Purification and Characterization of Invertase from *Torulaspora pretoriensis* YK-1

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Invertase was purified from *Torulaspora pretoriensis* YK-1 by acid treatment and column chromatography on DEAE-Toyopearl 650M and phenyl-Toyopearl 650M to homogeneity. The molecular weight of the purified enzyme was estimated to be 130,000 by SDS-polyacrylamide gel electrophoresis and 530,000 by gel filtration. The enzyme contained 50% molecular weight as carbohydrate. Properties of the invertase from *T. pretoriensis* was similar to external invertase from *Saccharomyces cerevisiae*.

Invertase (recommended name, β-fructofuranosidase; EC 3.2.1.26), which catalyzes the hydrolysis of β-D-fructofuranosyl linkages, is an important enzyme in yeasts for breadmaking. Sucrose is the principal fermentable sugar in sweet dough and white dough, so a yeast strain lacking external invertase cannot rapidly leaven such doughs. On the other hand, excess invertase activity often reduces the ability to leaven sweet dough because the hydrolysis of sucrose into glucose and fructose doubles the osmotic pressure around the cells.

*Torulaspora pretoriensis* IFO 10218 has been selected for its leavening ability and resistance to being frozen and thawed in dough. IFO 10218 cells readily agglutinate in suspension, so a mutant that does not agglutinate, YK-1, was produced for bakery use. YK-1 can be used in breadmaking in the usual method and in frozen-dough method.

The yeast *Saccharomyces cerevisiae* produces two forms of invertase. The external form is a glycosylated enzyme translated to the periplasmic space and the internal form is a carbohydrate-free enzyme remaining inside the cell. The production of these enzymes is controlled by glucose repression, and the level of the external form varies over a greater range than the internal form. As for other yeasts, invertases have been purified from *Shizosaccharomyces pombe*, *Schwanniomyces occidentalis*, and *Candida utilis*.

This paper reports purification and characterization of invertase from *T. pretoriensis* YK-1.

Yeast cells were cultured in a medium containing 1% yeast extract (Nakarai Tesque, Inc., Kyoto), 2% Polypepton (Nippon Seiyaku Co., Tokyo), and 2% glucose at 30°C with shaking (220 rpm) unless otherwise stated. Glucose in the medium was assayed with a high performance liquid chromatography.

The reaction mixture for the invertase assay contained 100 mM acetate buffer (pH 4.5), 150 mM sucrose, and the enzyme in a total volume of 0.25 ml. After incubation of the mixture at 30°C for 10 min, the reaction was stopped by the addition of 3,5-dinitrosalicylic acid reagent, and the reducing sugars produced were assayed. One unit was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent to glucose per minute under the above conditions.

We first investigated the subcellular location of the invertase in *T. pretoriensis* YK-1. Cells in the early exponential phase of growth (A_{600} = 0.8 to 1.2) were washed once with distilled water, suspended in 50 mM phosphate buffer (pH 7.2) containing 0.6 M KCl, 10 mM 2-mercaptoethanol, and Zymolyase 20T (3 U/ml), and incubated at 30°C for 2 h. After centrifugation of the suspension at 2000 × g for 5 min to collect protoplasts, the precipitate was washed once with 50 mM phosphate containing 0.6 M KCl. The combined supernatant including both the periplasmic space and cell walls was used as the external fraction. The protoplasts were suspended in the same buffer without KCl and vortexed vigorously for 1 min. This suspension was centrifuged at 2000 × g for 5 min to remove unbroken cells, and further centrifuged at 100,000 × g for 1 h. The resultant precipitate and supernatant were used as the membrane and cytosol fractions, respectively. The invertase activity was 91.3% in the external fraction, 5.5% in the membrane fraction, and 3.2% in the cytosol fraction. These results suggest that invertase is located in the periplasmic space, cell walls, or both, and that it hydrolyzes sucrose outside of the cell membrane.

To obtain a large amount of invertase, enzyme activity was monitored in the cells grown on glucose as the carbon source. The activity of the cell suspension increased gradually and reached a constant value some time after 16 h (Fig. 1). Glucose in the medium was consumed by 12 h after inoculation. Changes in time in enzyme formation by the cells grown on sucrose was similar to that in the cells grown on glucose (data not shown), indicating that sucrose did not induce the production of invertase.

Invertase was then purified as described below. Protein was measured by the method of Bradford. Washed cells grown for 24 h (about 56 g, wet weight) were suspended in 60 ml of 100 mM acetate buffer (pH 4.5) and passed through a French pressure cell (1500 kg/cm²) at 4°C. The homogenate was centrifuged at 8000 × g for 20 min to remove cell debris. The resulting supernatant was used as the cell-free extract. The pH of the cell-free extract was 5.0.

![Fig. 1. Invertase Production and Glucose Consumption by *T. pretoriensis* YK-1 During Its Cell Growth.](image)

Yeast cells grown in a medium composed of 1% yeast extract, 2% Polypepton and 2% glucose at 30°C were collected, washed, and suspended in 100 mM acetate buffer (pH 4.5). The invertase activity of the cell suspension was assayed as described in the text except that the reaction was for 3 min. Symbols: •, invertase activity; ○, cell growth (cell weight); △, glucose concentration in the culture medium.

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extract was adjusted to 4.5 with 1 M acetic acid and left overnight at 4°C. The supernatant was collected after centrifugation at 8000 g for 30 min. The enzyme solution was put on a column (2.5 × 18 cm) of DEAE-Toyopearl 650 M equilibrated with the buffer. The column was washed with the buffer and eluted with a linear gradient of NaCl concentrations from 0 to 0.4 M in the buffer. Two peaks of invertase activity appeared on the chromatography (data not shown). The first peak, with less than 1% of the total activity, passed through the column in the absence of NaCl and contained the activity that hydrolyzed p-nitrophenyl α-D-glucopyranoside, and the second peak, with most of the invertase activity, was eluted with 0.3 M NaCl. The minor fractions hydrolyzing sucrose probably contained α-glucosidase, which hydrolyzes sucrose more than maltose. The major fractions were collected and dialyzed against buffer containing 20% ammonium sulfate. The dialyzed solution was put on a phenyl-Toyopearl 650 M column (1.5 × 6 cm) equilibrated with the buffer containing 20% ammonium sulfate. The column was washed with the same buffer, and eluted with a decreasing gradient of ammonium sulfate from 20% to 0%. The enzyme was recovered in the fractions containing 0% ammonium sulfate with 200-fold purification and a recovery of 49% (Table).

On SDS-polyacrylamide gel electrophoresis (PAGE), the purified enzyme showed a single smeared, wide band from a glycoprotein with a mean molecular weight of 130,000 (Fig. 2A). After treatment by endoglycosidase H by the method of Klein et al., the molecular weight was 66,000 (Fig. 2B). The percentage of carbohydrate in the enzyme was found to be 50% of the total weight of protein by the phenol-sulfuric acid method with mannose as a standard. The enzyme had a single asymmetrical peak in its elution from a column of Sephacryl S-200 (1.5 × 95 cm) equilibrated with 100 mM acetic buffer (pH 4.5), and its molecular weight was estimated to be 530,000 (Fig. 3). The molecular weight found by gel filtration was four-fold that found by SDS-PAGE, so the native enzyme seemed to be a tetrameric protein. The shoulders on the right side of the main peak seemed to arise from oligomers composed of less than four subunits.

The optimum pH and temperature were 4.5 to 5.5 and 45°C, respectively. The enzyme was stable from pH 4.5 to 6.8 at 4°C for 16 h, and inactivated above 35°C at pH 6.8 for 30 min. The residual activity after incubation at 35°C for 30 min in the presence of metallic salts and chemical reagents was assayed. The enzyme was activated 26% by Mn²⁺ and 17% by Co²⁺, respectively, and completely inhibited by Ag⁺, Hg²⁺, and Na-bromosuccinimide, each at 1 mM.

The purified invertase was incubated with some substrates, and glucose liberated was measured by the glucose-oxidase method. The enzyme hydrolyzed sucrose and raffinose, but not maltose, melibiose, lactose, or methyl-α-glucoside.

From Lineweaver–Burk double-reciprocal plots, the Kₐ values for sucrose and raffinose at pH 4.5 were calculated to be 48.3 and 120, and the Vₐₕ values (U/mg protein) for these substrates were calculated to be 7090 and 3270, respectively.

External invertase has been purified from Sacch. cerevisiae and its properties have been characterized in other laboratories. The Kₐ value of this enzyme is 25 to 26 for sucrose and 150 for raffinose. The enzyme contains 50% of the molecular weight as carbohydrate, and the molecular weight of the carbohydrate-depleted form is 60,000 by SDS-PAGE. The native enzyme is composed mainly of an octamer and contains a small amount of hexamer, tetramer, and dimer.

In conclusion, the properties of the enzyme from T. pretoriensis were similar to those of external invertase from Sacch. cerevisiae, showing that these two species were closely related, as is also indicated by the properties of α-glucosidase.

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References
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