Purification and characterization of the cell-wall-associated and extracellular α -glucosidases from *Saccharomycopsis fibuligera*

Vladimír REISER* and Juraj GAŠPERÍK

Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 842 51 Bratislava, Slovak Republic

Cell-wall-associated and extracellular α -glucosidases were purified to homogeneity from *Saccharomycopsis fibuligera* KZ growing on a medium containing cellobiose as the sole source of carbon; this substrate has the greatest inducing effect on the production of both forms of the enzyme. Depending on the source of carbon, 75–90 % of the enzyme is associated with cell wall, from which it can be completely released by 1 % Triton X-100 at 25 °C in 2 h. Both enzymes are glycoproteins in monomeric form with an apparent molecular mass of 132 kDa estimated by SDS/PAGE and 135 kDa estimated by gel filtration. N-linked carbohydrate accounts for 12 % of the total mass. Both forms exhibited optimum activity at pH 5.5 and seem to be stable in the pH range 4.0–8.0 on incubation at 4 °C for 24 h. The cell-wall-associated form had an optimum activity at 42.5 °C and was stable in the absence of substrate up to 30 °C, while the

extracellular form had optimal activity at 52.5 °C and was stable up to 40 °C. Both forms are unable to renature after thermal inactivation. The cell-wall-associated and extracellular α -glucosidases cleaved the same kind of substrates, from maltose to maltoheptaose, isomaltase and panose, although showing different rates of hydrolysis, and had little or no activity with polysaccharides. The extracellular form cross-reacts with antibody raised against the cell-wall-associated form, and both forms show the same peptide pattern after cleavage with chymotrypsin. The amino acid sequences of six peptides from both forms show marked similarity to those of *Schwanniomyces occidentalis* glucoamylase [Dohmen, Strasser, Dahlems and Hollenberg (1990) *Gene* **95**, 111–121] and some other related hydrolases.

INTRODUCTION

A number of enzymes are associated with the degradation of starch, which is an excellent and widely used raw material in many fermentation processes. α -Glucosidase is one of the enzymes that participates in the hydrolysis of this polysaccharide, mainly in the final step of degradation. α -Glucosidases (α -D-glucoside glucohydrolases; EC 3.2.1.20) hydrolyse terminal, non-reducing α -1,4-linked glucose residues in different substrates, releasing α -D-glucose. They degrade disaccharides and oligosaccharides quickly, relative to larger structures, and polysaccharides are attacked only slowly, if at all [1]. α -Glucosidase belongs to the important group of α -glycosidases and is widely distributed throughout the animal and plant kingdoms, where it is generally found in association with amylases. The enzyme also occurs in a considerable number of micro-organisms [1] and has potential applications in basic research and clinical biochemistry [2].

Several comparative studies on starch assimilation and amylase production by yeasts have been published [3–5]. Of special interest are the species exhibiting amylolytic potential that enable extensive starch hydrolysis. This is usually accomplished by the concerted action of extracellular α -amylase and glucoamylase [6], but could be enhanced by α -glucosidase. *Saccharomycopsis fibuligera* has been considered in the realm of ascomycetous yeast species as a good producer of extracellular amylolytic enzymes [4]. When cultivated on starchy materials it synthesizes and secretes α -amylase and glucoamylase into the medium [7]. In the course of α -amylase and glucoamylase isolation from the liquid medium of pilot-plant cultivations of *Sacch. fibuligera* KZ on the

starch waste product 'fine fibre', Gašperík and Hostinová [8] detected the presence of enzyme fractions having a higher affinity for maltose than for soluble starch. They characterized this enzyme as an α -glucosidase. Further, they found that both cell-associated and extracellular forms of α -glucosidase are produced by this yeast. Because of the fact that all three hydrolytic enzymes, i.e. α -amylase, glucoamylase and α -glucosidase, are synthesized by *Sacch. fibuligera* KZ strain, this model could be attractive for structure–function investigations of amylases and also for the study of the regulatory aspects of the induction of the synthesis of these enzymes and their synergism of action on different substrates. In this paper we report on the production, purification, characterization and comparison of the cell-wall-associated and extracellular forms of α -glucosidase from *Saccharomycopsis fibuligera* KZ.

MATERIALS AND METHODS

Materials

β -Mercaptoethanol, Triton X-100, Tween 20, SDS, from maltose to maltoseheptaose, isomaltose, sucrose, trehalose, methyl α -D-glucoside, 4-nitrophenyl α -D-glucoside, α -D-melezitose and pullulan were obtained from Serva (Heidelberg, Germany). Glucose and soluble starch were purchased from Lachema (Brno, Czech Republic); dextrin (food component with average size of 15 maltose units) from Amylon (Červená Řečice, Czech Republic); and cellobiose from The Research Centre for Chemistry (Institute of Chemistry, Bratislava, Slovak Republic). Panose, turanose and β -glucuronidase were from Sigma (St. Louis, MO,

U.S.A.); amylose from Koch-Light (Colnbrook, U.K.); amylopectin and chymotrypsin from Calbiochem (Los Angeles, CA, U.S.A.); and proteinase K from Boehringer (Mannheim, Germany). All other reagents were of the highest purity available. Hybond-C nitrocellulose membranes were from Amersham International.

Organism and growth conditions

Saccharomycopsis fibuligera KZ was originally supplied by Professor Klaushofer, Institute of Food Technology, Vienna, Austria. The inoculum was grown in a medium containing 2% (w/v) peptone, 1% (w/v) yeast extract and 3% (v/v) glycerol overnight at 28 °C on a rotary shaker at 140 rev./min (excentricity 3 cm).

Effect of different carbon sources on α -glucosidase production

A 1 ml sample of the inoculum prepared as described above was centrifuged for 5 min at 5000 *g*, washed with sterile distilled water and used to inoculate 100 ml of medium containing 2% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) of the appropriate sugar as the sole source of carbon, and cultivated under the same conditions as the inoculum. At 6 h intervals 1 ml of the suspension was collected and used to assay enzyme activity and to measure pH and cell mass. Cells from the suspension (0.5 ml) were harvested by filtration across a glass-fibre GF 83 filter (Whatman) and washed with distilled water. The cell mass was determined gravimetrically after drying filters to constant weight at 105 °C. The remaining 0.5 ml of the suspension was centrifuged for 5 min at 5000 *g* and the supernatant was used to measure the pH and extracellular α -glucosidase activity. The pellet was washed three times with distilled water and used to determine cell-wall-associated α -glucosidase activity.

Solubilization of cell-wall-associated α -glucosidase

A 5 ml sample of the cell suspension obtained after cultivation on cellobiose (see below) was centrifuged for 5 min at 5000 *g* and washed three times with sterile distilled water. The pellet was resuspended in the original volume of 0.1 M sodium acetate, pH 5.0, with the appropriate solubilization agent and incubated at 25 °C with stirring (150 rev./min). At 15 min intervals samples of 50 μ l were withdrawn, centrifuged for 3 min at 5000 *g* and used for the determination of α -glucosidase activity and protein concentration.

Enzyme purification

The inoculum of *Sacch. fibuligera* KZ was prepared as described above and used to inoculate 10 $\times$ 100 ml of medium containing 2% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) cellobiose. A 1 litre portion of cell suspension obtained after cultivation (60 h) at 28 °C on a rotary shaker (140 rev./min; excentricity 3 cm) was centrifuged for 30 min at 3000 *g*, and the supernatant was filtered across a 0.4 μ m filter (Sartorius, Göttingen, Germany) and used to isolate extracellular α -glucosidase. Cell-wall-associated α -glucosidase was isolated from the pellet after washing three times with distilled water and resuspending in a final volume of 500 ml of distilled water. Unless otherwise noted, all further procedures were carried out at 4 °C.

Cell-wall-associated α -glucosidase

Cell suspension (500 ml) was mixed with an equal volume of 2% (v/v) Triton X-100 in 0.2 M sodium acetate buffer, pH 5.0, and

incubated for 2 h at 25 °C with rigorous stirring. Cells were harvested by centrifugation for 20 min at 3000 *g*; the supernatant was filtered through a 0.4 μ m filter (Sartorius), concentrated and simultaneously equilibrated with 50 mM potassium phosphate buffer, pH 7.5 (basic buffer), by ultrafiltration using a 30 kDa cut-off PM-30 membrane (Amicon) to a final volume of 300 ml and applied to a column (2 cm $\times$ 17 cm) of Q Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with basic buffer. After removal of unbound material, the column was eluted with 200 ml of a linear gradient of 20–40 mM NaCl in the buffer used for equilibration. Fractions of 6 ml were collected and assayed for α -glucosidase activity. Fractions with enzyme activity were pooled and applied to an Ultrogel AcA 34 (LKB) column (5 cm $\times$ 46 cm) equilibrated with the basic buffer. The fractions with α -glucosidase activity were pooled and applied to an FPLC Mono Q HR 10/10 column (Pharmacia) equilibrated with the basic buffer. The active fractions (1 ml) from the final purification step were used for characterization of the cell-wall-associated α -glucosidase.

Extracellular α -glucosidase

Supernatant (800 ml) after removal of the cells was concentrated and equilibrated with the basic buffer under the same conditions as mentioned above to a final volume of 400 ml. The first (Q Sepharose Fast Flow column) and second (Ultrogel AcA 34 column) purification steps were performed under the same conditions as described for cell-wall-associated α -glucosidase. The active fractions from the final purification step were used for characterization of extracellular α -glucosidase.

Localization of α -glucosidase

For localization of α -glucosidase bound on the cell surface, the cell suspension for α -glucosidase isolation (see above) was used. The pellet after the third wash with sterile distilled water was resuspended in 2 ml of a solution containing 0.1 M sodium acetate, pH 5.0, 1 M sorbitol and 10 mg of β -glucuronidase, and incubated at 28 °C. At 30 min intervals, 50 μ l of the suspension was withdrawn and centrifuged for 5 min at 5000 *g*, and the supernatant was used for determination of the activity of the released α -glucosidase. Protoplasts for intracellular α -glucosidase determination were prepared according to the procedure for yeast transformation [9] and lysed in water containing 0.1% (w/v) SDS.

α -Glucosidase assay

The enzyme assay was carried out by incubating 5 μ l of the enzyme with 5 μ l of 2% (w/v) maltose in 0.2 M sodium acetate buffer, pH 5.0, at 40 °C for 30 min. Reaction was stopped by adding 15 μ l of ice-cold 1.66 M Tris buffer, pH 7.6. Released glucose was quantified using the Glucose dehydrogenase Assay Kit (Merck, Darmstadt, Germany). One α -glucosidase unit was defined as the amount of enzyme which cleaves 1 μ mol of maltose per min under standard conditions mentioned above.

Protein concentration

Protein concentration was estimated spectrophotometrically according to Kalb and Bernlohr [10].

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [11] using 7.5% gels in the Mighty Small II apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Proteins were detected

by staining the gels with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) [12] or by the silver-staining method of Blum et al. [13].

Deglycosylation

The N-glycosidic linkage bound carbohydrate moiety of purified α -glucosidases was determined by treatment with Endo H (endo- β -N-acetylglucosaminidase H of *Streptomyces plitacus*; Boehringer, Mannheim, Germany) as described by Trimble and Maley [14]. A 3 μ g sample of the enzyme in 10 μ l of 100 mM sodium acetate buffer, pH 5.5, in the presence of 0.02% (w/v) SDS was boiled for 3 min. After cooling, 1 munit of Endo H was added and incubated overnight at 37 °C.

Molecular mass determination

The molecular mass of the native purified α -glucosidase was determined by gel filtration on Superose 6 HR 10/30 (Pharmacia) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 0.15 M NaCl. The molecular mass of denatured protein was estimated by SDS/PAGE on a 7.5%-polyacrylamide gel. Molecular mass standards for chromatography and SDS/PAGE were obtained from Boehringer.

Sequence determination

The proteins were cleaved with CNBr/70% formic acid at 25 °C for 24 h, or with trypsin (Boehringer) for 16 h at 25 °C in 0.3 M sodium phosphate buffer, pH 8.0, in 4 M deionized urea. The digest was lyophilized and fractionated on a reversed-phase column (ODP50; Asahipak; 0.46 cm $\times$ 15 cm) by using a gradient of acetonitrile in 50 mM ammonium bicarbonate at a flow rate of 1 ml/min. Peptides were sequenced on an Applied Biosystems A470 gas-phase sequencer.

Computer analysis

The amino acid sequences of the peptide fragments were aligned by using the results of the BLAST Network Service searches for protein similarity [15].

Peptide mapping

Samples containing 0.5 μ g of α -glucosidase were treated with 0.03 μ g of chymotrypsin for 30 min at room temperature in the stacking gel as described by Cleveland et al. [16]. The peptides were separated by SDS/PAGE on a 15%-polyacrylamide gel.

Antibody preparation and Western blotting

Samples containing 100 μ g of purified protein were injected into rabbits every 28 days. After the fourth injection the rabbits were bled out and the serum was checked for the ability to cross-react with homologous antigen. Western blotting was done according to Gershoni and Palade [17] and the immune complexes were detected by alkaline phosphatase reaction (Amersham). Low-range prestained molecular mass standards were obtained from Bio-Rad (Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

Production of α -glucosidase

We conducted studies to determine the effect of various carbon sources on the production of α -glucosidase by *Saccharomycopsis*

Table 1 Maximal α -glucosidase production in *Sacch. fibuligera* KZ on different carbon sources

Each medium contained 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) of the appropriate sugar. Cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml of medium at 28 °C on a rotary shaker (140 rev./min; eccentricity 3 cm).

Source of carbon (2%, w/v)	Maximum α -glucosidase production (units/mg dry wt.)		Time* (h)
	Cell-wall-associated	Extracellular	
Glucose	21.1	3.6	18
Maltose	22.3	4.3	18
Sucrose	22.3	3.9	18
Cellobiose	88.2	22.2	60
Dextrin	22.5	5.5	36
Starch	38.2	7.8	36

* Time of cultivation when maximum production was observed.

fibuligera KZ. Various simple and complex carbohydrates were used. Table 1 shows maximum specific activity (expressed relative to the dry cell mass) and the time when this was observed following growth on the various media. The levels of both the cell-surface-bound and extracellular forms of α -glucosidase were similar in media containing glucose, maltose and sucrose as the sole source of carbon. In the presence of starch, which is used predominantly as an inducer of synthesis of amylolytic enzymes, we observed approximately 2-fold higher specific activity. Interestingly, we found the most intense induction effect in the medium containing cellobiose (4.5 times higher) in comparison with the medium containing maltose, which we expect to be a natural inducer of α -glucosidase synthesis. Kelly et al. [22] found that among various mono- and di-saccharides tested, cellobiose had the greatest effect on the production of α -glucosidase from *Lipomyces starkeyi*. Expressing the activity relative to the cell mass enables us to demonstrate that this increase is not caused simply by the higher cell density but by an elevated production of the enzyme. The time point at which maximum production was observed is almost identical to the beginning of the stationary phase of cultivation for all carbon sources used (results not shown). Cells enter the stationary phase when cultivated on cellobiose after a much longer time (approx. 60 h) than when cultivated on other sources of carbon. Presumably this also contributes to the higher specific activity, because of the longer time during which cellobiose can act as an inducer.

Distribution of α -glucosidase

Data indicate that most α -glucosidase (75–90%, depending on the source of carbon; results not shown) is located on the cell surface, although the activity of the extracellular α -glucosidase is contaminated with glucoamylase, which is also secreted into the medium. The contribution from glucoamylase is approximately one-tenth of the final activity recorded for extracellular α -glucosidase (J. Gašperik, unpublished work). To determine more precisely the location of the enzyme on the cell surface we incubated a washed suspension of the cells with a solution containing β -glucuronidase and measured in a time-dependent manner the activity of the enzyme released into the supernatant after harvesting the cells by centrifugation. After 4 h at 37 °C, when only about 2% protoplasts were formed, we detected in the supernatant about 99% of the original cell-surface-bound en-

Figure 1 Effect of temperature on solubilization of the cell-wall-associated α -glucosidase from *Sacch. fibuligera* KZ by Triton X-100

The conditions of the experiment were as described in the Materials and methods section. Temperatures used for solubilization are indicated.

zyme activity (results not shown). These results indicate that α -glucosidase is associated with the surface of the cell wall rather than with the periplasmic space or the cellular membrane, so that the designation 'cell-wall-associated α -glucosidase' would be more accurate. This is similar to other yeast α -glucosidases, since those with an activity on malto-oligosaccharides are usually associated with the cell wall [6]. Measurement of enzyme activity after lysis of the protoplasts shows that there is no significant quantity of α -glucosidase located intracellularly (1.5 %).

Solubilization of cell-wall-associated α -glucosidase

In order to isolate cell-wall-associated α -glucosidase, we tested the effects of different agents on its solubilization. Enzyme activity was measured as a criterion of effectiveness, and specific activity was determined as a criterion of selectivity. The maximal relative values of α -glucosidase activity released from the cell wall by SDS (1 %, w/v), Triton X-100 (1 %, v/v), Tween 20 (1 %, v/v), proteinase K (100 μ g/ml), β -mercaptoethanol (0.05 M) and KCl (0.5 M) were 16.4, 98.0, 88.0, 37.5, 75.8 and 63.2 % respectively. Among the agents used, Triton X-100 was also the most selective (results not shown). The release of the enzyme by Triton X-100 depended on solubilization temperature (Figure 1).

The degree of solubilization was proportional to the temperature; maximal enzyme release (measured as the enzyme activity) was most rapidly achieved at 37 °C, but subsequently a considerable decrease, probably due to denaturation of the enzyme under these conditions, was observed.

Purification

Cell-wall-associated α -glucosidase

A four-step purification protocol was required to purify cell-wall-associated α -glucosidase. The enzyme was released from the cell wall of the washed cell suspension obtained after cultivation of *Sacch. fibuligera* KZ in a medium containing cellobiose as the sole source of carbon (see the Materials and methods section). The concentration step was designed for ease of handling and to utilize a partial purification effect of ultrafiltration. The first purification step on a Q Sepharose Fast Flow column was the most effective, as a substantial increase in specific activity was observed. The next purification step was gel filtration on an Ultrogel AcA 34 column. Finally, a homogeneous preparation was obtained by rechromatography on a Mono Q HR 10/10 column.

Extracellular α -glucosidase

Extracellular α -glucosidase was isolated from the supernatant after cultivation of *Sacch. fibuligera* KZ in a medium containing cellobiose as the sole source of carbon (see the Materials and methods section). Ultrafiltration was followed by ion-exchange chromatography on a Q Sepharose Fast Flow column and gel filtration on Ultrogel AcA 34. Most impurities (mainly pigments) were removed by the final purification step. Table 2 summarizes the relevant purification data.

Molecular mass of α -glucosidase

The molecular mass of the native purified α -glucosidase was determined by gel filtration on a Sepharose 6 HR 10/30 (FPLC system) column. For both forms of α -glucosidase an apparent molecular mass of 135 kDa was calculated from a calibration curve obtained by gel filtration of standard proteins. To reveal the subunit composition, the molecular mass was also estimated under denaturing conditions by SDS/PAGE. Coomassie Blue staining revealed one band having a molecular mass of about 132 kDa (Figure 2). This value is in good agreement with the molecular mass determined by gel-filtration chromatography and suggests that the native enzyme is a monomer.

Table 2 Purification of α -glucosidases from *Sacch. fibuligera* KZ

Experimental details are described in the Materials and methods section.

Purification step	Cell-wall-associated α -glucosidase			Extracellular α -glucosidase		
	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)
Crude enzyme	14320	354	40	3810	3620	1
Ultrafiltration	12810	298	43	3540	1880	2
Q Sepharose Fast Flow	6810	72	95	2230	153	15
Ultrogel AcA 34	5330	48	111	1050	6	175
Mono Q HR 10/10	3710	23	161	—	—	—

Figure 2 Molecular mass determination of glycosylated and deglycosylated α -glucosidases from *Sacch. fibuligera* KZ

Samples of purified cell-wall-associated (lanes 2 and 3) and extracellular (lanes 4 and 5) α -glucosidases were separated by SDS/PAGE on 7.5%-polyacrylamide gels before (lanes 2 and 4) and after (lanes 3 and 5) treatment with Endo H. Molecular mass standards were loaded in lane 1. Experimental conditions were as described in the text; the gel was stained with Coomassie Brilliant Blue.

Carbohydrate content

Both forms of α -glucosidase are glycoprotein in nature, as they are sensitive to Endo H. After treatment under denaturing conditions, both the extracellular and cell-wall-associated α -glucosidase forms moved as a single band of identical molecular mass (116 kDa), indicating that their carbohydrate moieties bound through N-glycosidic linkages form 12% of the total mass of the protein (Figure 2).

Physicochemical properties

Figure 3(a) shows pH profiles of the α -glucosidase activities. Both enzymes reached maximum activity at pH 5.5, although some differences between them could be observed. The extracellular form of the enzyme appeared to be more active at low pH values. The effect of pH on inactivation of both forms of enzyme was also measured. The data obtained suggest that both are similarly stable, with 80% of the maximal activity retained between pH 4.0 and 8.0 after 24 h of incubation at 4 °C (results not shown).

Figure 3(b) presents the effect of temperature on the activity of α -glucosidase. The temperature optima of the enzyme differed considerably. The cell-wall-associated form was most active at 42.5 °C, while the extracellular form reached maximal activity at 52.5 °C. Thermal inactivation in the presence of the substrate was also more pronounced for the extracellular form, and was similar to that observed for the cell-wall-associated form only between 60 and 70 °C.

The profiles of thermal inactivation and subsequent renaturation of the two enzymes in the absence of substrate also differed. Cell-wall-associated α -glucosidase was stable up to 30 °C; at higher temperatures the activity declined very sharply. The extracellular form seems to be more stable, as it was inactivated at a temperature higher than 40 °C (Figure 4). Aliquots of enzyme solution after thermal inactivation were renatured at 25 °C for 24 h. Both forms were similar in their inability to renature (results not shown), in contrast to glucoamylase from the same strain which displayed extensive renaturation properties [18].

Substrate specificity

As can be seen from Table 3, both forms of the enzyme displayed highest activity towards maltose and had no significant activity on most polysaccharides tested. The α -glucosidases displayed the

Figure 3 Effects of pH (a) and temperature (b) on the activity of α -glucosidase from *Sacch. fibuligera* KZ

Enzyme activities of cell-wall-associated (○) and extracellular (●) α -glucosidases are expressed as percentages of the maximum value observed. (a) A solution containing 0.01 unit/ml enzyme was incubated at various pH values for 30 min at 40 °C. Buffers used [29]: 0.05 M KCl/HCl buffer (Clark and Lubs solution), pH 1.0–2.2; 0.05 M potassium hydrogen phthalate/HCl buffer (Clark and Lubs solution), pH 2.2–4.0; 0.1 M citric acid/0.02 M Na_2HPO_4 buffer (McIlvaine solution), pH 2.6–7.6; 0.02 M Na_2HPO_4 /0.02 M NaH_2PO_4 buffer, pH 6.5–8.0; 0.04 M sodium 5:5-diethylbarbiturate/HCl buffer (Britton and Robinson solution), pH 7.5–10.0. (b) Enzyme mixture containing 0.01 unit/ml enzyme, 0.1 M sodium acetate buffer, pH 5.5, and 1% (w/v) maltose was incubated at various temperatures for 30 min.

ability to hydrolyse $\alpha(1\text{--}4)$ and $\alpha(1\text{--}6)$ sugar linkages, but showed low or no activity on $\alpha(1\text{--}2)$ and $\alpha(1\text{--}3)$ sugar linkages, or on aryl and alkyl α -D-glucosides. Because there is little information on the secretion from yeasts and/or yeast-like fungi of α -glucosidases that are active on higher malto-oligosaccharides [19,21] and starch [22], a remarkable feature of both forms was their ability to hydrolyse lower malto-oligosaccharides. Both α -glucosidases were similar in their ability to digest the same kind of substrates, but considerable differences between them could be observed in the rates of hydrolysis.

Comparison of the two forms of α -glucosidase

After treatment with Endo H, the cell-wall-associated and extracellular α -glucosidases gave rise to bands with the same

Figure 4 Effect of temperature on the stability of α -glucosidase from *Sacch. fibuligera* KZ

Cell-wall-associated (○) and extracellular (●) α -glucosidase activities are expressed as percentages of the maximum activity determined. Enzyme activities were assayed under standard conditions (see the Materials and methods section). The residual enzyme activity was measured after incubation of the enzyme mixture containing 0.01 unit/ml enzyme in distilled water for 10 min at various temperatures.

molecular mass. In order to establish the extent of sequence similarity between them, we carried out a comparative analysis of the two proteins.

Figure 5 Peptide mapping of glycosylated and deglycosylated α -glucosidases from *Sacch. fibuligera* KZ

Samples of purified cell-wall-associated glycosylated (lane 2) and deglycosylated (lane 4), and extracellular glycosylated (lane 3) and deglycosylated (lane 5), α -glucosidases after cleavage with chymotrypsin were subjected to separation by SDS/PAGE on a 15%-polyacrylamide gel. Molecular mass standards were loaded in lane 1. Experimental conditions are described in the text; the gel was silver-stained.

In order to investigate whether the two proteins share common epitopes in the molecule, a polyclonal antibody was raised against the cell-wall-associated enzyme. The serum was tested immunochemically on Western blots. The same bands were seen for both forms of the enzyme, indicating that the antibody raised against cell-wall-associated α -glucosidase cross-reacts with extracellular α -glucosidase (results not shown). Subsequently, peptide mapping of glycosylated and deglycosylated forms of the two proteins was carried out using chymotrypsin. As shown in Figure 5, the patterns of peptides generated were almost identical. Some

Table 3 Substrate specificity of the cell-wall-associated and extracellular α -glucosidases from *Sacch. fibuligera* KZ

Substrates were present at 2% (w/v) in 0.1 M sodium acetate, pH 5.5. Relative activities are expressed as percentages (%) relative to the enzyme activity displayed with 2% (w/v) maltose as substrate under standard conditions

Substrate	Relative activity (%)		Sugar linkage type	No. of sugar units
	Extracellular	Cell-wall-associated		
Maltose	100.0	100.0	$\alpha(1-4)$	2
Maltotriose	31.4	45.3	$\alpha(1-4)$	3
Maltotetraose	20.3	40.9	$\alpha(1-4)$	4
Maltopentaose	25.3	39.7	$\alpha(1-4)$	5
Maltohexaose	32.2	36.2	$\alpha(1-4)$	6
Maltoheptaose	28.4	27.0	$\alpha(1-4)$	7
Isomaltose	32.7	21.7	$\alpha(1-6)$	2
Panose	19.0	12.4	$\alpha(1-4), \alpha(1-6)$	3
Turanose	1.0	1.0	$\alpha(1-3)$	2
Melezitose	0	0	$\alpha(1-2), \alpha(1-4)$	3
Methyl α -D-glucoside	0	0	Alkyl α -D-glucoside	1
Phenyl α -D-glucoside	0	0	Aryl α -D-glucoside	1
Trehalose	0	0	Glucopuranoside	2
Sucrose	0	0	Fructosyl α -D-glucoside	2
Starch	0.8	0	$\alpha(1-2), \alpha(1-4), \alpha(1-6)$	Polymer
Amylose	0	0	$\alpha(1-4)$	Polymer
Amylopectin	1.6	0.4	$\alpha(1-6)$	Polymer
Pullulan	0	0	$\alpha(1-6)$	Polymer

Figure 6 Comparison of amino acid sequences of peptide fragments from *Sacch. fibuligera* KZ cell-wall-associated and extracellular α -glucosidases with that of *Schwan. occidentalis* glucoamylase

A *Sacch. fibuligera* KZ α -glucosidase fragment similar to *Schwan. occidentalis* glucoamylase amino acid sequence 357–369 was obtained after cleavage with CNBr. All other fragments were generated after hydrolysis with trypsin. The dashed line represents the *Schwan. occidentalis* (S.o.g.) glucoamylase sequence. The approximate positions of cell-wall-associated (■) and extracellular (□) *Sacch. fibuligera* (S.f.a.) α -glucosidase peptide fragments similar to this sequence are shown. Amino acids identical in the sequence are indicated by ★; conservative amino acids are indicated by —.

S.f.a.	GAISQEPYVWESVAEA	VGDALVVTVPVLEQGVDTVKG	GLIVAXDN
C.t.a.	804 GAIAQEPFRWDSVANA 819 *** _ _ _ _ _	872 FGDDLLVSPVLEPNVTQIKA 891 _ _ _ _ _	955 GLIVNLND 962 ****
A.n.a.	456 STIPQEPYRWASVIEA 471 * _ _ _ _	523 VGPALMVVPLEPLVNTVKG 542 ** _ _ _ _ _	
H.i.		736 WGPALLITPVLKQGADTVSA 755 _ _ _ _ _	815 GLIVALGE 822 *****
H.s.		699 WGPALMVVTPVLEPYVQTVNA 718 _ _ _ _ _	778 KLIVAADD 885 **** *
Rb.i.		736 WGPALLITPVLQGAETVSA 755 _ _ _ _ _	815 GLIIALND 822 *** _
Rb.s.		699 WGPALMVVTPVLEPYTVVRG 718 _ _ _ _ _	778 KLIVAADD 885 **** *
R.s.i.		287 WGPALLITPVLKPGVENVSA 303 _ _ _ _ _	359 GLIVALDD 372 *****
H.l.a.		746 WGEALLITPVLQAGKAEVTG 765 _ _ _ _ _	

Figure 7 Sequence similarity of the peptide fragments from *Sacch. fibuligera* KZ α -glucosidases to other related hydrolases

S.f.a., *Saccharomycopsis fibuligera* α -glucosidase; C.t.a., *Candida tsukubaensis* α -glucosidase; A.n.a., *Aspergillus niger* α -glucosidase; H.i., human isomaltase; H.s., human sucrase; Rb.i., rabbit isomaltase; Rb.s., rabbit sucrase; R.s.i., rat sucrose-isomaltase; H.l.a., human lysosomal α -glucosidase. Amino acids identical in the sequence are indicated by ★; conservative amino acids are indicated by —.

differences in the intensity and mobility of certain fragments could be observed, particularly in the case of the deglycosylated forms (lanes 4 and 5). These data suggest the possibility of some structural differences between the two forms. The question that arises is whether such similar proteins have such different enzymic properties, particularly thermal stability in the presence and absence of the substrate and substrate specificity, due to differences in co-/post-translational modification or due to the presence of isoenzymes in the cell.

We also determined the sequences of the peptide fragments from the two α -glucosidase forms, but were not able to determine the N-terminal amino acid sequences because they were blocked. The results of a global sequence search, which revealed masked sequence identities between the *Schwanniomyces occidentalis* glucoamylase [23] and both cell-wall-associated and extracellular α -glucosidases from *Sacch. fibuligera* KZ (Figure 6), enables us to suppose considerable similarity between the two forms of α -glucosidase at the amino acid level. Because the *Schwan.*

occidentalis glucoamylase is not similar to other glucoamylases which have been sequenced thus far, this model would be excellent for investigation of differences in the mode of action of α -glucosidase and glucoamylases on different substrates. The sequences of the three peptide fragments are similar to those of microbial α -glucosidases from *Candida tsukubaensis* [20] and *Aspergillus niger* [24] and also to those of other related hydrolases, e.g. sucrase-isomaltases from human [25], rabbit [26] and rat [27], and human lysosomal α -glucosidase [28] (Figure 7).

We thank Ralf Bischoff, Division for Molecular Biology of Mitosis, German Cancer Research Center, Heidelberg, Germany, and Tony Houthave, EMBL, Heidelberg, Germany, for protein sequencing. This work was supported by a research grant (2/999 134) from the Grant Agency of the Slovak Academy of Sciences.

REFERENCES

- Kelly, C. T. and Fogarty, W. M. (1983) *Process. Biochem.* **18**, 6–12
- Haeghele, E. O., Schaich, E., Rauscher, E., Lehmann, P., Buerk, H. and Wahlefeld, A. W. (1982) *Clin. Chem.* **28**, 2201–2205

- 3 De Mot, R., Andries, K. and Verachtert, H. (1984) *Syst. Appl. Microbiol.* **5**, 106–118
- 4 De Mot, R., Demeersman, M. and Verachtert, H. (1984) *Syst. Appl. Microbiol.* **5**, 421–432
- 5 Akaki, M., Kawamura, R. and Yamada, T. (1984) *J. Agric. Chem. Soc. Jpn.* **58**, 153–159
- 6 De Mot, R. (1990) in *Yeast: Biotechnology and Biocatalysis* (Verachtert, H. and De Mot, R., eds.), pp. 163–222, Marcel Dekker, Inc., New York
- 7 Sukhumavashi, J., Kato, K. and Harada, T. (1975) *J. Ferment. Technol.* **53**, 559–565
- 8 Gašperík, J. and Hostinová, E. (1990) *Biológia (Bratislava)* **45**, 1013–1019
- 9 Beggs, J. D. (1978) *Nature (London)* **275**, 104–109
- 10 Kalb, V. F., Jr. and Bernlohr, R. W. (1977) *Anal. Biochem.* **82**, 362–371
- 11 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 12 See, Y. P. and Jackowski, G. (1990) in *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), pp. 1–22, IRL Press, Oxford
- 13 Blum, H., Bein, H. and Gross, H. J. (1983) *Electrophoresis* **8**, 93–99
- 14 Trimble, R. B. and Maley, F. (1984) *Anal. Biochem.* **141**, 515–522
- 15 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- 16 Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- 17 Gershoni, J. M. and Palade, G. E. (1982) *Anal. Biochem.* **124**, 396–405
- 18 Gašperík, J. and Hostinová, E. (1993) *Curr. Microbiol.* **27**, 11–14
- 19 Kinsella, B. T., Larkin, A., Bolton, M. and Cantwell, B. A. (1991) *Curr. Genet.* **20**, 45–52
- 20 Kinsella, B. T., Hogan, S., Larkin, A. and Cantwell, B. A. (1991) *Eur. J. Biochem.* **202**, 657–664
- 21 Saha, B. C. and Bothast, R. J. (1993) *Curr. Microbiol.* **27**, 73–77
- 22 Kelly, C. T., Moriarty, M. E. and Fogarty, W. M. (1985) *Appl. Microbiol. Biotechnol.* **22**, 352–358
- 23 Dohmen, R. J., Strasser, W. M., Dahlems, U. M. and Hollenberg, C. P. (1990) *Gene* **95**, 111–121
- 24 Kimura, A., Takata, M., Sakai, O., Matsui, H., Takai, N., Takayamagi, T., Nishimura, I., Uozumi, T. and Chiba, S. (1992) *Biosci. Biotechnol. Biochem.* **56**, 1368–1370
- 25 Chantret, I., Lacasa, M., Chevalier, G., Ruf, J., Islam, I., Mantei, N., Edwards, Y., Swallow, D. and Rousset, M. (1992) *Biochem. J.* **285**, 915–923
- 26 Hunziker, W., Spiess, M., Semenza, G. and Lodish, H. F. (1986) *Cell* **46**, 227–234
- 27 Broyart, J. P., Hugot, J. P., Perret, C. and Porteu, A. (1990) *Biochim. Biophys. Acta* **1087**, 61–67
- 28 Hoefsloot, L. H., Hoogeveen-Westerveld, M., Kroos, M. A., van Beeumen, J., Reuser, A. J. J. and Oostra, B. A. (1988) *EMBO J.* **7**, 1697–1704
- 29 McKenzie, H. A. and Dawson, R. M. C. (1969) in *Data for Biochemical Research: pH, Buffers, and Physiological Media* (Dawson, R. M. C., Elliott, D. C., Elliott W. H. and Jones, K. M., eds.), pp. 475–508, Oxford University Press, Oxford