Characterization of the invertase from *Pichia anomala*

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Synthesis of invertase (EC 3.2.1.26) in *Pichia anomala* is controlled by the carbon source in the culture medium. The enzyme was purified to homogeneity from *P. anomala* cells fully de-repressed for invertase synthesis and shown to be a multimeric glycoprotein composed of identical subunits with an apparent molecular mass of 86.5 kDa. The carbohydrate moiety accounts

for approx. 30 % of the total mass of the molecule and consists of manno-oligosaccharides N-linked to the polypeptide. Most of the characteristics of the enzyme analysed in this study were similar to those previously reported for other yeast invertases, with the remarkable exception of its thermal sensitivity which appears after 15 min incubation at temperatures above 32 °C.

INTRODUCTION

Non-conventional yeasts, belonging to genera other than *Saccharomyces* or *Schizosaccharomyces*, are receiving increasing attention as subjects for biochemical and molecular biological studies because of their wide metabolic diversity and the development of procedures that allow their genetic manipulation [1–5]. The *Pichia* genus contains the largest number of species [6] and consequently includes some of the more attractive organisms from both the academic and industrial points of view. Some *Pichia* species have been shown to be excellent hosts for the expression of heterologous genes, with production yields equivalent to or higher than those obtained with *Saccharomyces* [7].

A heterologous protein that has been expressed in a *Pichia* system is the invertase (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) from *Saccharomyces cerevisiae* [8]. This enzyme has been intensively studied yielding valuable information on the structure [9], biosynthesis [10,11] and secretion [12,13] of yeast glycoproteins. The invertase produced by *Pichia pastoris* from the SUC2 gene of *S. cerevisiae* has also been characterized [14,15] and its analysis has revealed that it is only core-glycosylated, in a similar way to the invertase produced by the *sec18* mutants of *S. cerevisiae* [16]. It is not clear whether this limited degree of glycosylation is also true for authentic *P. pastoris* glycoproteins (non-heterologous products), as none of these proteins has yet been purified and characterized.

In order to gain information on the glycoproteins produced by a *Pichia* species we decided to purify and characterize the invertase from *Pichia anomala*. The choice of this organism was based on its ability to ferment sucrose (and consequently to produce invertase) as well as on its potential as a yeast amenable to genetic analysis because of its heterothallic nature with known strains of stable opposite mating types [17].

MATERIALS AND METHODS

Materials

Reagents for gel electrophoresis and protein assay were obtained from Bio-Rad Laboratories. DEAE-Sephacel was from Pharmacia. Endoglycosidase H was kindly donated by Dr. Frank Maley, New York State Department of Health, Albany, NY, U.S.A. Proteins used as molecular-mass markers, *S. cerevisiae* invertase, raffinose, 2,3,5-triphenyltetrazolium chloride, glucu-

ronidase, sorbitol, Coomassie Blue R-250, Bromophenol Blue and glycine were purchased from Sigma. All other reagents were of analytical grade.

Organism and growth conditions

P. anomala CBS 5759 (NRRL Y-366, NCYC 432, ATCC 8168) was obtained from the Mycothèque de l'Université Catholique de Louvain (MUCL), Louvain-la-Neuve, Belgium, and maintained in YEP–agar medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) agar] containing 2 % (w/v) glucose as carbon source. Growth for invertase production was carried out in liquid YEP medium, containing 3 % (w/v) glucose, for 14–16 h, at 28 °C, in an orbital shaker at 200 rev./min; the cells were then harvested by low-speed centrifugation, re-inoculated into fresh medium containing 2 % (w/v) raffinose as carbon source, at an initial A_{600} of 2.5, and shaken for a further 3.5 h, at the same speed and temperature.

Subcellular fractionation

Cells from early-exponential-phase cultures were converted into protoplasts by treatment with glucuronidase as described previously [2]. Protoplasts were washed with 0.1 M sodium acetate buffer, pH 4.5, containing 1.2 M sorbitol and then lysed by resuspension in the same buffer without sorbitol followed by mechanical shaking in a Vortex mixer at maximum speed. Lysates were fractionated into membrane (pellet) and cytosol (supernatant) fractions by centrifugation at 100 000 *g*, for 30 min, at 4 °C.

Enzyme purification

The cells from 12 litres of culture in raffinose medium were harvested, washed twice in ice-cold distilled water and once more with 0.1 M sodium acetate buffer, pH 4.5, resuspended in 100 ml of the same buffer and broken by mechanical shaking with glass beads (0.45 mm diameter) in a Vortex mixer at maximum speed. All further purification steps were performed at 4 °C. The crude extract was spun first at 5000 *g* for 5 min and then at 40 000 *g* for 30 min. The pH of the supernatant of the second centrifugation was readjusted to 4.5 and then solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly, with continuous stirring, to 85 % satn. After standing overnight in the cold, the precipitated material was removed by

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centrifugation at 40 000 *g* for 30 min. The supernatant, containing most of the invertase activity, was dialysed against 0.1 M sodium acetate buffer, pH 4.5, and then applied to a DEAE-Sephacel column (1.5 cm × 20 cm) equilibrated in 50 mM sodium acetate buffer, pH 6.0. The column was washed with 200 ml of this buffer and then eluted with 150 ml of a linear gradient of 10–400 mM NaCl in the same buffer. Fractions exhibiting invertase activity were pooled, dialysed and rechromatographed in the same way, except that the elution was performed with 300 ml of a linear gradient of 10–250 mM NaCl in the same buffer.

The purified invertase was extensively dialysed against 0.1 M sodium acetate buffer, pH 4.5, and stored at –20 °C after the addition of glycerol to a final concentration of 40 % (v/v).

Enzyme assay

Invertase activity was measured by the method of Goldstein and Lampen [18]. One unit of invertase is the amount of enzyme that hydrolyses sucrose to yield 1 µmol of glucose/min at 30 °C and pH 4.5.

Invertase activity on substrates other than sucrose was determined by measuring the reducing sugars released from the substrate [19,20].

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [21]. When it was necessary to avoid denaturing conditions for the samples, SDS was omitted from both the gel and the buffers. Linear gradient SDS/PAGE (3–12 % gels) was carried out as described in [22].

Proteins were detected either by staining the gels with Coomassie Brilliant Blue R-250 [22] or by the AgNO₃ method of Morrissey [23]. Invertase activity was detected *in situ* in the gels as previously described [24,25].

Carbohydrate analysis

Individual sugars were identified by g.l.c. of their alditol acetate derivatives [26] in a Hewlett–Packard 5710A chromatograph. Samples for sugar analysis, containing inositol (1 mg/ml) as internal standard, were hydrolysed in 2 M trifluoroacetic acid at 121 °C, for 1 h, in sealed evacuated tubes. Neutral sugars were identified on a 3 % OV-225 on 80/100 chromosorb G AW-DMCS column at 200 °C; injector and detector temperatures were 250 °C and 300 °C respectively and the carrier gas was He at 30 ml/min.

Other methods

Protein concentration was measured by the bicinchoninic acid method [27]. Enzymic deglycosylation with endoglycosidase H was performed as described previously [28].

RESULTS AND DISCUSSION

Invertase production by *P. anomala*

As for other previously studied yeast species (*S. cerevisiae* [29], *Schizosaccharomyces pombe* [30]) the synthesis of invertase by *P. anomala* was found to be influenced by the carbon source in the culture medium. Figure 1 shows that a high concentration of glucose in the culture medium completely repressed production of the enzyme, whereas the use of 2 % (w/v) raffinose as carbon source allowed derepression of invertase synthesis. Culture in

Figure 1 Synthesis of invertase by *P. anomala*

Cells were grown overnight in YEP medium containing 3 % (w/v) glucose, as described in the Materials and methods section, and then transferred to fresh medium containing 3 % (w/v) glucose (●) or 2 % (w/v) raffinose (△), as carbon source. At the indicated times, samples were withdrawn and the invertase activity in the cells was determined.

medium containing glucose concentrations lower than 0.2 % (w/v) or 2 % (w/v) sucrose also permitted synthesis of the enzyme, although to a lower extent than in raffinose medium (not shown). In all cases, after attainment of the maximum derepression level, a decrease in invertase activity in the cells was observed which was not the consequence of a gradual inactivation produced by temperature or low pH, as similar patterns of invertase production were obtained in cultures grown at lower temperatures and/or in media buffered at pH 5.5 (not shown).

This decline in invertase activity was not due to secretion of the enzyme into the extracellular medium either, as all the activity in the cultures remained associated with the cells. On subcellular fractionation, invertase was found to be associated almost exclusively with the cell wall. About 95 % of total cell activity was solubilized by glucuronidase during the conversion of cells into protoplasts, and the remaining 5 % was associated with the particulate fraction when protoplasts were lysed and fractionated into membranes and cytosol. Most of the enzyme was also obtained in soluble form when cells were broken down by mechanical shaking with glass beads, followed by differential centrifugation of the cell extract, indicating lack of covalent linkages between invertase and cell wall structural components.

Purification of invertase

Table 1 summarizes relevant data of the purification procedure, which started with the preparation of a crude cell extract from cultures grown under conditions that allowed maximum derepression of invertase synthesis. The first purification step took advantage of the fact that the invertase from *P. anomala* remains soluble in concentrated (up to 85 % satn.) (NH₄)₂SO₄ solutions, probably because of its carbohydrate content (see below). The binding of invertase to the anion exchanger DEAE-Sephacel allowed its separation from all associated proteins in two successive chromatographic steps eluted with different NaCl gradients, with a final activity yield higher than in other previously published purifications of different yeast invertases [31–33].

Molecular mass and carbohydrate composition

Analysis of the purified invertase by SDS/PAGE revealed a single band with a mobility corresponding to a molecular mass of 86.5 kDa. An apparent molecular mass of about 254 kDa was calculated for the native purified enzyme from its mobility in

Table 1 Purification of *P. anomala* invertase

For experimental details see the Materials and methods section.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery yield (%)
Crude extract	668	19280	29	—	100
(NH ₄) ₂ SO ₄ precipitation	123	18645	152	5	97
First DEAE-Sephacel chromatography	37	13625	368	13	93
Second DEAE-Sephacel chromatography	6	8895	1482	51	46

Figure 2 Gel electrophoresis of purified *P. anomala* invertase: effect of deglycosylation

Samples of purified *P. anomala* invertase containing 5 µg of protein were electrophoresed, before (lane 2) and after (lane 3) treatment with endoglycosidase H, on an SDS/linear-gradient polyacrylamide (3–15%) gel slab, after which the gel was stained with Coomassie Brilliant Blue. Numbers on the left indicate the molecular mass of the markers (lane 1), from top to bottom: myosin, β-galactosidase, phosphorylase b, BSA, catalase and aldolase.

non-denaturing PAGE with the following molecular-mass markers: urease hexamer (540 kDa), urease trimer (270 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) and ovalbumin (45 kDa). However, a molecular mass of 350 kDa was calculated for purified invertase filtered through a Sepharose CL-6B column calibrated with the following markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (220 kDa) and aldolase (158 kDa). These results suggest that native *P. anomala* invertase could be a multimeric enzyme comprised of three or four subunits of identical size.

Treatment of denatured invertase with endoglycosidase H for 18 h resulted in a shift in the apparent molecular mass to 61 kDa, as determined by SDS/PAGE (Figure 2), indicating the presence of N-linked carbohydrate contributing approx. 30% to the total mass of the molecule, assuming that both glycosylated and deglycosylated forms of the enzyme behave similarly in the gel. At least six different diffuse bands could be observed on SDS/PAGE of the enzyme treated with the glycosidase for shorter periods of time (5, 10, 20 and 45 min), which can be interpreted as the gradual elimination of a similar number of N-linked oligosaccharides (not shown). Besides N-acetylglucosamine, which should constitute the N-linkages, the only mono-saccharide identified by g.l.c. in the sugar moiety of the enzyme was mannose.

Figure 3 Effect of pH on the stability of invertase

Samples containing 0.015 unit of purified invertase were incubated for 4 h in 20 mM citric acid/phosphate buffer, at the indicated pH, after which the pH was readjusted to 4.5 and the remaining activity determined.

Effect of pH and temperature on invertase activity and stability

The optimum pH was determined by incubating purified invertase with sucrose in 0.1 M citric acid/phosphate buffer, at pH 2.5–7.5. The enzyme exhibited maximum activity at pH 4.5 and a sharp decrease in activity was found at pH values lower than 4.0 or higher than 6.5 (not shown). More than 90% of maximum invertase activity was retained after a 4 h incubation at pH values in the range 4.5–6.5, and the enzyme was highly unstable at pH values below 4.0 (Figure 3).

The optimum temperature for invertase activity was 38 °C (assayed on sucrose at pH 4.5 for 10 min) and the enzyme was rather unstable at temperatures above 32 °C (Figure 4), with a half-life at 37 °C of only 15 min. Invertase activity was also quickly lost after successive rounds of freezing at –20 °C and thawing to room temperature as well as when pure enzyme solutions, previously dialysed against distilled water, were lyophilized and then redissolved in cold 0.1 M sodium acetate buffer, pH 4.5.

Whereas the behaviour of *P. anomala* invertase with respect to pH is similar to that found for other yeast invertases, its thermosensitivity and relatively low optimum temperature are unusual characteristics that differentiate it from the invertases from *S. cerevisiae* [34], *Sch. pombe* [35] and *Schwanniomyces occidentalis* [32]. Although various studies have established that glycosylation increases the resistance of yeast invertase to thermal inactivation [34,36], the lower carbohydrate content of the *P. anomala* enzyme, compared with that of invertases from *S.*

Figure 4 Effect of temperature on the stability of invertase

Samples such as those in Figure 3 were incubated for 15 min in 0.1 M sodium acetate buffer, pH 4.5, for 15 min at the indicated temperature after which individual tubes were quickly cooled to 4 °C and the remaining activity was determined.

Figure 5 Lineweaver-Burk plot of hydrolysis of sucrose (a) and raffinose (b) by purified *P. anomala* invertase

Straight lines were calculated by the least-squares method.

cerevisiae [31] and *Sch. pombe* [33], cannot be the reason for its higher thermolability, because its level of glycosylation is similar to that of the core-glycosylated form of the *S. cerevisiae* invertase, and core glycosylation appears to be sufficient to stabilize the latter against thermal denaturation [36,37].

Substrate-specificity and kinetic properties

The invertase purified from *P. anomala* was fully active on sucrose and displayed hydrolytic activity with raffinose that was 28 % of that observed with sucrose. The enzyme was not active with inulin or melezitose.

The effect of substrate concentration on invertase activity was examined with both sucrose and raffinose using the standard conditions of the assay described in the Materials and methods section (30 °C and pH 4.5). With both substrates the enzyme obeys the Michaelis-Menten equation (Figure 5). The K_m values for sucrose and raffinose were 16 mM and 82 mM respectively, about 40 % lower than those reported for *S. cerevisiae* invertase on the same substrates under similar assay conditions [38], indicating a higher affinity of *P. anomala* invertase for both sucrose and raffinose.

To summarize, we have purified the invertase from *P. anomala* to homogeneity and determined some of its relevant structural and kinetic characteristics. This is, to our knowledge, the first authentic (non-heterologous) glycoprotein produced by a *Pichia* species ever to have been purified and characterized.

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