaccharide.

The same pattern of morphological alterations seen for *H. grisea* in response to sorbose was reported for various other fungi. These alterations were attributed to modifications of the cell wall composition, mainly a reduction of the percentage of ß-1,3-glucan, but also diminished incorporation of glucose into other polymers (Barnett and Lilly 1951; Crocken and Tatum 1968; Bisaria et al. 1989; El-Shafei 1997). Supposedly, these chemical modifications physically weaken the cell wall, impairing the growth in the form of long filaments. However, this altered morphology could also result from changes in the reactions involved in the synthesis of the cell wall or in its relative rates (Crocken and Tatum 1968).

The expression of ß-glucosidases in different fungi is regulated at the transcriptional level by induction, and different inducers have been identified, including cellobiose and α- and β-lactose, but also D-xylose, D-galactose, β-gentibiose, and sophorose (Knowles et al. 1987; Kubicek 1992; Ilmén et al. 1997; Ding et al. 2007). Differently from *H. grisea* var. *thermoidea*, some fungi are able to utilize sorbose as sole carbon source, sometimes producing good levels of extracellular ß-glucosidases (Lilly and Barnett 1953; Sahoo et al. 1986; Cai et al. 1998). However, *T. reesei* QM9414 (Bisaria et al. 1986; Nanda et al. 1986) and *P. decumbens* (Wei et al. 2011) also grew poorly in sorbose.

The effects of sorbose on the production and secretion of the multifunctional ß-glucosidase by *H. grisea* were markedly dependent on the inducer sugar, since increased extracellular levels of the enzyme were obtained in the presence of cellobiose but lower intra- and extracellular levels were verified with lactose or glucose. Differently from our results, the production of extracellular ß-glucosidases by *T. reesei* C-5 in glucose was enhanced about 1.5-fold by the addition of sorbose (Sahoo et al. 1986). Dependency on inducer was also observed in *V. volvacea*, with strong suppression of the extracellular levels of an endoglucanase by sorbose in lactose-induced but not in cellobiose-induced cultures (Zhang et al. 2011).

The net increase of total ß-glucosidase and ß-galactosidase activities in response to the addition of sorbose to cellobiose-containing cultures of *H. grisea* strongly suggested a stimulation of the synthesis and not only the secretion of the multifunctional ß-glucosidase. In contrast to our results, the extracellular levels of ß-glucosidase in *T. reesei* cultures in cellobiose were increased about 4-fold by the addition of 5 % sorbose but the intracellular levels were lowered, suggesting a stimulation of the secretion of the enzyme only (Bisaria et al. 1986).

The stimulatory effect of sorbose on the synthesis and secretion of the multifunctional ß-glucosidase by *H. grisea* was dependent on the presence of a good inducer of the enzyme, since the production of other enzymes was unaffected. Thus, it may not be attributed to a nonspecific increase of the secretion of extracellular enzymes related to the higher number of hyphal tips associated with the morphological alterations, as proposed by others (Sahoo et al. 1986; Nanda et al. 1986; Bisaria et al. 1989). In contrast to our results, Sahoo et al. (1986) observed an increase of the extracellular levels of acid phosphatases, but not amylases, in response to the addition of sorbose to *T. reesei* cultures in glucose. However, an inducer-specific effect of sorbose was also observed in *T. reesei*, since the ketohexose greatly increased the extracellular levels of cellulases in media containing cellulose or cellobiose, but not starch (Nanda et al. 1986; Chen and Wayman 1993). Unlike *T. reesei*, *H. grisea* is a good producer of extracellular ß-glucosidases, and cellobiose is not a good inducer of the production of cellulases by this fungus, which justifies the lack of response of endoglucanase production to sorbose addition.

Contrasting with the mainly cytosolic and extracellular distribution of *H. grisea* enzyme, most ß-glucosidase is bound to the cell wall in *T. reesei* and *V. volvacea* (Kubicek 1981, 1983; Nanda et al. 1982; Bisaria et al. 1986; Cai et al. 1998; Ding et al. 2007). Moreover, the increase in the extracellular levels of the enzyme in cultures of these fungi in media containing sorbose is accompanied by a decrease in the cell wall-bound and intracellular fractions, with constant total activity (Mishra and Tatum 1972; Kubicek 1981, 1983; Sahoo et al. 1986; Nanda et al. 1986). Thus, the extracellular levels of the enzyme are apparently enhanced as a consequence of its release from the cell wall, attributed to the reduction in the wall ß-1,3-glucan content caused by sorbose, since most ß-glucosidase is normally tightly associated to cell

wall glucan (Nanda et al. 1982, 1986; Kubicek 1983; Bisaria et al. 1986, 1989; Messner et al. 1990). Evidently, this is not the case with *H. grisea*, and the enhancement of extracellular ß-glucosidase levels in response to sorbose must involve different molecular mechanisms.

Similar to that as observed for *H. grisea*, the presence of sorbose in the culture medium reduced the efficiency of utilization and the specific consumption and uptake rates of different sugars by some fungi (Crocken and Tatum 1968; Sternberg and Mandels 1980; Sahoo et al. 1986; Nanda et al. 1986). Moreover, slow metabolism and uptake of sorbose were also observed in *N. crassa* (Crocken and Tatum 1968) and *T. reesei* (Nanda et al. 1986).

The lowered rates of glucose uptake and cellobiose breakdown and/or uptake observed in *H. grisea* cultures in media supplemented with glucose or cellobiose in the presence of sorbose strongly suggest that the stimulation of the synthesis of the multifunctional ß-glucosidase could involve at least two mechanisms: prolonged presence of the inducer, cellobiose, and lower catabolite repression by glucose. Decreased rates of cellobiose breakdown and/or uptake in the presence of sorbose have also been considered determinant factors for the increase in cellulases production by *T. reesei* (Sternberg and Mandels 1979; Sahoo et al. 1986; Nanda et al. 1986; Bisaria et al. 1989).

Taken together, our results show that cellobiose is a good inducer of a multifunctional ß-glucosidase in *H. grisea*. The addition of sorbose to the culture medium containing cellobiose greatly enhanced the extracellular levels of the enzyme, while the cytosolic and cell-wall fractions remained constant, demonstrating that the ketohexose stimulated both the synthesis and the secretion of the enzyme. The enhancement of enzyme production was dependent on the presence of cellobiose as inducer, and consequently may not be attributed to a nonspecific increase in the secretion of extracellular enzymes due to the paramorphogenic alterations caused by sorbose. Moreover, the increased extracellular levels of the enzyme may not be ascribed to an increased release from the fungal cell-wall due to chemical alterations, since it is mostly intracellular, not cell-wall bound. However, sorbose greatly delays glucose uptake and cellobiose uptake and/or hydrolysis in *H. grisea* cultures, and the sustained presence of cellobiose associated to decreased catabolite repression by glucose may explain the stimulation of the multifunctional ß-glucosidase production.

The cell wall recognizably plays an important role in the control of the synthesis and secretion of exported proteins. This is well exemplified in the wall-less "slime" mutant of *N. crassa* that does not present glucan synthase activity (Leal-Morales and Ruiz-Herrera 1985), and is unable to control the synthesis and secretion of several enzymes, including invertases, alkaline proteases, aryl-ß-glucosidases, and cellobiases. Interestingly, alkaline proteases and invertases were produced constitutively by this mutant of *N. crassa*, but aryl-ß-glucosidases and cellobiases required induction by cellobiose (Pietro et al. 1989), as observed here for *H. grisea* grown in the presence of sorbose. This favors the idea that disorders in the periplasmic space have not affected the control of induction of ß-glucosidases, in either *N. crassa* or *H. grisea*.

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