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The Comparison of the Characteristics of Partially Purified Internal Invertase by Mating Type in the Heterobasidiomycetous Yeast

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이담자 효모균의 성접합형에 따른 세포내 Invertase의 성질 비교

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Abstract — This work was carried out to study physiological characteristics of *Rhodosporidium toruloides* cells having two different mating types. The mating type A produces internal, cell wall-bound, and external invertases while type a produces only two invertases except external invertase. Comparing their characteristics after partial purification of internal invertases from both mating type cells, invertase from type a has decreased 15% of invertase activity only by Mn^{2+} while invertase from type A has been increased 11% of invertase activity by Zn^{2+} and decreased 15% of invertase activity by Mn^{2+}. On the effect of enzyme inhibitor, invertase of type a was inhibited from 12% to 57% by 2-mercaptoethanol, sodium dodecyl sulfate, phenol, but invertase of type A was slightly inhibited only by phenol. The thermal stability of both invertases has showed steep inactivation at above 80℃ and their optimal temperatures were similar at 60℃. Invertase from type A showed stability only on condition of acid from pH 3 to 6 and its opimal pH was 5.0, while invertase from type a showed stability at the wide range of pH 3~10 and its optimal pH was 4.0. And the K_m values of invertases from type A and type a were 2.5×10^{-3} M and 3.4×10^{-3} M, respectively.

The yeast, *Rhodosporidium toruloides*, has a sexual generation in life cycle and haploid strains in this yeast are classified into two mating types (type A and type a) (1). In generally, each of these cells ordinarily vegetative grows by budding. When haploid cells of the opposite types are mixed with each other, each cell forms mating tube, which recognizes the tube of mating partner by sexual conjugation, followed by cell-to-cell fusion. Consequently, the ovoid form of haploid cells becomes filamentous diploid cells.

At the initial stage of the mating process, type A cells have secreted mating pheromone, rhodotorucine A, which induces mating tube formation of type a cells (2). The sexual differentiation is characterized by the arrest of the vegetative growth in the G1 phase of the cell division cycle and subsequently the formation of an elongated mating tube (2).

α-factor (3) and a-factor (4, 5) secreted by type α cell and type a cell which are mating types induce the sexual differentiation of each cell on ascomycetous yeast *Saccharomyces cerevisiae* as well (4-6). After receiving sexual pheromone, the bud formation of these yeast cells is repressed by the arrest of G1 phase in the cell division cycle (7). The cell surface grows differently from budding and the physiological changes occur internally and externally in cells, while highly polymerized substances such as RNA and protein except DNA continue to be synthesize.

Key words: *Rhodosporidium toruloides*, invertase, mating type
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In recent studies, we have purified receptor (trigger peptidase) of sexual pheromone (rhodotorucine A) which has protease activity among cell membrane bound protein of mating type a (8), and we have reported physiological characteristics of this protein in cells by reconstitution in phospholipids liposome (9). Glycoprotein trigger peptidase didn't exist in sexual pheromone producing type A but only existed in pheromone-receiving type a.

With this experiment based on these results, we have compared their characteristics on internal invertase as target protein in order to study physiological characteristics of cells having two different mating types.

Materials and Methods

Microorganisms

Rhodosporidium toruloides IFO 0559-M-919 (haploid, mating type a, ovoid cell form, orange-colored colony) and *Rhodosporidium toruloides* IFO 0880-M-1057 (haploid, mating type a, long ovoid cell form, yellow-colored colony) stocked in our laboratory were used as strains which produce invertases (2).

Cultivation

The basal medium (YPG medium) has contained 0.4% yeast extract, 0.5% polypeptone, 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 50 μg of chloramphenicol per m*l*. The seed culture was incubated at 28℃ with reciprocal shaking for 24 hr. The main culture was performed in a 500 m*l*-flask with 200 m*l* of YPG medium and the seed culture broth was inoculated at a concentration of 1×10^6 cells per m*l*. The culture was done by continuous reciprocal shaking for 3 days.

Preparation of cell free extracts

Cells (mating type A and a) were grown at 28℃ in 500 m*l*-flasks which contained 200 m*l* of YPG medium in a reciprocal shaking incubator (K.M.C.-8480S) for 3 days and were washed twice by suspension in 200 m*l* of 10 mM phosphate buffer (pH 7.0) (buffer A), followed by centrifugation. All of the following operations were performed at 0℃ to 4℃. The washed cells have suspended in 200 m*l* of buffer A and disrupted with sonicator (Sonics and Materials inc. Model SM-15), then the cell debris was removed by centrifugation at 11,000×g for 10 min. The supernatant was used for crude internal invertase preparation.

Partial purification of internal invertase

The crude enzyme solution was adjusted to pH 4.0 with HCl and frequently stired at 4℃ for 20 hr. Acid precipitated proteins were removd by centrifugation at 11,000×g for 20 min. The supernatant solution was adjusted to pH 7.0. With NaOH before applying it to a column of DEAE-Sephadex A-50 (4 by 25 cm) equilibrated with 10 mM phosphate buffer (pH 7.0). After the column was washed with the same buffer until the optical density of the washing solution at 280 nm is decreased to below 0.1, elution was performed with 400 m*l* of NaCl (0 to 0.5 M) of linear gradient in the same buffer. Active fractions collected were partially purified internal invertase.

Assay of invertase

2% sucrose prepared in acetate buffer (pH 4.0) was incubated with invertase at 30℃ for 30 min. The amount of reducing sugars converted by enzyme was estimated by the method of Somogyi-Nelson (10). One unit of enzyme activity was defined as the amount of enzyme required to increase one μg reducing sugar per minute at 30℃ and pH 4.0 for 30 min.

Results and Discussion

The confirmation of the location of invertase on each mating type

We have examined the location of invertase on this strain because it is known that the invertase of ascomycetous yeast *Saccharomyces cerevisiae* has two kinds of internal and cell wall bound invertase (11-16). Internal crude invertase fraction, supernatant which is obtained by centrifugation (11,000×g) at 4℃ with disrupted cells, was gained to measure enzyme activity. And then, the pellet was washed 3 times with 10 mM phosphate buffer (pH 7.0) and suspended with the same buffer. This cell-debris

Table 1. Invertase activity of each cell location on mating type A and type a

Mating type	Invertase		
	Internal	Cell wall-bound	External
type A[1)]	+	+	+
type a[2)]	+	+	−

+: present, −: absent
[1)] pheromone producer, [2)] pheromone target cell

Table 2. Internal invertase activity in *Rodosporidium toruloides* mating type A and type a

Mating type	Invertase activity (μ/mg)
type A[1)]	532
type a[2)]	98

[1)] pheromone producer, [2)] pheromone target cell

solution was used to measure cell wall-bound invertase activity. External invertase activity was confirmed by the measurement of enzyme activity in cultivated solution.

As shown in Table 1, we had known that internal, cell wall-bound, and external invertase have all existed in mating type A.

On the contrary, in type a, internal and cell wall-bound invertase existed but external invertase didn't exist. As a result, there were conspicuous difference between mating type A cells and a cells as shown in Table 2.

We have came to the conclusion that there was big difference on enzyme activity as well as location when we have compared their intercharacteristics on invertase as target protein on two different mating type cells. This indicates that there is the clear difference in chracteristics of protein factor which dominates physiological activites according to mating type even in the same strains.

Effect of metal ions and organic compounds

As shown in Table 3, the enzyme of mating type A showed that apporoximatly 11.1% of invertase activity is increased by Zn^{2+} and 42.2% of invertase activity is decreased by Mn^{2+}, but the invertase of mating type a cell didn't show any change only except 15.4% of decrease by Mn^{2+}.

The effect of internal invertase by several organic

Table 3. Effect of metal ions on the internal invertase activity

Reagent (1 mM)	Relative activity (%)	
	type A	type a
None	100.0	100.0
$CaCl_2$	100.0	100.0
$MgSO_4$	100.0	100.0
$ZnSO_4$	111.1	100.0
$CoCl_2$	100.0	100.0
$MnCl_2$	57.8	84.6

Table 4. Effect of organic compounds on the internal invertase activity

Reagent (1 mM)	Relative activity (%)	
	type A	type a
None	100.0	100.0
EDTA	100.0	100.0
2-mercaptoethanol	120.0	46.1
SDS*	93.3	42.3
Phenol	82.2	88.4

*Sodium dodecyl sulfate

compounds (enzyme inhibitor) was that invertase produced by type a was inhibited from 11.6% to 57.7% by 2-mercaptoethanol, sodium dodecyl sulfate (SDS), phenol (shown in Table 4). But invertase of type A has showed 20% of increasing effect with 2-mercaptoethanol while has showed some inhibition with only phenol. We suggest that this result is the physiologically characterizing index on cells which have two different mating types.

Thermal stability and effect of temperature

The protein of the both enzyme solutions prepared in 0.1 M sodium acetate buffer (pH 4.0) were kept for 20 min at various temperatures and assayed residual activities. As seen in Fig. 1-A, the invertases producted by two mating type cells have maintained high invertase activity until 70℃, but both invertases have shown steep inactivation at 80℃. The optimal temperatures for both invertases were 60℃ similarly (shown in Fig. 1-B). On the thermal stability and optimal temperature of both invertases, there were nearly no difference.

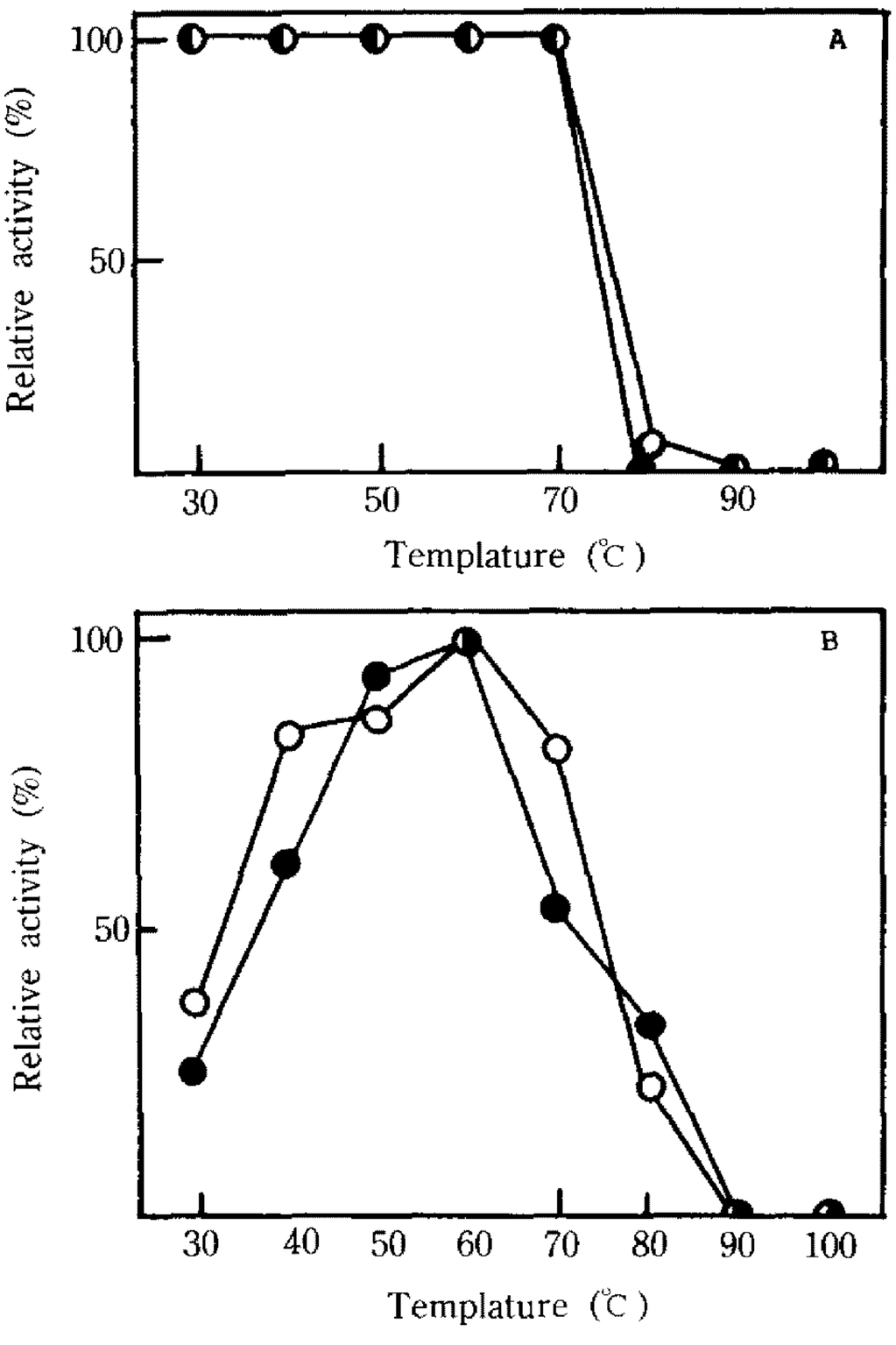

Fig. 1. Effects of temperature and thermal stability on internal invertase.
A: Thermal stability of internal invertase from mating type A and type a.
B: Effect of temperature on internal invertase from mating type A and type a.
—○—; mating type A invertase activity, —●—; mating type a invertase activity.

Optimal pH and pH stability of enzyme

After both enzyme solutions at various pHs had been incubated at 4℃ for 24 hr, we could know pH stability by measuring the remaining activities. As shown Fig. 2-A, the stability range of invertase produced by type a was narrow from pH 3 to pH 6 while taht of invertase produced by type A was very wide from pH 3 to pH 10.

As shown in Fig. 2-B, the optimal pH was 4.0 for invertase produced by type A and 5.0 for invertase produced by type a, respectively.

Michaelis-Menten constants

The Michaelis-Menten constants for the sucrose-

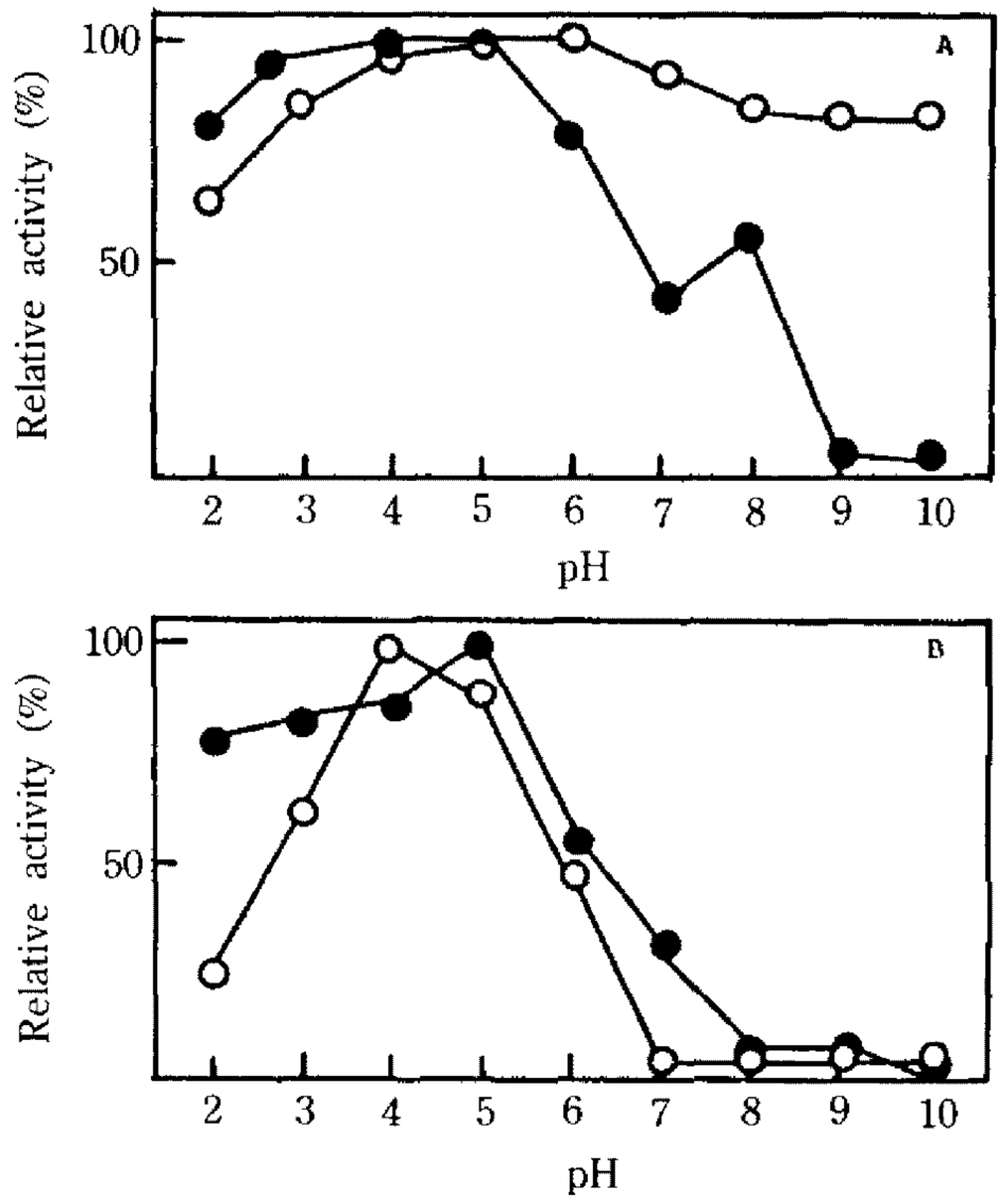

Fig. 2. Effect of pH stability for invertase activity.
A: Effect of pH on internal invertase stability.
B: Optimal pH on internal invertase.
—○—; mating type A invertase activity, —●—; mating type a invertase activity.

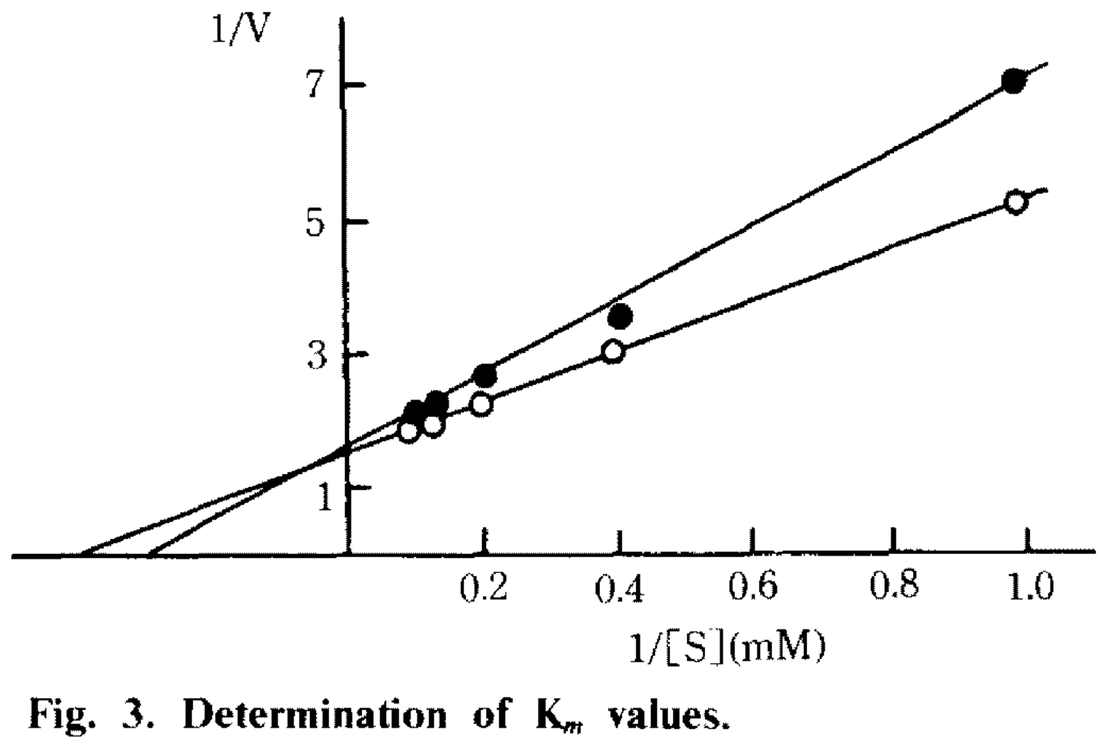

Fig. 3. Determination of K_m values.
—○—; mating type A invertase, —●—; mating type a invertase

hydrolyzing activities of the both enzymes are determined from Lineweaver-Burk plots, as shown in Fig. 3. The K_m values for sucrose of type A and type a producing invertase calculated. The K_m values of invertase from type A and type a were 2.5×10^{-3} M and 3.4×10^{-3} M, respectively.

요 약

서로 다른 성접합형 A와 a를 가지는 *Rhodosporidium toruloides*가 생산하는 invertase를 조사한 결과, 접합형 A는 세포내, 세포벽 결합, 세포의 분비성 invertase의 3가지를 생산하였으나, 접합형 a는 세포외 invertase를 생산하지 않는 접합형 특이성이 있는 것을 알았다. 양 접합형 세포로부터 세포내 invertase를 부분정제하여 이들의 성질을 비교한 결과, 접합형 A 세포 유래의 효소는 Zn^{2+}에 의하여 11%의 활성 상승효과와 Mn^{2+}에 의한 42.2%의 감소를 보이는 반면, a세포 유래의 효소는 Mn^{2+}에 의한 15%의 활성 감소만을 보였다.

효소 저해제의 효과에서 a세포의 invertase는 2-mercaptoethanol, sodium dodecyl sulfate, phenol에만 약간 저해를 받을 뿐이었다. 이들 양 효소의 열안정성은 모두 80℃ 이상에서 급격히 실활되는 양상을 보였으며 최적 온도가 60℃로 둘 다 비슷하였다.

a세포 유래의 invertase는 pH 3에서 pH 10까지 넓은 pH의 범위에서 안정하며 최적 pH가 4.0인 반면, A세포의 invertase는 pH 3에서 pH 6까지 산성의 조건에서만 안정하였으며 최적 pH는 5.0으로 나타났다. 그리고, A세포와 a세포 유래 invertase의 K_m값은 각각 2.5×10^{-3} M과 3.0×10^{-3} M이였다.

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