A cytosolic trehalase from the thermophilic fungus *Humicola grisea* var. *thermoidea*

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A cytosolic trehalase was purified from the thermophilic fungus *Humicola grisea* var. *thermoidea*. The apparent molecular mass of the purified native enzyme was estimated to be 360 kDa by gel filtration chromatography. A single polypeptide band of approximate molecular mass 120 kDa was detected by SDS-PAGE, suggesting that the native enzyme was composed of three identical polypeptides. The carbohydrate content of the enzyme was estimated to be 12%. The pI of the enzyme, estimated by electrofocusing, was about 4.0. The purified trehalase was specific for trehalose and its activity was stimulated by manganese, calcium and cobalt and inhibited by aluminium chloride, copper sulfate, ADP and ATP. Sugars such as cellobiose, lactose, sucrose, fructose and maltose also showed some inhibitory effect which was abolished in the presence of calcium. The purified cytosolic trehalase exhibited an apparent K_m of 0.86 mM, a pH optimum of 5.5 and an optimum temperature of 60 °C. Treatment with calcium induced disaggregation of the enzyme into three components. Apparently, calcium-induced disaggregation was not a prerequisite for calcium-mediated activation of the enzyme. The disaggregated trehalase was more labile to temperature than the native enzyme. The properties of the two *H. grisea* trehalases: the cytosolic enzyme and the conidial one, were compared.

Keywords: *Humicola grisea* var. *thermoidea*, cytosolic trehalase, conidial trehalase

INTRODUCTION

Trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) is a non-reducing disaccharide which is found in a large variety of organisms including bacteria, yeasts, filamentous fungi, plants and insects (Elbein, 1974). Trehalose serves as an endogenous carbon reserve for a number of physiological functions (Panek, 1963; Panek & Souza, 1964; Hey-Ferguson *et al.*, 1973; Hirokasu & Shimoda, 1981; Thevelein, 1984) or as a protectant against several forms of environmental stress (Van Laere, 1989).

Trehalose is hydrolysed to glucose by the enzyme trehalase (α , α -trehalase glucohydrolase, EC 3.2.1.28). The biochemical properties of this enzyme, which has been isolated from different sources, are highly variable (Elbein, 1974). Fungal trehalases can be grouped into two distinct categories: the so-called non-regulatory trehalases which have an acid pH optimum and high heat stability, and the so-called regulatory trehalases which have a neutral pH optimum and low thermal stability (Thevelein, 1984). Some fungi such as *Saccharomyces cerevisiae* (Mittenbühler & Holzer, 1988; App & Holzer, 1989) and *Schizo-*

saccharomyces pombe (De Virgilio *et al.*, 1991) possess both types of trehalase.

Among thermophilic fungi the genus *Humicola* is reported to be a good source of trehalase (Prasad & Maheswari, 1978; Zimmerman *et al.*, 1990). An extracellular trehalase activity associated with *H. grisea* var. *thermoidea* conidia was characterized in a previous study from our laboratory (Zimmerman *et al.*, 1990). We considered it worthwhile to investigate the possibility that another trehalase exists in *H. grisea*, in addition to the enzyme which accumulates on the surface of conidia.

Here we report the purification and partial biochemical characterization of a cytosolic trehalase from *H. grisea* var. *thermoidea*. The properties of this cytosolic trehalase were compared with those of the conidial enzyme, in order to improve our knowledge about the trehalase system of this filamentous fungus.

METHODS

Organism and growth conditions. The *Humicola* strain was isolated from Brazilian soil and classified as *H. grisea* var. *thermoidea* (Peralta *et al.*, 1990). The fungus was maintained in

Fig. 1. DEAE-Trisacryl chromatography of cytosolic trehalase. The enzyme activity recovered from the Fractogel column was applied to a DEAE-Trisacryl column (15.0 × 1.0 cm) equilibrated with 50 mM sodium acetate buffer pH 5.5, and eluted with a gradient of the same buffer (50–500 mM) (---) at a flow rate of 20 ml h⁻¹. The arrows indicate the pooled fractions. ●, Trehalase activity; ○, A₂₈₀.

the laboratory on slants of solid oatmeal baby food (Peralta *et al.*, 1990). Conidia from 10-d-old cultures were inoculated into liquid medium of the following composition: 0.2% sodium nitrate, 0.1% potassium phosphate monobasic, 0.05% magnesium sulfate, 0.8% yeast extract (Difco), 0.1% potassium chloride, 1.0% mannitol. One millilitre of trace elements solution (0.1% FeSO₄ · 7H₂O and 0.30% ZnSO₄) was added to 100 ml of culture medium and the pH was adjusted to 6.0 with diluted NaOH. The cultures were incubated at 45 °C with agitation (140 oscillations min⁻¹).

Extraction and purification of cytosolic trehalase. The mycelia grown as described above were harvested by filtration, rinsed with distilled water, blotted on filter paper and stored at -15 °C until use. The mycelial mass (100 g) was ground in a mortar with acid-washed sand at 0 °C. Cell disruption was continued for an additional 30 min after addition of 10 ml cold 100 mM sodium acetate buffer, pH 5.5 (acetate buffer) (g mycelium)⁻¹. The slurry was centrifuged at 10000 *g* for 20 min and the supernatant (crude extract) was saved. Solid ammonium sulfate was added to the crude extract to achieve 75% saturation and the mixture was stirred for 30 min. The suspension was stored overnight in a cold room. Precipitated protein was collected by centrifugation at 25000 *g* for 30 min, redissolved in 40 ml acetate buffer and dialysed overnight against 5 litres of the same buffer. The dialysed solution was heated at 60 °C for 20 min and then transferred to an ice bath. Coagulated protein was spun down (3000 *g*) and the clear supernatant was lyophilized, dissolved in 5.0 ml acetate buffer and applied onto a Fractogel 65 (S) column (40 × 3 cm) equilibrated and eluted with the same buffer. Fractions with trehalase activity were pooled and applied to a DEAE-Trisacryl (M) column (15 × 1 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.5, and eluted with a linear gradient of the same buffer (50–500 mM) (Fig. 1). Fractions containing trehalase activity were pooled, dialysed against distilled water and lyophilized. After the DEAE-Trisacryl step, the enzyme preparation appeared homogeneous.

Enzyme assay. Cytosolic trehalase was routinely assayed in 50 mM sodium acetate buffer, pH 5.0, as described by Zimmermann *et al.* (1990) and the glucose released was measured as reducing sugar (Miller, 1959). When kinetic parameters, substrate specificity or inhibition by other carbohydrates were investigated, glucose was determined by the glucose oxidase assay (Bergmeyer & Bernt, 1974). Kinetic parameters (*K_m* and *V_{max}*) were analysed as described by Zimmermann *et al.* (1990). One unit of trehalase activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of trehalose min⁻¹, at 60 °C and pH 5.5.

Electrophoresis. PAGE was carried out at pH 8.9 in 6% (w/v) acrylamide gels, according to Davis (1964). SDS-PAGE was performed by the method of Laemmli (1970) using 6% acrylamide. Gels were stained with Coomassie blue. Molecular mass standards were: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase B (97.4 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Activity staining was performed by the method of Gabriel & Wang (1969), except that glucose oxidase (30 U per gel) was fixed in the gels. Isoelectric focusing using Pharmalyte pH 2.5–5.0 was carried out in rod gels according to O'Farrell *et al.* (1977). The 6% gels contained 5% (v/v) carrier ampholyte. Twenty five micrograms of purified trehalase were loaded on the gel (0.6 × 13.0 cm). After focusing at 500 V for 6 h, the pH gradient was measured by cutting one gel into 5 mm-thick slices and extracting each slice with 1.0 ml 25 mM KCl. Trehalase activity staining was performed as described by Gabriel & Wang (1969).

Fractogel column chromatography. The molecular mass of the purified cytosolic trehalase was estimated by Fractogel 55 (F) gel filtration. A sample of trehalase (0.8 ml) was loaded onto a Fractogel column (47.0 × 1.8 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. Molecular mass markers were: thyroglobulin (670 kDa); urease (480 kDa); β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). The column was eluted at 5 °C at a flow rate of 12 ml h⁻¹. The eluate was collected in 1.2 ml fractions.

Determination of neutral carbohydrate and protein. Total neutral carbohydrate was estimated by the phenol-sulfuric acid method of Dubois *et al.* (1956) using glucose as standard, and protein was estimated by the Lowry method using bovine serum albumin as standard.

Determination of the trehalase N-terminal amino acid. The purified trehalase was dansylated using the procedure described by Gray (1972). Dansylated protein (250 μg) was hydrolysed with 6 M HCl at 110 °C for 24 h. After removal of HCl, the hydrolysate was dissolved in 25 μl 25% (v/v) aqueous pyridine. Five microlitres of the solution were spotted on a 5 × 5 cm precoated polyamine sheet. Chromatography was carried out using 1.5% (v/v) formic acid in water, in the first direction, and benzene/acetic acid (9:1, v/v) in the second direction at 90° to the first one. After chromatography the dansylated amino acids were identified under ultraviolet light. Chromatography of a mixture of dansylated amino acid standards was carried out in a similar fashion.

Reagents. Trehalose, AMP, ADP, ATP, glucose oxidase, phenazine methosulfate, nitro blue tetrazolium, molecular mass markers for protein and dansylated amino acids were purchased from Sigma. Polyamine layer sheets were from Gallard-Schlesinger.

Table 1. Purification of cytosolic trehalase from *H. grisea* var. *thermoidea*

Step	Total volume (ml)	Total protein (mg)	Total units*	Specific activity [U (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
Crude extract	280	953	319	0.33	100	1.0
Ammonium sulfate (75 %) precipitate	35	316	158	0.50	49	1.5
Heat shock (60 °C)	35	107	150	1.40	47	4.2
Fractogel	66	21	103	4.90	32	14.8
DEAE-Trisacryl	25	0.3	32	106.66	10	323.2

*One unit of trehalase activity is the amount of enzyme that catalyses the hydrolysis of 1 µmol trehalose min⁻¹.

RESULTS

Purification and biochemical characterization of cytosolic trehalase activity

The enzyme was extracted from 17-h-old mycelia grown in mannitol as the carbon source; under these conditions the specific activity of the cytosolic trehalase was found to be the highest. Table 1 summarizes a typical purification of the enzyme. After DEAE-Trisacryl chromatography

Table 2. Effect of various cations on cytosolic trehalase activity

The results are expressed as means ± SD for at least three replicates.

Salt	Concn (mM)	Specific activity [U (mg protein) ⁻¹]	Activation (-fold)
Control		107 ± 11	—
CaCl ₂	5	310 ± 19	2.9
MgCl ₂	10	128 ± 7	1.2
CoCl ₂	1	187 ± 17	1.7
MnCl ₂	7.5	450 ± 36	4.2
NH ₄ Cl	1	107 ± 9	1.0
CuSO ₄	1	18 ± 3	0.2
ZnCl ₃	1	102 ± 12	0.9
Al ₂ Cl ₃	1	0	—
BaCl ₃	1	120 ± 12	1.1

Fig. 2. Isoelectric focusing of *H. grisea* cytosolic trehalase. (a) Gel stained with Coomassie blue; (b) gel stained for activity. A piece of wire (W) indicates the cathode side.

the specific activity of the cytosolic trehalase was about 107 U (mg protein)⁻¹ and a purification of 323-fold was achieved. Non-denaturing- and SDS-PAGE of the purified enzyme showed a single protein band after staining with Coomassie blue. Fractogel 55 (F) gel filtration indicated that the native protein had a molecular mass of approximately 360 kDa. However, on SDS-PAGE, the purified enzyme exhibited a single band of molecular mass approximately 120 kDa, suggesting that the enzyme was formed of three subunits of molecular mass approximately 120 kDa. The N-terminal amino acid of the cytosolic trehalase was found to be glycine, and the carbohydrate content of the protein was estimated as 12% using glucose as standard. The isoelectric point of the cytosolic trehalase was 4.0 (Fig. 2).

Catalytic properties

The effect of several cations on the activity of the cytosolic trehalase was investigated (Table 2). Activation was observed in the presence of manganese chloride (4.2-fold),

Table 3. Inhibition of *Humicola* trehalase by nucleoside phosphate

Nucleoside phosphate (10 mM)	CaCl ₂ (mM)	Specific activity [U (mg protein) ⁻¹]	Inhibition (%)
None	0	107 ± 9	—
AMP	0	102 ± 12	5
ADP	0	52 ± 7	51
ATP	0	35 ± 5	67
None	5	310 ± 15	—
AMP	5	282 ± 33	9
ADP	5	67 ± 10	78
ATP	5	47 ± 8	85

calcium chloride (2.9-fold) and cobalt chloride (1.7-fold). Ammonium chloride had no effect and zinc chloride, magnesium chloride and barium chloride had only slight effects on the enzyme activity. Conversely, copper sulfate and aluminium chloride inhibited the enzyme activity about 83 % and 100 %, respectively.

The effect of pH on the cytosolic trehalase activity was investigated using 50 mM sodium acetate (pH 3.0–6.0) and 50 mM Tris/HCl (pH 6.0–9.0) buffers. The enzyme showed maximal activity at pH 5.5. The optimum temperature for trehalose hydrolysis was 60 °C, in either the presence or the absence of 5.0 mM calcium chloride.

In a previous study (Zimmerman *et al.*, 1990) we demonstrated that the conidial trehalase purified from *H. grisea* is markedly inhibited by inorganic phosphate, ADP and ATP. To ascertain whether the nucleoside phosphates would similarly affect the cytosolic trehalase, the effects of AMP, ADP and ATP were tested (Table 3). At 10 mM ADP and ATP inhibited the enzyme activity by 51 % and 67 %, respectively. A half-maximal effect of ATP on the activity of trehalase was observed at 3.0 mM. The inhibitory effects of ADP and ATP were also

Table 5. Kinetic constants of cytosolic trehalase

Treatment	K _m (mM)	V _{max} [U (mg protein) ⁻¹]
Control	0.87 ± 0.08	392 ± 36
CaCl ₂ (5.0 mM)	0.89 ± 0.04	740 ± 27
MnCl ₂ (7.5 mM)	0.83 ± 0.06	821 ± 65
ATP (0.5 mM)	1.38 ± 0.23	96 ± 14
ATP (1.0 mM)	2.94 ± 0.62	89 ± 19

observed in the presence of calcium where they almost entirely abolished the calcium activating effect (Table 3). In contrast, AMP only slightly inhibited trehalase activity, in either the presence or absence of calcium.

The purified cytosolic trehalase was highly specific for trehalose as a substrate and did not hydrolyse cellobiose, lactose, sucrose, maltose or raffinose. However, its activity was inhibited by cellobiose, lactose, sucrose, fructose and maltose (Table 4). This inhibition was abolished in the presence of calcium, which also stimulated trehalase activity 3.0–3.5-fold (Table 4).

Kinetic studies

The K_m values of the cytosolic trehalase, in either the presence or absence of calcium, were essentially the same (Table 5). In contrast, calcium or manganese increased the V_{max} 1.9- and 2.1-fold, respectively. The K_m values obtained in the presence of ATP suggested that the inhibitory effect of the nucleotide involved both non-competitive and competitive mechanisms, since both K_m and V_{max} were affected.

Mechanism of trehalase activation by calcium

Aggregates of trehalase have been described previously for the enzyme purified from *Neurospora crassa* (Hecker & Sussman, 1973). To ascertain whether the effect of calcium

Table 4. The effect of various carbohydrates on cytosolic trehalase activity

Addition	Activity (U ml ⁻¹)	Activation (-fold)	Inhibition (%)
Control	79 ± 10	—	—
Cellobiose (10 mM)	36 ± 3	—	54
Lactose (10 mM)	25 ± 4	—	68
Sucrose (10 mM)	36 ± 5	—	54
Fructose (10 mM)	43 ± 7	—	45
Maltose (10 mM)	36 ± 4	—	54
CaCl ₂ (5 mM)	279 ± 14	3.5	—
CaCl ₂ (5 mM) + Fructose (10 mM)	279 ± 12	3.5	—
CaCl ₂ (5 mM) + Maltose (10 mM)	257 ± 10	3.2	—
CaCl ₂ (5 mM) + Cellobiose (10 mM)	261 ± 18	3.3	—
CaCl ₂ (5 mM) + Lactose (10 mM)	249 ± 10	3.1	—
CaCl ₂ (5 mM) + Sucrose (10 mM)	277 ± 13	3.5	—

Fig. 3. Effect of calcium on the aggregation state of trehalase. After treatment with 10 mM calcium chloride, the trehalase sample was submitted to 6% PAGE at pH 8.9. Three micrograms of trehalase per gel was applied. The gels were stained for trehalase activity. (a) Control. (b) Sample treated with calcium. Calcium was omitted in the composition of the gels and the running buffer. A piece of wire (W) indicates the cathode side.

Fig. 5. Gel electrophoresis of trehalase Form III recovered from the Sephacryl S-200 column (Fig. 4). Form III was dialysed and analysed by 6% PAGE before (a) and after (b) addition of 10 mM calcium chloride. Electrophoresis was run at pH 8.9 and the gels were stained for trehalase activity. A piece of wire (W) indicates the cathode side.

Fig. 4. Gel filtration in Sephacryl S-200 of calcium-treated purified trehalase. The column (47.0 × 1.5 cm) was equilibrated and eluted with 50 mM sodium acetate buffer pH 5.5, with (●) or without (○) 100 mM calcium chloride. Fractions (1.2 ml) were collected at a flow rate of 12 ml h⁻¹.

on trehalase activity was correlated with formation or dissociation of aggregates, the enzyme activity of calcium-treated samples was tested after electrophoresis in polyacrylamide gels. Fig. 3 shows that the sample treated with

calcium dissociated into three main polypeptide components with trehalase activity, suggesting that calcium induced disaggregation of the enzyme. This was also observed by gel filtration on Sephacryl S-200 (Fig. 4). The elution profile of a trehalase sample pretreated with 10 mM calcium chloride showed at least three peaks of activity, which were designated as Form I, Form II and Form III, following the order of outlet from the column. Form III, which might be the monomer, showed either one or three activity bands, respectively, when analysed by PAGE after dialysis or with calcium during electrophoresis (Fig. 5a, b), demonstrating that the effect of calcium was reversible.

Thermostability studies of the disaggregated trehalase forms

Fig. 6 shows the thermostability curves obtained at 70 °C with the trehalase activity peaks eluted from the Sephacryl S-200 column. Forms I and II exhibited practically the same behaviour, with half-lives of about 6 min. However, Form III was more labile than Forms I and II, exhibiting a half-life of about 2.5 min.

Fig. 6. Thermal denaturation at 70 °C of the disaggregated trehalase forms. Aqueous solutions of the trehalase forms were heated in a water bath at 70 °C. Samples were withdrawn at the indicated times, chilled and tested for residual trehalase activity. ●, Form I; △, Form II; ○, Form III; (▲) native enzyme not treated with calcium.

DISCUSSION

The thermophilic fungus *H. grisea* var. *thermoidea* produced two trehalases with high specificity for the substrate. One of these enzymes occurs at the outer proteinaceous layer of conidia, and it has been characterized previously (Zimmerman *et al.*, 1990). In the present study we described the properties of the second *H. grisea* trehalase, a cytosolic enzyme with a specific activity of 0.33 U (mg protein)⁻¹ in the crude extract, much lower than that of the conidial trehalase [9.1 U (mg protein)⁻¹]. Both *H. grisea* trehalases possess remarkable thermal stability, a property which facilitates their purification (see Methods).

The purified cytosolic trehalase exhibited an apparent molecular mass of about 360 kDa by gel filtration, but in SDS-PAGE the enzyme migrated as a single polypeptide with an approximate molecular mass of 120 kDa, suggesting that the native enzyme was formed of three seemingly identical subunits. Another example of an oligomeric trehalase is the *Neurospora crassa* enzyme (Hecker & Sussman, 1973). Further evidence to support the assumption that it consists of identical subunits was that glycine was the only N-terminal amino acid found, and that a single band was found after electrofocusing and staining of the gels either for activity or protein (Fig. 2). It should be mentioned that the N-terminal amino acid of the conidial enzyme was also found to be glycine (data not shown).

The molecular structures of the cytosolic and conidial trehalases apparently differ. The latter exhibits an apparent molecular mass of 580 kDa and separates on SDS-PAGE into three polypeptides of 105, 98 and 84 kDa, which suggest a hexameric structure (Zimmerman *et al.*, 1990). A further structural difference between the two *H. grisea* trehalases is in carbohydrate content: 12% for the

cytosolic enzyme and 56% for the conidial form (Zimmerman *et al.*, 1990).

Despite the differences in structure, the two trehalases had similar optima for temperature (60 °C) and pH (5.5 and 5.6 for the cytosolic and the conidial trehalases, respectively). Both enzymes were inhibited by inorganic phosphate, ADP and ATP and were activated by manganese, calcium and cobalt, the cytosolic trehalase being more active in the presence of manganese rather than calcium (Table 2). In contrast, the two *H. grisea* trehalases exhibited different sensitivity to several metal ions, for example copper and aluminium ions strongly inhibited the cytosolic enzyme (Table 2) but were without effect on the one from conidia (Zimmerman *et al.*, 1990). The cytosolic trehalase activity was inhibited by some carbohydrates (Table 4), which had no effect on the activity of the conidial enzyme (Zimmerman *et al.*, 1990). The K_m values of the two *H. grisea* trehalases also differed. That for the cytosolic trehalase (0.86 mM) was much lower than that of the conidial enzyme (2.5 mM; Zimmerman *et al.*, 1990).

Treatment with calcium induced disaggregation of the cytosolic trehalase, as clearly demonstrated by the separation of calcium-treated enzyme samples in SDS-PAGE (Fig. 3), or by molecular sieve chromatography (Fig. 4). Under the two conditions the calcium-treated enzyme dissociated into at least three components. The effect of calcium was fully reversible by dialysis (Fig. 5). A likely possibility was that the binding of calcium relaxed the interactions among protein monomers, thus shifting the equilibrium of the molecules toward the dissociated forms. Another important effect of calcium was to increase trehalase V_{max} about twofold (Table 5). Nevertheless, the K_m and V_{max} values of the three peaks separated by gel filtration (Fig. 4), assayed in the presence of calcium (not shown), were practically the same and very close to that of the native purified enzyme assayed in the presence of calcium (Table 5). This result suggested that disaggregation of the enzyme oligomers might not be a prerequisite for calcium-mediated activation.

The thermostability of the calcium-treated trehalase forms separated by gel filtration, indicated that the disaggregated protein was more labile to temperature than the native enzyme (Fig. 6). There are other examples in the literature of the influence of the aggregation state on the stability of enzyme molecules (Metzenberg, 1964; McFeters *et al.*, 1969; Saddoff, 1970). With the *H. grisea* trehalase this property might be of physiological relevance, as this fungus is well adapted to high temperature environments.

The extracellular and the cytosolic trehalases of *H. grisea* had the same N-terminal amino acid, and as the former is larger and has a higher carbohydrate content than the intracellular enzyme, we would suggest that both may share the same polypeptides, but that trehalase secretion and additional glycosylation of the protein result in different aggregated enzyme forms. Thus, the differences noted in K_m , inhibition by metal ions and sugars could be explained by the different degrees of glycosylation and molecular organization of the two enzymes. In this

respect, Buttner *et al.* (1991), studying the external and internal β -glucosidases and glucoamylase from the yeast *Arxula adenivorans*, suggested that glycosylation combined with dimerization may be responsible for the physical and chemical differences observed between the intracellular and extracellular forms of the enzymes.

The biological role of the cytosolic trehalase of *H. grisea* is not immediately obvious. A likely possibility is that the intracellular enzyme is a storage precursor of the extracellular one. Considering their acid pH optima and their elevated thermoresistance, the *H. grisea* trehalases should be grouped among the so-called non-regulatory trehalases (Thevelein, 1984). Nevertheless, both enzymes exhibited some *in vitro* properties (e.g. sensitivity to ions, sugars, ADP and ATP) which could represent potentially regulatory parameters for modulation of their activity *in vivo*. Further studies may clarify this interesting question.

To conclude, and taking into account our previous studies on *H. grisea* var. *thermoidea* enzymes (Zimmerman *et al.*, 1990; Peralta *et al.*, 1990; Monti *et al.*, 1991), we consider that this organism is a good source of potentially useful enzymes and represents a convenient model to investigate protein export and glycosylation mechanisms in filamentous fungi.

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REFERENCES

- App, H. & Holzer, H. (1989). Purification and characterization of neutral trehalase from yeast ABS1 mutant. *J Biol Chem* **264**, 17583–17588.
- Bergmeyer, H. U. & Bernt, E. (1974). In *Methods of Enzymatic Analysis*, vol. 3, pp. 1205–1215. Edited by H. U. Bergmeyer. New York: Verlag-Chemie/Academic Press.
- Büttner, R., Bode, R. & Birnbaum, D. (1991). Comparative study of external and internal β -glucosidases and glucoamylase of *Arxula adenivorans*. *J Basic Microbiol* **31**, 423–428.
- Davis, B. J. (1964). Disc electrophoresis. *Ann NY Acad Sci* **121**, 404–427.
- De Virgilio, C., Müller, J., Boller, T. & Wiemkem, A. (1991). A constitutive, heat shock-activated neutral trehalase occurs in *Schizosaccharomyces pombe* in addition to the sporulation-specific acid trehalase. *FEMS Microbiol Lett* **84**, 85–90.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- Elbein, A. D. (1974). The metabolism of α,α -trehalose. *Adv Carbohydr Chem Biochem* **30**, 227–256.
- Gabriel, O. & Wang, S.-F. (1969). Determination of enzymatic activities in polyacrylamide gels. I. Enzymes catalyzing the conversion of nonreducing substrates to reducing products. *Anal Biochem* **27**, 545–554.
- Gray, W. R. (1972). End-group analysis using dansyl chloride. *Methods Enzymol* **25**, 121–138.
- Hecker, L. I. & Sussman, A. S. (1973). Activity and heat stability of trehalase from the mycelium and ascospores of *Neurospora*. *J Bacteriol* **115**, 582–591.
- Hey-Ferguson, A., Mitchell, M. & Elbein, A. D. (1973). Trehalose metabolism in germinating spores of *Streptomyces hygroscopicus*. *J Bacteriol* **116**, 1084–1085.
- Hirokasu, I. & Shimoda, C. (1981). Changes in trehalose content and trehalase activity during spore germination in fission yeast, *Schizosaccharomyces pombe*. *Arch Microbiol* **129**, 19–20.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- McFeters, G. A., Sandine, W. E., Becker, R. R. & Elliken, P. R. (1969). Some factors affecting association–dissociation of β -galactosidase from *Streptococcus lactis* 792. *Can J Microbiol* **15**, 105–110.
- Metzenberg, R. L. (1964). Enzymically active subunits of *Neurospora* invertase. *Biochim Biophys Acta* **89**, 291–302.
- Miller, G. H. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–429.
- Mittenbühler, K. & Holzer, H. (1988). Purification and characterization of acid trehalase from *suc-2* mutant. *J Biol Chem* **263**, 8537–8543.
- Monti, R., Terenzi, H. F. & Jorge, J. A. (1991). Purification and properties of an extracellular xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. *Can J Microbiol* **37**, 675–681.
- O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133–1142.
- Panek, A. (1963). Function of trehalose in baker's yeast (*Saccharomyces cerevisiae*). *Arch Biochem Biophys* **100**, 422–425.
- Panek, A. D. & Souza, N. O. (1964). Purification and properties of baker's yeast trehalase. *J Biol Chem* **239**, 1671–1673.
- Peralta, R. M., Terenzi, H. F. & Jorge, J. A. (1990). β -D-Glycosidase activities of *Humicola grisea*: biochemical and kinetic characterization of a multifunctional enzyme. *Biochim Biophys Acta* **1033**, 243–249.
- Prasad, A. R. S. & Maheswari, R. (1978). Purification and properties of trehalase from the thermophilic fungus *Humicola lanuginosa*. *Biochim Biophys Acta* **525**, 162–170.
- Sadoff, H. L. (1970). Heat resistance of spore enzymes. *J Appl Bacteriol* **33**, 130–140.
- Thevelein, J. M. (1984). Regulation of trehalose mobilization in fungi. *Microbiol Rev* **48**, 42–59.
- Van Laere, A. (1989). Trehalose, reserve and/or stress metabolite? *FEMS Microbiol Rev* **63**, 201–210.
- Zimmerman, A. L. S., Terenzi, H. F. & Jorge, J. A. (1990). Purification and properties of an extracellular conidial trehalase from *Humicola grisea* var. *thermoidea*. *Biochim Biophys Acta* **1036**, 41–46.

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