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# Purification and Characterization of Internal Invertase in *Rhodosporidum toruloides* Mating Type a Cells

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Abstract

The internal invertase of *Rhodosporidium toruloides* mating type a cells was purified to a single band on SDS-PAGE from cell-free extract by acid precipitation, ion exchange chromatography and gel filtration. The determined molecular weight of the purified enzyme was about 95,000 by gel filtration and 100,000 daltons on SDS-polyacryamide gel electrophoresis. This enzyme didn't show any activity change by several metal ions except 15.4% decrease by $Mn^{2+}$ and was strongly inhibited by 2-mercaptoethanol and SDS. The invertase maintained its activity at high level until 70°C, but inactivated at 80°C almost completely. The optimal temperature and pH of the enzyme were about 60°C and pH 5.0, respectively. The stable pH range of invertase was narrow from pH 3.0 to 6.0. The Km value and isoeletric point of enzyme were $3.4\times10^{-3}$M, pH 5.5, respectively.

**Key words**: *Rhodosporidium toruloides*, invertase, yeast mating type, intracelluar invertase, invertase purification

## INTRODUCTION

Conjugation between haploid cells of the two compatible mating types, A and a, of the heterobasidiomycetous yeast, *Rhodosporidium toruloides*, is mediated by the mating pheromone secreted by the haploid cells of each mating type(1) Before the conjugation, the vegetative cells differentiate to gamete cells responding to the mating pheromone produced by the opposite mating type cells.

The sexual differentiation is characterized by the arrest of the vegetative growth in the $G_1$ phase of the cell division cycle and protrusion of a long mating tube which grows toward the partner cells. Rhodotorucine A, a mating pheromone produced by the mating type A cells, has the following sequence: Tyr-Pro-Glu-Ile-Ser-Trp-Thr-Arg-Asn-Gly-Cys-(S-farnesyl)OH(2). Both the farnesyl and the peptidyl moieties are indispensable for the biological activity of the pheromone(3).

The studies on the mode of pheromone action have revealed a unique signaling mechanism that requires hydrolysis of rhodotorucine A at the surface of mating type a cells as the trigger reaction for the pheromone action(4). We have found that the receptor has protease activity, receives pheromone of rhodotorucine A, and has high mating-type-specificity present in only mating type a cells (5-8). The receptor didn't exist in sexual pheromone producing type A but existed only in pheromone-receiving type a. The receptor has endopeptidase activity and is a glycoprotein. Therefore, it is regarded that some differences of physiological characteristics exist in each mating strain(type A and a). For the purpose of grasping the mating type specific characteristics, we have studied invertase as a target protein.

Invertase(EC 3.2.1.26) is widely distributed throughout plant, animals and various microorganisms. In particular, many studies on yeast invertase have been carrying out until now.

Recently, we have purified the internal invertase from *R. toruloides* mating type A(9). In this study, we describe the purification of internal invertase from mation type a cell and compare the enzymatic characteristics with mating type A invertase.

## MATERIALS AND METHODS

### Microorganism

*Rhodospridium toruloides* IFO 0880-M-1057(haploid mating type a, ovoid cell form, orange colored colony)

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stocked in our laboratory was used as a strain which produce a invertase(10).

### Cultivation

We have used YPG medium(11) containing 50μg of chloramphenicol per ml on this cultivation. The seed culture was incubated at 28°C with reciprocal shaking for 24hr. The main culture was performed in a 500ml-flask with 200ml of YPG medium and then the seed culture broth was inoculated at the concentration of $1\times10^{6}$cells per ml. The culture was done by the continuous reciprocal shaking at 28°C for 3 days.

### Preparation of crude internal enzyme fraction

Mating type a cells($1\times10^{8}$cells per ml) cultivated in YPG medium at 28°C with shaking were harvested by centrifugation at 8,000×g, and the cells were washed twice with 10mM phosphate buffer(pH 7.0) (buffer A), followed by centrifugation at 4°C. The washed cells were suspended in 200ml of buffer A and disrupted by sonicator (Model SM 51., sonics and materials Inc), then the cell debris was removed by centrifugation at 11,000×g 10min. The supernatant was used for the crude internal invertase preparation.

### Invertase assay

2% sucrose prepared in acetate buffer(pH 4.0) was incubated with invertase at 30°C for 30min. The amount of reducing sugars converted by enzyme was estimated by the method of Somogyi-Nelson. One unit of enzyme activity was defined as the amount of enzyme required to increase one μg reducing sugar per minute at 30°C and pH 4.0 for 30min.

### Determination of molecular weight

The molecular weight of the purified enzyme was determined by gel filtration of sephadex G-200. The standard proteins used for calibration were chymotrypsinogen(M.W. 25,700), alcohol dehydrogenase(M. W. 40,000), eggalbumin(M.W. 45,000), bovine serum albumin(M.W. 66,000) and gulcose oxidase(M.W. 150,000).

The subunit molecular weight of enzyme was estimated by SDS-polyacrylamide gel(10%) electrophoresis.

### Measurement of isoelectric point

The isoelectric point of the purified invertase was measured by 5% polyacrylamide disc gel isoelectric focusing 0.2% ampholine(prepared by mixing pH 3.5~10 and 2.5~4 ampholites in the ratio of 1 : 2). Three identical gels (0.8×8cm) were prepared, one for the calibration of pH gradient, another for the detection of protein, and the other for the detection of invertase activity. 100μg of purified invertase was loaded on a column and electrophoresis was done at the constant voltage 100V per column at 4°C for 20hr.

### Chemicals

DEAE-Sephadex A-50, Sephadex G-200, chymotrypsinogen, alcohol degydrogenase, egg albumin, bovine serum albumin, glucose oxidase and low marker protein were purchased from Sigma Chemical Co.

Other chemicals were obtained from commercial sources.

## RESULT AND DISCUSSION

### Purification of internal invertase

The mating type a of *R. toruloides* was grown on YPG medium. The crude enzyme solution from the disrupted cells was adjusted to pH 4.0 with HCl and frequently stirred at 4°C for 20hr. The acid precipitated protein was removed by centrifugation(11,000×g, 20min) and the supernatant protein was adjusted to pH 7.0 with NaOH before applying to a column.

After dialyzed against 0.01M sodium phosphate buffer (pH 7.0) for 20hr, the protein was applied to a column(3 by 30cm) of DEAE-Sephadex A-50 containing NaCl(0~0.2M concentration). The elution pattern is given in Fig. 1. The first DEAE-Sephadex A-50 column chromatography has taken an yield of 46% in terms of total activity(Table 1).

The active fractions from the previous step were dialyzed and loaded on a column of sephadex G-200(Fig. 2). The concentrated sample was then applied to a gel filtration of Sephadex G-200(2.3 by 90cm) equlibrated with the same buffer(pH 5). This purification step has increased specific activity by about 10 folds of ion exchange step (Table 1).

The active fractions from the gel filtration were applied a column of second DEAE-Sephadex A-50 and mono Q(FPLC) column chromatography. After the mono Q (FPLC) column chromatography, the purity of the internal invertase was increased to 183.3 folds in the specific enzyme activity of the mating type a cell compared with crude extract(as shown in Table 1).

**Table 1. Purification of internal invertase from mating type a cell**

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Supernatant from sonication | 2,046 | 2,704 | 1.3 | 1 | 100 |
| Acid(pH 4) precipitation | 1,056 | 2,233 | 2.1 | 1.6 | 83 |
| First DEAE-Sephadex A-50 Column chromatography | 169 | 1,246 | 7.3 | 5.5 | 46 |
| Gel filtration on Sephadex G-200 | 14 | 947 | 67.6 | 51.2 | 35 |
| Second DEAE- Sephadex A-50 column chromato-graphy | 9 | 729 | 81.0 | 61.4 | 27 |
| Mono Q(FPLC) column Chromatography | 1 | 242 | 242.0 | 183.3 | 9 |

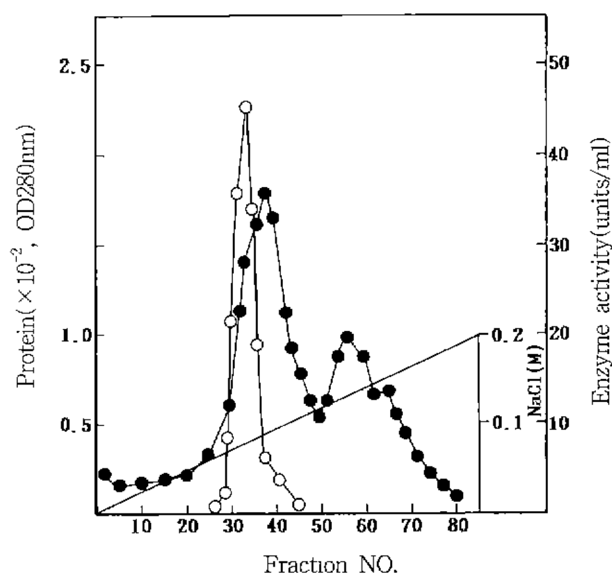


**Fig. 1. Internal invertase purification using a DEAE-Sephadex A-50 column chromatography.**
●—● : Absorbance at 280nm
○—○ : Enzyme activity

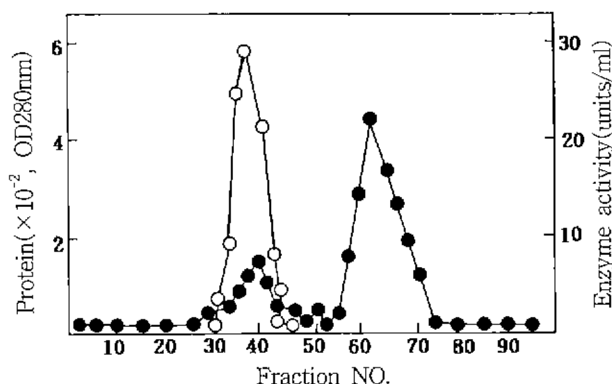


**Fig. 2. Profile of gel filtration on Sephadex G-200.**
●—● : Absorbance at 280nm
○—○ : Enzyme activity

## Homogeneity of the purified enzyme

The purified enzyme was subjected to electrophoresis at pH 9.5 on polyacrylamide gel(10%) using the discontinous buffer method. Under these conditions the enzyme appeared as a single protein band when stained with coomassie blue R-250(Fig. 3).

## Molecular weight of internal invertase

The molecular weight of the internal invertase was determined under nondenaturing and denaturing conditions. The activity of internal invertase was eluted in a fraction corresponding to molecular size of approximately 95,000 daltons by gel filtration through Sephadex G-200 (Fig. 4). The Sephadex G-200 fractions showing the enzyme activity were pooled and further analyzed by SDS-PAGE.

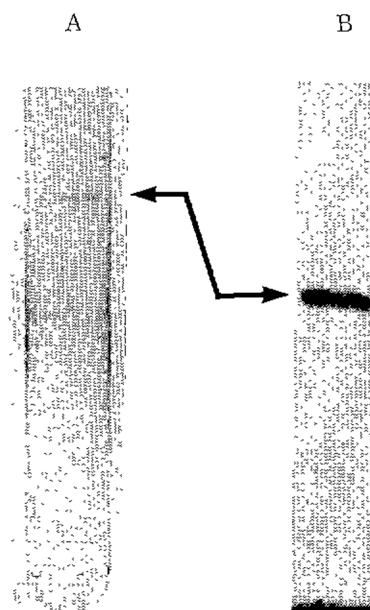


**Fig. 3. Polyacrylamide gel disc electrophoresis(A) and SDS-PAGE(B) of the purified interal invertase in mating type a.**

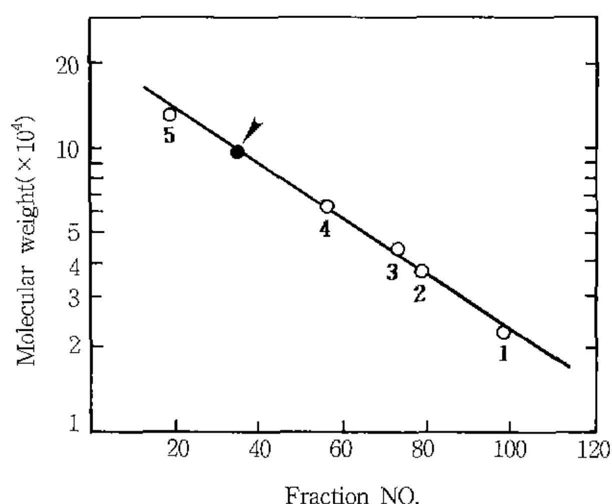


**Fig. 4. Determination of molecular weight of internal invertase by gel filtration on sephadex G-200.**
Molecular weights of standard proteins are 1; chymotrypsinogen(25,700), 2; alcohol dehydrogenase(40,000), 3; egg albumin(45,000), 4; bovine serum albumin(66,000), 5; glucose oxidase(150,000).

The coomassie blue stained protein pattern on SDS-PAGE shown in Fig. 5 demonstrates that the internal invertase obtained was essentially homogeneous and that the enzyme possibly consists of a single polypeptide chain with a molecular weight of 100,000(Fig. 5).

## Effect of metal ions and organic compounds

As shown in Table 2, the internal invertase of mating type a cell didn't show any activity change by several metal ions, only except 15.4% decrease by $Mn^{2-}$.

The serveral organic compounds(enzyme inhibitor) inhibited the invertase activity from 11.6% to 57.7% by 2-mercaptoethanol, sodium dodecyl sulfate(SDS), and phenol (Table 3).

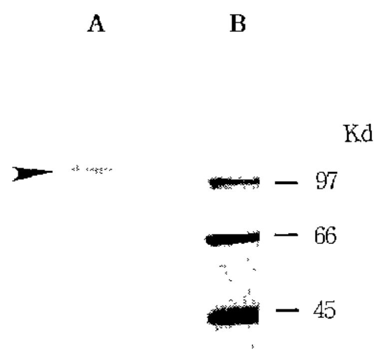


**Fig. 5. SDS-polyacrylamide gel electrophoresis of purified internal invertase.**
A: Purified internal invertase
B: Low marker protein

**Table 2. Effect of metal ions on the internal invertase activity from type a cell**

| Reagent(1mM) | Relative activity(%) |
|---|---|
| None | 100.0 |
| $CaCl_2$ | 100.0 |
| $MgSO_4$ | 100.0 |
| $ZnSO_4$ | 100.0 |
| $CoCl_2$ | 100.0 |
| $MnCl_3$ | 84.6 |

**Table 3. Effect of organic compounds on the internal invertase activity from mating type a cell**

| Reagent(1mM) | Relative activity(%) |
|---|---|
| None | 100.0 |
| EDTA | 100.0 |
| 2-Mercaptoethanol | 46.1 |
| SDS | 42.3 |
| Phenol | 88.4 |

## Thermal stability and effect of temperature

The enzyme solution prepared in 0.1M sodium acetate buffer(pH 4.0) was kept for 20min at various temperatures and assayed residual activities. As shown in Fig. 6, the invertase maintained high activity until 70°C, but showed almost complete inactivation at 80°C.

The optimal temperature for invertase was 60°C(Fig. 6).

## Optimal pH and stability of enzyme

After enzyme solutions at various pHs were incubated at 4°C for 24hr, pH stability was evaluated by measuring the remaining activity.

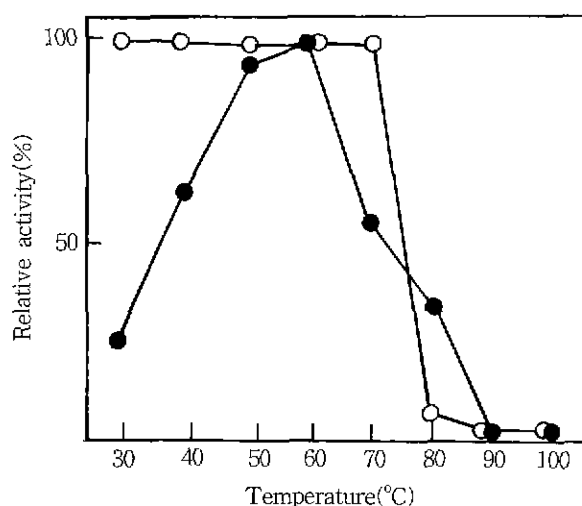


**Fig. 6. Effect of temperature and thermal stability on internal invertase from mating type a cell.**
●—●: Optimal temperature
○—○: Thermal stability

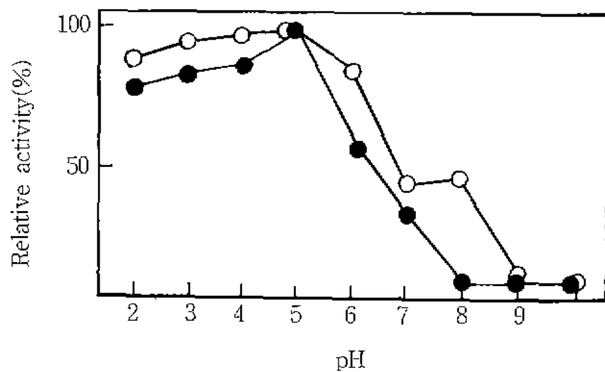


**Fig. 7. pH stability and optimal pH for invertase activity from mating type a cell.**
●—●: pH stability
○—○: Optimal pH

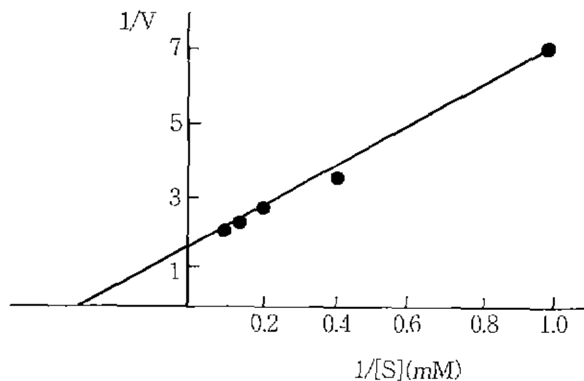


**Fig. 8. Determination of Km value.**

As shown in Fig. 7, the stability range of invertase was narrow from pH 3 to pH 6. And optimal pH was 5.0 for internal invertase produced by mating type a cell.

### Michaelis-Menten constants and isoelectric point

The Michaelis-Menten constants for the sucrose hydrolyzing activity of the enzyme is determined from Lineweaver-Burk plots, as shown in Fig. 8. The Km value for sucrose of mating type a producing invertase was calculated. The Km value of enzyme was $3.4\times10^{-3}$M.

The isoelectric point of internal invertase from a cell was determined to be about pH 5.5 by polyacrylamide disc gel isoelectric focusing.

## ACKNOWLEDGEMENTS

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