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Invertase from a strain of *Rhodotorula glutinis*

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Abstract

An invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) from *Rhodotorula glutinis* was purified by ammonium sulfate fractionation, gel filtration and anion exchange chromatography. Invertase molecular weight was estimated to be 100 kDa by analytical gel filtration and 47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular mass determinations indicated that the native enzyme exists as a homodimer. It is a glycoprotein that contains 19% carbohydrate. The enzyme attacks β-D-fructofuranoside (raffinose, stachyose and sucrose) from the fructose end. It has a K_m of 0.227 M and a V_{max} of 0.096 μmol/min with sucrose as a substrate. Invertase activity is stable between pH 2.6 and 5.5 for 30 min, maximum activity being observed at pH 4.5. The activation energy was 6520 cal/mol. The enzyme is stable between 20 and 60 °C. Mg^{2+} and Ca^{2+} ions stimulated invertase activity 3-fold, while Fe^{2+}, K^+, Co^{2+}, Na^+ and Cu^{2+} increased activity about 2-fold. The transfructosylation reaction could not be observed. This enzyme is of particular interest since it appears to have a high hydrolytic activity in 1 M sucrose solution. This fact would make the enzymatic hydrolysis process economically efficient for syrup production using by-products with high salt and sugar contents such as sugar cane molasses.

Keywords: *Rhodotorula glutinis*; *Hemiascomycetidae*; Food industry; Enzymatic characterization; Invertase; β-Fructofuranoside fructohydrolase; Invert sugars

1. Introduction

Acid invertase is an important industrial enzyme with applications in the production of non-crystallizable invert sugars and soft-centered chocolates (Wiseman, 1979). Invertase hydrolyses sucrose into a mixture of glucose and fructose under sucrose concentrations lower than 10% (wt./vol.) and has transfructosylating activity under sucrose concentrations higher than 10% (wt./vol.). β-D-fructosyl-transferase (FTase, EC 2.4.1.9) obtained from plants and fungi (Chen and Liu, 1996) has a similar activity. Invertase has been widely studied, especially in the yeasts *Saccharomyces cerevisiae* and *Schwanniomyces occidentalis* (Fontana et al., 1992; Costaglioli et al., 1997) and in filamentous fungi such as *Aureobasidium* sp. ATCC 20524 (Hayashi et al., 1992b) and *Aspergillus niger* (Romero-Gómez et al., 2000).

The yeast *Rhodotorula* produces large amounts of fats (Prescott and Dunn, 1962; Rose and Harrison, 1970) and has been used for the production of single-cell proteins from ethanol, acetic acid and acetaldehyde (Hofer and Misra, 1978; Yech, 1996). Recently, epoxide hydrolase (EH) has been described by Kronenburg et al. (1999) in this yeast; however, as far as we know, it has not been studied as a possible producer of invertase.

It is the aim of this paper to report our investigation concerning the physical and kinetic properties of an invertase produced by *Rhodotorula glutinis* in order to identify its possible economic advantages in industrial processes such as syrup production.

2. Results and discussion

2.1. Invertase production

The biosynthesis of the enzyme was strongly influenced by the sugar used as the main carbon source. Among the sugars tested (fructose, glucose, lactose,

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Table 1
Purification scheme of the invertase from *Rhodotorula glutinis*. The values given are the means from at least two experiments

Purification steps	Total activity (μkat)	Total protein (mg)	Specific activity (μkat/mg protein)	Purification factor	Yield (%)
Crude extract	0.32	7.80	0.04	1.00	100
Ammonium sulfate (40–80%)	0.05	0.60	0.08	2.00	15.6
Sephadex G-150	0.04	0.20	0.20	5.00	12.5
DEAE-Sephacell	0.03	0.15	0.20	5.00	9.4

maltose, raffinose and sucrose) only the sucrose-grown and the raffinose-grown cultures possessed invertase activity, indicating that the enzyme is substrate inducible. Similar results were obtained with invertases from *S. occidentalis* (Costaglioli et al., 1997) and *A. niger* (Rubio et al., 1997). Under the conditions described above, invertase production in sucrose culture reached a maximum after incubation for 36 h. At this time, the total specific activity of the invertase was 0.04 μkat/mg protein. However, enzyme synthesis declined with age, as did the concentration of free glucose in the medium. In this respect, invertase synthesis in *R. glutinis* is similar to that of *S. cerevisiae*, where production of the enzyme is regulated by the concentration of glucose present in the medium (Fontana et al., 1992).

R. glutinis did not grow on carboxymethylcellulose, cellobiose or starch. This indicates that it did not form cellulases, β-glucosidases or amylases.

2.2. Purification and characterization of invertase

The purification of invertase from *R. glutinis* is shown in Table 1. The specific activity of the final purified preparation was 0.20 μkat/mg protein, representing a total purification factor of 5. The invertase yield obtained by this purification procedure is low; nevertheless, our objective was to purify and study the enzyme properties. The elution profiles from DEAE Sephacell chromatographic column, from which a homogeneous enzyme was eluted with a linear gradient of (1 M) NaCl, showed a single peak with a symmetrical distribution of activity. The molecular weight estimated by gel filtration was 100 kDa. However, SDS-PAGE of the enzyme revealed a single protein band, whose estimated molecular weight was 47 kDa. This difference suggested the presence of subunit structures in the enzyme, similar to those reported for *S. cerevisiae Y2180* and *A. niger* invertases (Babczinski, 1980; Rubio and Maldonado, 1995). The carbohydrate content of the enzyme was 19% (carbohydrate/protein, wt./wt.), a value similar to the carbohydrate content (20%) of *Aspergillus japonicus* invertase (Hayashi et al., 1992a) but lower than that of *Aureobasidium* sp. *ATCC* 20524 invertases (30–53%) (Hayashi et al., 1992b).

2.3. Effect of pH and temperature

The optimum pH of the enzyme on the hydrolysis of sucrose was 4.5. It maintained 48% of its relative activity after 30 min at pH 3.0 but at pH 8.0, 93% of the relative activity was lost. Invertase is stable under acidic conditions between pH 2.6 and 5.5, and maintains 90% of its residual activity at pH 6.0 for 30 min (Fig. 1).

The activity of the enzyme was determined between 20 and 70 °C. The activation energy, calculated from the Arrhenius plot, was 6520 cal/mol. Invertase from *Rhodotorula* was stable at 60 °C while its half-life was 30 min. This stability was higher than that exhibited by the invertase from *Azotobacter chroococcum,* whose half-life at 60 °C was 3 min (De la Vega et al., 1991). Stability decreased 33% at 70 °C. Results suggested that in these conditions bacterial contaminations decreased.

2.4. Effect of metal ions on invertase activity

Table 2 shows that Ca^{2+} and Mg^{2+} stimulated invertase activity about 3-fold while Fe^{2+}, Co^{2+}, Na^{+}, Cu^{2+} and K^{+} increased it about 2-fold as compared to enzyme activity in the absence of such ions (control). This effect of metal ions could be important in the use of raw materials with a high salt content. In contrast, these

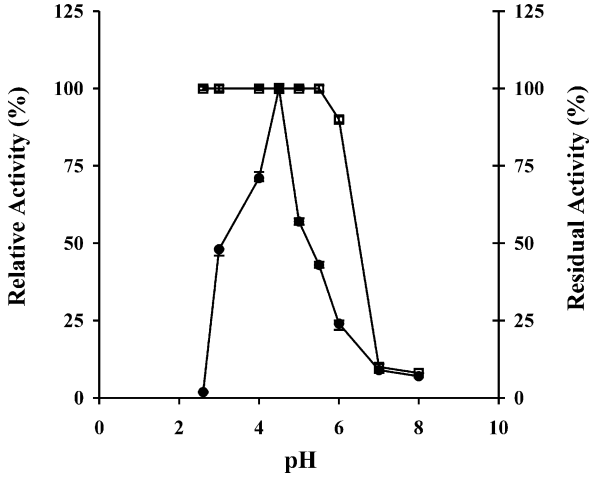

Fig. 1. Effect of pH on the activity (relative activity ●) and stability (residual activity □) of the invertase from *Rhodotorula glutinis*. The values given are means of three experiments done in duplicate.

Table 2
Effect of various metal ions on the activity of the invertase from *Rhodotorula glutinis*. The values given are means of three experiments done in duplicate

Ions	Relative activity (%)
Cu^{2+}	200
Ca^{2+}	340
Mg^{2+}	350
K^{+}	100
Hg^{2+}	100
Na^{+}	200
Co^{2+}	170
Fe^{2+}	240
Control	100

metal ions inhibited invertase activity in other microorganisms such as *A. japonicus* (Hayashi et al., 1992a) and *A. chroococcum* (De la Vega et al., 1991). Hg^{2+} does not affect invertase activity, suggesting that tryptophan and cysteine are not essential for its activity, a fact that would make it different from invertases from *S. cerevisiae* and *A. niger* (Hoshino and Momose, 1966; Rubio and Maldonado, 1995).

2.5. Kinetic parameters and specificity for substrate

A Lineweaver–Burk plot of the enzyme affinity for sucrose gave a straight-line plot ($y = 3.3118\ x + 14.763$; R^2 is 0.968) from which the K_m was calculated as 0.227 M and the V_{max} of the enzyme for sucrose was 0.096 μmol/min (Fig. 2). These values were similar to that obtained with the invertase from *A. japonicus* (Hayashi et al., 1992a) and seemed to be larger than those from *S. occidentalis*, 0.020 M (Klein et al., 1989) and *A. niger*, 0.060 M (Rubio and Maldonado, 1995).

The enzyme was able to hydrolyze raffinose, stachyose and sucrose, but not cellobiose, lactose, melezitose, maltose, inulin and trehalose. These results suggest that the enzyme is a β-D-fructofuranosidase fructohydrolase, able to attack the β-D-fructofuranosides from the fructose end.

2.6. Effect of sucrose concentration on transfructosylating activity

1 M sucrose is a syrup with a low free water content. The reaction catalyzed by invertase at 60 °C shows that the enzyme activity is not affected by 1 M sucrose solution, which implies that its activity does not require large amounts of free water. It is known that FTases can act on sucrose by cleaving the β-1,2 linkage and transferring the fructosyl moiety to a suitable acceptor molecule such as sucrose. By contrast, FFases commonly possess both hydrolytic and transfructosylating activity in sucrose concentrations higher than 10% (wt./vol.) and demonstrate the only hydrolytic activity in sucrose concentrations lower than 10% (wt./vol.). In order to determine whether the enzyme produced by *R. glutinis* possessed high transfructosylating activity, we examined it with various initial sucrose concentrations (0.15–1 M) in the reaction mixture. Fig. 3 shows that the hydrolytic activity of the invertase increases with the increase in sucrose concentration in the reaction medium. Chromatographs from these samples show no oligosaccharide production, a fact that indicates that the invertase has no transfructosylating activity in the assayed concentrations. In contrast, fungal invertase has more transfructosylating activity with a 1 M sucrose solution (Hayashi et al., 1993; Chen and Liu, 1996).

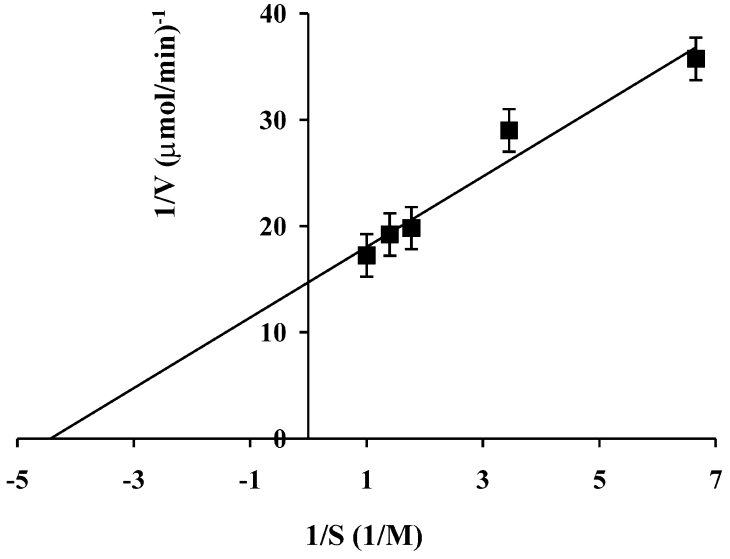

Fig. 2. Lineweaver–Burk plot of initial velocity against sucrose concentration. The values given are means of three experiments done in duplicate.

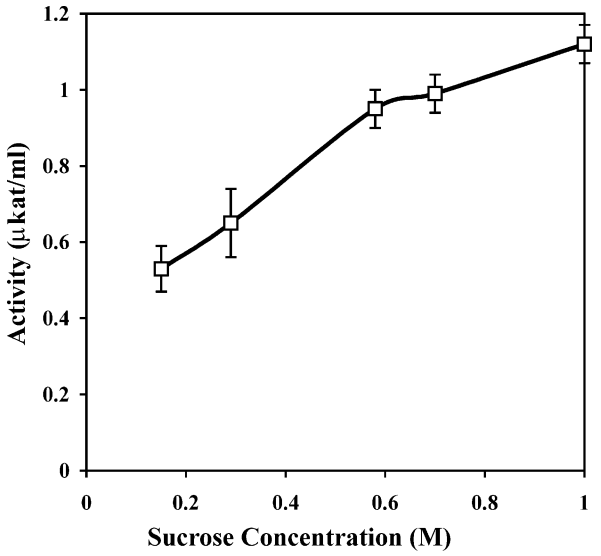

Fig. 3. Effect of the sucrose concentration on the hydrolytic activity of the invertase from *Rhodotorula glutinis* (R^2, 0.954). The values given are means of three experiments done in duplicate.

In brief, the invertase isolated from *R. glutinis* is a β-fructofuranosidase (invertase, EC 3.2.1.26). Interesting properties were found such as its stability at 60 °C, its activation by ions (Ca^{2+}, Mg^{2+}, K^+, Co^{2+}, Fe^{2+}, Na^+ and Cu^{2+}) and its lack of transfructosylating activity on a 1 M sucrose solution, Therefore, invertase from *R. glutinis* may have a potential for industrial application in syrup production because of its strong hydrolytic activity in a 1 M sucrose solution, in constrast to other fungi.

3. Experimental

3.1. Enzyme production

R. glutinis strain CIM 1001 was obtained from our own laboratory culture collection. The strain was grown in liquid Czapek medium. The pH was adjusted to 4.5 with 0.1 N HCl. Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated with 2.6×10^6 cells/ml. The culture was incubated for 36 h at 30 °C on a reciprocating shaker (250 rpm). The carbon sources tested were carboxymethylcellulose, cellobiose, fructose, glucose, lactose, maltose, raffinose, starch and sucrose at 0.5% (wt./vol.).

3.2. Enzyme assays

The reaction mixture is 0.25 ml of 0.40 M sucrose solution, 0.20 ml of 0.20 M acetic acid–sodium acetate buffer (pH 4.5) and 0.05 ml of enzyme solution. Enzymatic activity was determined at 35 °C and pH 4.5, measuring the reducing sugars released with the Somogyi–Nelson reagent (Somogyi, 1952). One μkat was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of sucrose per second under the above conditions. The soluble protein was measured by the method of Bradford (1976), with bovine serum albumin as a standard. The carbohydrate content of the enzyme was measured by the phenol-sulfuric acid method (Dubois et al., 1956).

3.3. Enzyme purification

The yeast was isolated from a 36 h old-sucrose culture by centrifuging at 15,000 *g* for 20 min. Intracellular enzyme was prepared by resuspending the cell in 0.2 M acetic acid–sodium acetate buffer (pH 4.5) plus 1.4 mM of 2-mercaptoethanol and disrupting in a ballistic disintegrator Braun B (Melsungen A.G.). Disrupted cells were centrifuged at 15,000 *g* for 10 min and the supernatant solution was used as an intracellular enzyme source (crude extract). Extracellular enzymatic activity was tested in the culture broth. The crude extract was first precipitated with solid ammonium sulfate (40% saturation). The precipitate obtained was collected by centrifugation at 15,000 *g* for 20 min, and dissolved in 0.2 M acetic acid–sodium acetate buffer with 1.4 mM of 2-mercaptoethanol. Solid ammonium sulfate was added to the supernatant solution to achieve 80% saturation. Precipitated proteins were collected again by centrifugation at 15,000 *g* for 20 min and dissolved in 1 ml of the same buffer. The invertase activity was in the 80% fraction, which was dialyzed overnight against the same acetate-buffer. Purification was effected by gel filtration (Sephadex G-150 column; 45×2.5 cm) equilibrated and eluted with 0.2 acetic acid–sodium acetate buffer (pH 4.5) containing 1.4 mM of 2-mercaptoethanol. The active fractions (11–17) were pooled and applied to a DEAE-Sephacell column (11×1.5 cm), and then eluted with a linear gradient from 0 to 1 M NaCl solution in the same buffer. Fractions (13–16) containing invertase activity were pooled. The purified extract was dialyzed against 0.2 M acetic acid–sodium acetate buffer plus 1.4 mM of 2-mercaptoethanol and stored at 4 °C.

3.4. Estimation of molecular weight

The molecular weight (M_r) was estimated, using a 45×2.5 cm column of Sephadex G-150, equilibrated and eluted with 0.2 M acetate buffer (pH 4.5) plus 1.4 mM of 2-mercaptoethanol. The standard proteins were bovine serum albumin (67,000), ovoalbumin (45,000), alkaline phosphatase (100,000) and catalase (250,000). SDS-PAGE was performed in 9% acrylamide gels, which were calibrated using a proteins standard kit (36,000–205,000) from Sigma. For detecting proteins, gels were stained with an alkaline silver nitrate solution.

3.5. Effect of pH and temperature on enzyme activity and stability

Invertase activity was examined in a pH range of 2.6–8.0. Activity value was obtained by adding to the reaction mixture 0.2 glycine-HCl buffer (pH 2–3), 0.2 M acetate buffer (pH 4–5) or 0.2 M phosphate buffer (pH 6–8). The temperature was tested in the range of 20–70 °C at pH 4.5. In order to investigate the stability of the enzyme at various pHs, the enzyme solutions were pre-incubated for 30 min at each pH. In the heat stability testing of the enzyme, enzyme solutions (pH 4.5) were preincubated at various temperatures (25–80 °C) for 30 min. The residual activity of the treated enzyme was assayed under the standard assay conditions. The activation energy was calculated from the Arrhenius plot.

3.6. Effect of metal ions on invertase activity

The following ions: Ca^{2+}, Mg^{2+}, K^{+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Na^{+} and Fe^{2+} (all as chlorides) were added to the reaction mixture at a 5 mM concentration (pH 4.5 with 1 N HCl).

3.7. Measurement of K_m and V_{max}

Kinetic constants K_m and V_{max} were determined by Lineweaver–Burk plots, as a function of the sucrose concentration (0.05 to 1 M) at pH 4.5.

3.8. Substrate specificity

The substrates used in the reaction mixtures were: 0.02 M cellobiose, lactose, maltose, melezitose, raffinose, stachyose, sucrose, trehalose and 1% inulin.

3.9. Effect of sucrose concentration on transfructosylating activity

Invertase was incubated in the reaction mixture with different sucrose concentrations (0.15, 0.29, 0.58, 0.70 and 1 M) for 3 h at pH 4.5. Aliquots (100 μl) were removed and spotted directly on the paper. Paper chromatography was carried out with *n*-butanol:pyridine: water (52:33:15) as solvent for 30 h. Sucrose, D-glucose and D-fructose were used as migration markers. The sugars were detected by dipping the chromatogram in an alkaline silver nitrate solution (Trevelyan et al., 1950).

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