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Purification of Internal Invertase in *Rhodosporidium toruloides* Mating Type A Cells

Yong-Kee Jeong† and Beung-Ho Ryu*

Dept. of Microbiology, Dong Eui University, Pusan 614-714, Korea
**Dept. of Food Science and Technology, Kyung Sung University, Pusan 608-736, Korea*

Abstract

The internal invertase was purified from cell free extract of *Rhodosporidium toruloides* IFO 0559-M-919 by acid precipitation, ion-exchange chromatography and gel filtration to the unique enzyme protein on disc electrophoresis. We have found out that molecular weight of purified internal invertase was 90,000 by gel filtration and the purified enzyme was protein with 4 homogeneous subunits appearing as single band of 22,000daltons on SDS-polyacrylamide gel electrophoresis.

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

INTRODUCTION

Heterobasidiomycetous yeast *Rhodosporidium toruloides*[1] has compatible mating type A and a. In the life cycle of the *R. toruloides*, the alteration from the sexual to asexual mode of growth is achieved by conjugation of two yeast from haploid cells compatible mating types.

The sexual cell interaction is mediated by mating-type-specific mating pheromones secreted by the haploid cells[2].

For that reason, specificity for the opposite cell of both cells is very high. On our study recently, we have found out that the receptor which has protease activity and receives pheromone of rhdotorucine A which mating type A produce has high mating-type-specificity existing in only mating type a[3].

Therefore, it is regarded that there will be some difference of physiological characteristics depending on mating-type-specificity in each mating strain (type A and a). For the purpose of grasping the above, we have studied invertase which is glycoprotein with as target protein.

Invertase (EC 3.2.1.26) is widely distributed throughout plants[4-7], animals, and various microorganisms[8-22].

In particular, many a study on invertase from yeast have been carrying out until now since Santiago and Robert had begun to study[21,22]. We, on this study, have studied purification of internal invertase with a view to comparing with the specificity of invertase existing in each mating type.

First of all, we are going to report the result, for the internal invertase of mating type A has purified to the single band on SDS-PAGE.

MATERIALS AND METHODS

Microorganism

Rhodosporidium toruloides IFO 0559-M-919 (haploid mating type A, ovoid cell form, orange-colored colony) stocked in our laboratory were used as strains which produce a invertase[2].

† To whom all correspondence should be addressed

Cultivation

We have used YPG medium[23] contained 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 1×10^6 cells per ml. The culture was done by the continuous reciprocal shaking at 28° C for 3days.

Preparation of crude internal enzyme fraction

Mating type A cells (1×10^8 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 (Sanics and Materials Inc., Model SM15), 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 requird 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), egg albumin (M. W. 45,000), bovin serum albumin (M. W. 66,000) and glucose oxidase (M.W. 150,000). The subunit molecular weight of enzyme was estimated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide.

Chemicals

DEAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-200, chymotrypsinogen, alcohol dehydrogenase, 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 in 500-ml shake flasks, each containing 200ml of YPG medium under the conditions as given in the "Method". 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 it to a column.

After dialyzed against 0.01M sodium phosphate buffer (pH 7.0) for 20hr, the protein was applied to a column (4 by 25cm) of DEAE-Sephadex A-50 containing NaCl (0~0.6M concentration). The elution pattern is given in Fig. 1.

The DEAE - Sephadex A-50 column chromatography has taken an yield of 47% in terms of total activity(Table 1). The active fractions from the previous step were dialyzed and loaded on a SP-Sephadex C-50 column (2.5 × 18cm) that had been equilibrated with the same buffer. The column was eluted with buffer A containing 0.2M NaCl. This step slightly has increased specific activity. The active fractions from SP-Sephadex C-50 column chromatography were pooled and dialyzed against buffer A (pH 5). Proteins were concentrated about 1/15 of original volume with freezing dryer.

Table 1. Purification of internal invertase from mating type A cells

Purification step	Volumn (ml)	Total protein (OD 280nm)	Total activity (unit)	Specific activity (unit/OD280nm)	Purification (fold)
Supernatant from sonicator	201	5587.2	60600	10.8	1.0
Acid(pH4) precipitation	165	2793.6	49692	17.8	1.6
Dialysis	153	1808.5	40785	22.6	2.1
First DEAE-Sephadex A-50 column chromatography	66	188.6	28549	151.3	14.0
SP-Sephadex C-50 column chromatography	32	52.3	9516	182.0	16.9
Gel filtration on Sephadex G-200	20	10.3	7803	757.6	70.1
Second DEAE-Sephadex A-50 column chromatography	17	5.8	6710	1156.9	107.1

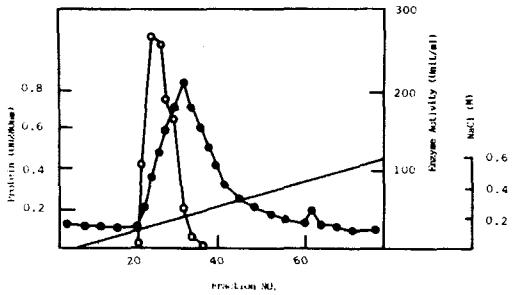

Fig. 1. Internal invertase purification using a DEAE-Sephadex A-50 column chromatograpohy.
●——● ; absorbance at 280nm
○——○ ; enzyme activity

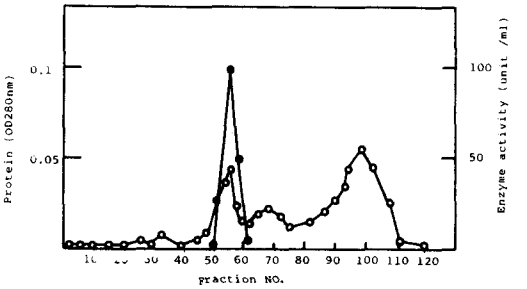

Fig. 2. Gel filtration on Sephadex G-200.
●——● ; enzyme activity
○——○ ; absorbance at 280nm

The concentrated sample was then applied to a column of Sephadex G – 200 (2.3 by 90cm) equilibrated with the same buffer (pH 5). This purification step has increased specific activity of about 4 folds (Table 1). The gel filtration pattern is shown in Fig. 2.

The active fractions from the gel fitration were applied to a column of second DEAE-Sephadex A-50 (1 by 18cm). After the second DEAE-Sephadex A-50 column chromatography, the purification of the internal invertase was achieved 107 folds relative to the specific enzyme activity of the mating type A cell(as shown in Table 1).

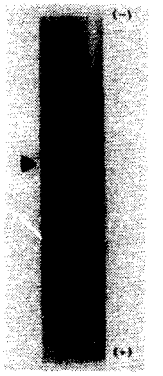

Fig. 3. Polyacrylamide gel disc electrophoresis of the purified internal invertase.

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 has appeared as one protein band when stained with Coomasii blue R-250.

The characteristics of polyacrylamide electrophoresis are shown in Fig. 3. It has verified the extreme purification of the internal invertase in mating type A cell.

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 a molecular size of approximately 90,000 daltons by gel filtration through Sephadex G 200 (Fig. 4).

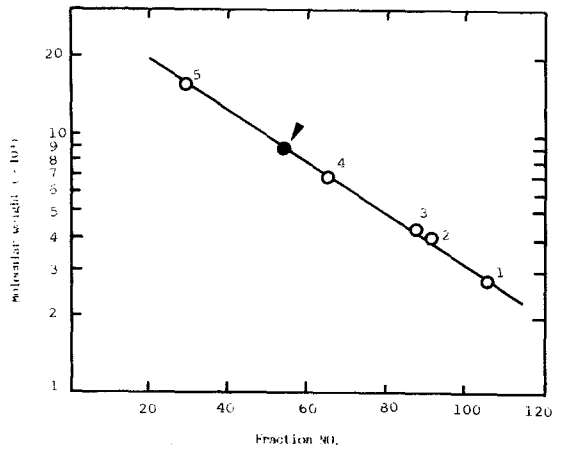

Fig. 4. Determination of moleculer weight of internal invertase by gel filtration on Sephadex G-200.
Molecular weight of standard protein are 1 ; chymotrypsinogen (25,700), 2 ; alcohol dehydrogenase (40,000), 3 ; egg albumin (45,000), 4 ; bovine serum albumin (66,000), 5 ; glucose oxidase (150,000)

The Sephadex G 200 fractions containing the enzyme were polled and further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The Coomasii blue stained protein pattern on SDS-PAGE shown in Fig. 5-B demonstrats that the internal invertase obtained was essentially homogeneous and that the enzyme possibly consists of a single (Fig. 5-A and 5-B).This result indicates that the invertase consist of four similar subunits, each having a molecular weight of 2,200.

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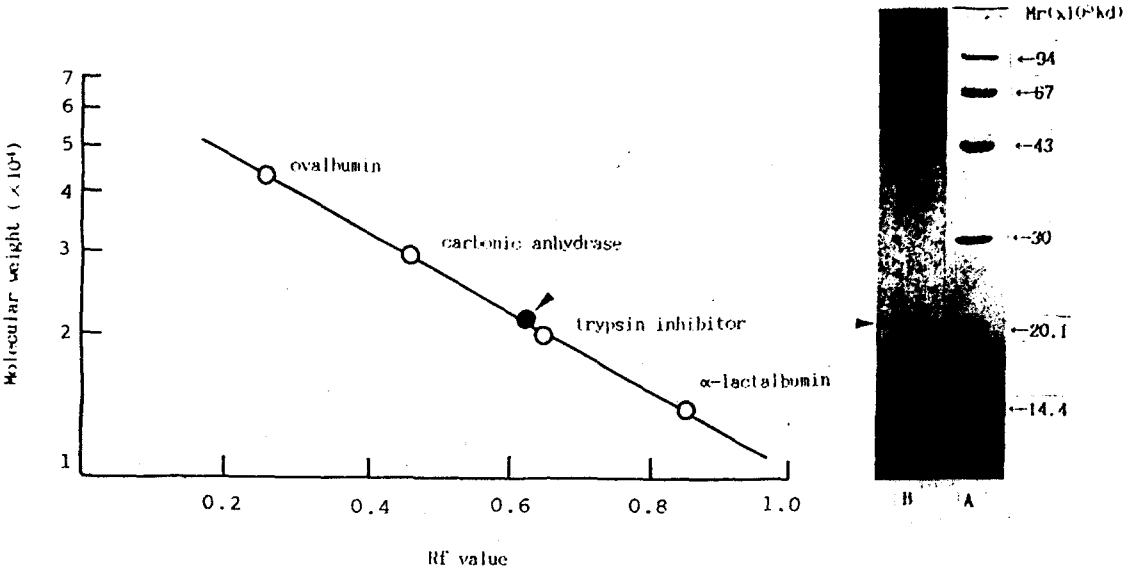

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified internal invertase.
A ; low marker protein, B ; purified internal invertase

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*Rhodosporidium toruloides*의 접합형 A 세포내 Invertase의 정제

정영기† · 류병호*

동의대학교 자연과학대학 미생물학과
*경성대학교 공과대학 식품공학과

요 약

이담자 효모균 *Rhodosporidium toruloides*의 mating type A 세포에서 세포내 invertase를 정제하였다. 세포내 invertase는 배양 세포의 파쇄액을 산침전 시킨 후 그 상등액으로부터 DEAE-Sephadex A-50, SP-Sephadex C-50 column chromatography와 Sephadex G-200 gel fitration 등의 과정을 거쳐 polyacrylamide gel disc 전기영동상 단일 효소 단백질까지 정제되었다. 정제효소의 분자량은 gel filtration에 의하여 90,000이었고, SDS-PAGE상에서는 22,000 dalttons에서 단일 band를 보여 단일종의 subunit가 4개로 구성된 단백질로 추정된다.