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Comparative Studies of Invertase Isozymes Produced by *Rhodotorula glutinis* K-24

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Rhodotorula glutinis K-24가 생산하는 Invertase Isozymes 군에 관한 비교 연구

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***Rhodotorula glutinis* K-24 was found to produce internal, cell wall bound, and external invertase. Internal invertase was purified by column chromatographies on DEAE-Sephadex A-50, SP-Sephadex C-50, gel filtration on Sephadex G-200 and isoelectric focusing. Cell wall bound invertase was partially purified by the following procedures; column chromatography on DEAE-Sephadex A-50 and gel filtration on Sephadex G-100. Optimum pH and temperature for enzymatic activities of internal and cell wall bound invertase were pH 3.0 and 60°C, respectively. Both enzymes were inhibited by $HgCl_2$, $AgNO_3$, $MnSO_4$, and sodium dodecylsulfate. The molecular weights of internal and cell wall bound invertases were estimated to be 310,000 and 67,000, respectively. Other physicochemical properties of the both enzymes were similar.**

Invertase (EC 3.2.1.26) is widely distributed throughout plants (1-11), animals (12), and various microorganisms (13-25). This enzyme catalyzes two reactions, the hydrolysis of the sucrose to yield fructose and glucose and a reaction which transfers the fructose moiety to acceptor molecule. Most of the invertase preparations described in the past have had varying amounts of carbohydrate with them. Generally, invertase are known as glycoproteins. However, the content of glycomoiety has been reported to vary somewhat according to the source and to the origin of the enzyme.

Various yeasts are known to produce cell wall bound and internal invertase, but not external invertase. Only a small amounts of invertase in the case of *Saccharomyces cerevisiae* was reported to be liberated in medium when the cells were incubated at concentration of glucose lower than 1.0%, due to its catabolite repression (25).

The role of cytoplasmic membrane in the secretion of enzyme by microorganisms (26-29) and animal cells (30) has aroused great interest in recent years. Yeast invertase is an attractive system with which this phenomenon is studied.

In our study, *Rhodotorula glutinis* K-24 was employed as a producer of various invertase isozymes. It was found that this yeast produce not only the large amount of cell wall bound and internal invertase but also invertase in medium (31). The invertase activity in culture medium significantly increased in high concentration of glucose with the cell growth. These facts clearly indicate that *Rh. glutinis* is not subjected to

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catabolite repression for the secretion of external invertase. From this point of view, *Rh. glutinis* is particularly interesting microorganism, compared with other yeasts. Additionally, this yeast also contains a large amounts of invertase in cell wall. This enzyme is solubilized from cell wall by the action of its lytic enzyme. In other yeasts, it was reported that cell wall bound invertase was localized in the intermural space, therefore the presence of glucosamine and mannan in this enzyme suggests the possibility that the enzyme may be covalently attached to a cell wall component.

This paper describes the purification and some properties of internal and cell wall bound invertases.

Materials and Methods

Microorganisms

Rh. glutinis K-24 and *Bacillus pumilus* stocked in our laboratory were used as strains which produced invertases and cell wall lytic enzyme.

Cultivation

The basal medium contained 7.0% glucose, 2.0% polypeptone, 0.5% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 4 ppm $MnSO_46H_2O$, 4 ppm $FeSO_4.7H_2O$, and 2 ppm thiamin. The seed culture was incubated at 25°C with continuous reciprocal shaking for 3 days. The main culture was performed in 10 *l* fermentor (B. Braun Melsurgen Co.) and the seed culture was inoculated at a concentration of 2% (V/V). The culture was continuously aerated at the rate of 10 *l* per min and agitated at 300 rpm, the fermentation temperature being maintained at 25°C. The cultivation was stopped at the maximum point of cell growth.

Preparation of cell free extracts

Cells were harvested by centrifugation at 10,000g for 10 min, washed twice, suspended in 1 *l* of 0.01 M sodium phosphate buffer (pH 7.0), and then disrupted with a Dyno Mill Disintegrator at below 10°C. The disrupted solution was adjusted to pH 5.5 by acetic acid solution of 30% and the mixture was kept at 4°C for 24 hr. Then the cell debris was removed by centrifugation at 10,000g for 10 min. The supernatant was used for internal invertase preparation and the precipitate was used for preparation of cell wall. That is, unbroken cell and cytoplasmic materials were separated from walls by differential centrifugation until the walls were virtually free from these materials.

Assay of invertase

Sucrose solution (2%) prepared in acetate buffer (pH 4.0) was incubated with invertase at 30°C for 10 min. The amount of reducing sugars converted by enzyme was estimated by the method of Somogyi-Nelson (32). One unit of enzyme activity was defined as the amount of enzyme increasing one of optical density at 660 nm for 10 min.

Solubilization of cell wall bound invertase

Cell wall lytic enzyme purified from *B. pumilus* by the method in the literature (33) was used for solubilization of cell wall bound invertase. The separated cell walls were suspended in acetate buffer, pH 5.5 and incubated at 37°C for 2hr with cell wall lytic enzyme. Insoluble materials in the reaction mixture were removed by centrifugation. The supernatant was used for the preparation of cell wall bound invertase.

Sugar composition

Determination of total sugar content was performed by phenol sulfuric acid method of Dubois *et al.* (34).

Estimation of molecular weight

Gel filtration by the method of Andrews (35) was applied to the estimation of molecular weights of the invertases. A column of Sephadex G-200 was employed and protein concentration was assayed by measuring the optical density at 280 nm. Elution was carried out with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The following proteins were employed as molecular weight markers; apoferritin (M.W. 443,000), alcohol dehydrogenase (M.W. 150,000), bovine serum albumin (M.W. 67,000), ovalbumin (M.W. 44,000), lysozyme (M.W. 14,000). Subunit molecular weight was estimated by the method of Weber and Osborn (34).

Measurement of isoelectric point

Isoelectric point of the invertase was determined by the method of Vesterverg (36). The enzyme solution was dialyzed against distilled water before being used. A 110 m*l* column (LKB) was employed for isoelectric focusing using 1% Ampholine (prepared by

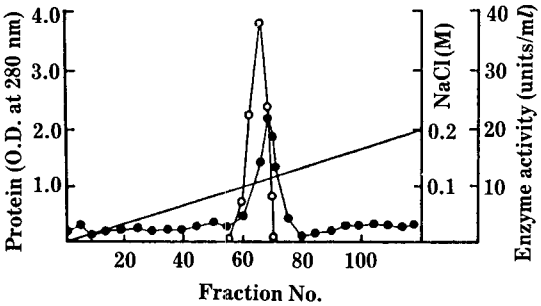

Fig. 1. Elution profile of ion exchange chromatography on DEAE – Sephadex A-50.

● – ●; absorbance at 280 nm, ○ – ○; enzyme activity

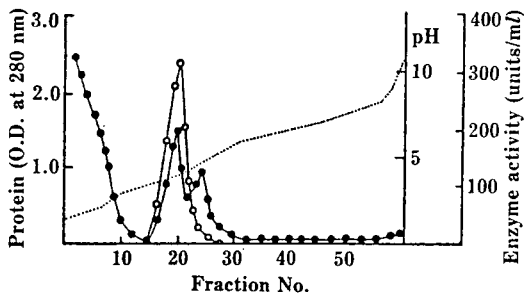

Fig. 2. Elution profile of isoelectric focusing on Ampholine gradient column.

● – ●; absorbance at 280 nm, ○ – ○;enzyme activity, ...; pH gradient

mixing pH 2.5-4.0 and 5.0-8.0 Ampholines in the ratio of 1:1). It was carried out for 72 hr, 700 V, at 4°C. The eluate was collected as 3 m*l* fraction.

Chemicals

Sephadex G-200, Sephadex G-100, SP-Sephadex C-50, DEAE-Sephadex A-50, apoferritin, alcohol dehydrogenase, bovine serum albumin, ovalbumin, and lysozyme were purchased from Sigma Chemical Co. Other chemicals were obtained from commercial sources.

Results

Purification of internal invertase

The crude enzyme solution from the disrupted cell suspension was dialyzed with 0.01 M sodium phosphate buffer (pH 7.0). DEAE-Sephadex A-50, previously equilibrated with the same buffer, was added to the enzyme solution. After 2 hr with frequent stirring, almost the internal invertase was adsorbed. The supernatant was discarded and the ion-exchanger was washed twice with 1 *l* of fresh buffer. The adsorbed internal invertase was eluted with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl. The purification in this step was approximately 5 fold. The pooled eluates from batch adsorption were dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, and applied to a column (3 × 30 cm) of DEAE-Sephadex A-50. The enzyme was eluted by the linear gradient with same buffer, containing NaCl (0-0.2 M concentration). The elution pattern is given in Fig. 1. DEAE-Sephadex column chromatography afforded a purification of 50 fold in terms of specific activity (Table 1).

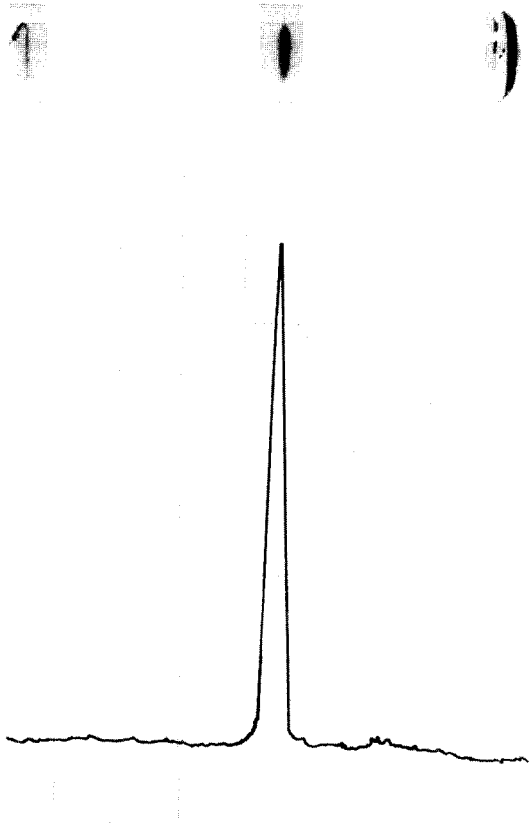

Fig. 3. Polyacrylamide gel disc electrophoresis and densitometric tracing of the purified internal invertase.

The active fractions from the previous step were pooled. concentrated, and dialyzed against 0.01 M sodium citrate buffer, pH 3.8. The precipitate was removed by centrifugation at 10,000g for 10 min. The supernatant was concentrated and loaded on a

SP – Sephadex C-50 column (2×20 cm) that had been equilibrated with same buffer. The column was eluted using same buffer, for this enzyme had lower partition coefficient (α) than one. This purification step increased specific activity of about 2 fold (Table 1).

The pooled eluates from SP-Sephadex C-50 column chromatography were dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, concentrated to 2 m*l* solution and then loaded on a Sephadex G-200 column (2.5×90 cm) which had been equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. Elution was done isocratically with same buffer containing 0.1 M NaCl. This step slightly increased specific activity.

The active fractions from the gel filtration were subjected to isoelectric focusing. As seen from Fig. 2, the enzyme activity is found as a single peak. The isoelectric point of the enzyme was pH 4.0.

Homogeneity of the purified enzyme

The purified enzyme was subjected to electrophoresis at pH 9.5 on polyacryamide gel (10%) using the discontinuous buffer method. Under these conditions the enzyme appeared as one protein band when stained with Amido Black 10 B. Densitometric tracing mode and the characteristic of PAGE are shown in Fig. 3. It verified the extreme purification of the internal invertase.

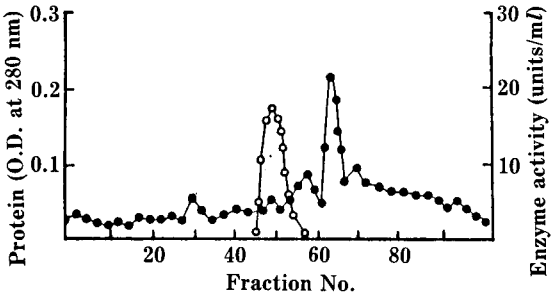

Fig. 4. Gel filtration on Sephadex G-100.
● – ● ; absorbance at 280 nm, ○ – ○ ; enzyme activity

Purification of cell wall bound invertase

The purified cell wall was hydrolyzed by its lytic enzyme and released in reaction solution about 80% of cell wall bound enzyme. The enzyme activity in solution reached maximum after 2 hr of reaction, containing a large amount of sugars. After centrifu-

Table 1. Purification procedures of internal and cell wall bound invertase from *Rh. glutinis* K-24.

	Purification step	Volume (m*l*)	Total protein (OD 280× m*l*)	Total activity (Units)	Specific activity (Units/OD 280)	Yield (%)
(A)	Homogenate	1070	10421.4	53500.0	5.1	100
	Dialysis	1080	4210.0	45360.0	10.8	85
	Batch DEAE-Sephadex A-50 column chromatography	190	752.4	32580.0	48.5	68
	DEAE-Sephadex A-50 column chromatography	60	92.8	24990.0	269.3	47
	SP-Sephadex C-50 column chromatography	120	39.6	19500.0	492.4	36
	Gel filtration on Sephadex G-200	20	25.2	15000.0	595.2	28
	Isoelectric focusing	21	5.3	7665.0	1446.2	14
(B)	Crude enzyme	175	437.5	656.2	1.5	100
	Dialysis	180	189.0	540.0	2.9	83
	DEAE-Sephadex A-50 Column chromatography	107	48.2	331.7	6.9	51
	Gel filtration on Sephadex G-100	24	8.4	129.6	15.4	20

A; Internal invertase purification procedure
B; Cell wall bound invertase purification procedure

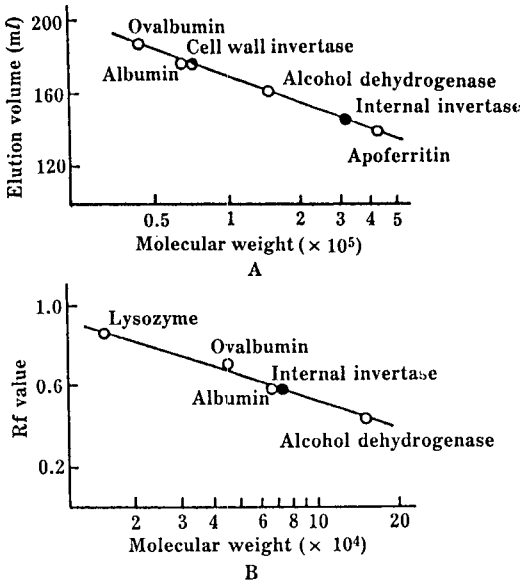

Fig. 5. Determination of molecular weight of internal and cell wall bound invertases.
A; Molecular weight of internal and cell wall bound invertase estimated by gel filtration on Sephadex G-200.
B; Subunit molecular weight of internal invertase estimated by SDS polyacrylamide gel electrophoresis.

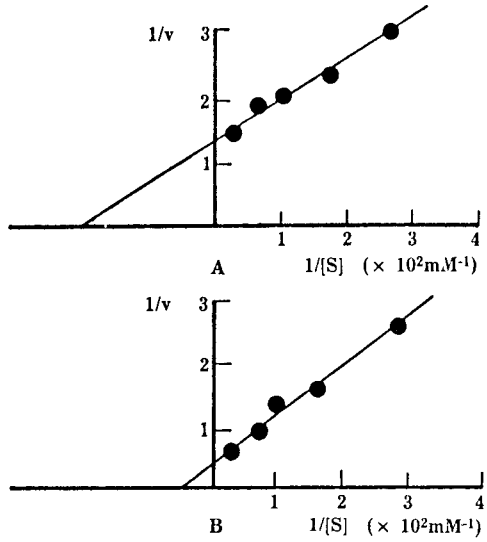

Fig. 6. Determination of K_m values.
A; Internal invertase
B; Cell wall bound invertase

gation, the supernatant was used in following purification procedures. The enzyme solution was dialyzed against 0.01 M sodium phosphate buffer, pH 7.0 and applied to a column (3×30 cm) of DEAE-Sephadex A-50.

The active fractions from DEAE-Sephadex A-50 column chromatography were pooled, concentrated by ultrafiltration and loaded to a Sephadex G-100 column (2.5×90 cm) that had been equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The elution pattern is given in Fig. 4. The active fractions were shown as a single peak, but not purified homogeneously. This enzyme solution was used to illustrate the enzymatic and physicochemical properties of the cell wall bound invertase without further purification. The steps of a typical purfication of both enzyme, internal and cell wall bound invertase, are given in Table 1. The specific activity of the final preparation increased 238 fold for internal invertase and 10 fold for cell wall invertase.

Molecular weights

Gel filtration of the purified both enzymes on Sephadex G-200 was carried out in order to estimate their molecular weights. As seen in Fig. 5, The molecular weights of internal and cell wall bound invertases are estimated to be 310,000 and 67,000, respectively. The subunit molecular weight of internal invertase estimated by SDS-polyacrylamide gel electrophoresis was 70,000 as shown in Fig. 5. This result indicates that the internal invertase consists of four similar subunits, each having a molecular weight of 70,000. The subunit molecular weight of cell wall bound invertase was not estimated.

Michaelis-Menten constants

The Michaelis-Menten constants for the sucrose hydrolyzing activities of the both enzymes are determined from Lineweaver-Burk Plots, as shown in Fig. 6. The K_m values for sucrose of internal and cell wall bound invertase were calculated to be 4.3×10^{-3}M and 2.1×10^{-2}M, respectively.

Effect of pH and pH stability for enzyme activity

As shown in Fig. 7, the both enzyme activities are maximum at pH 3.0, identically. After the both enzyme solutions at various pHs had been incubated at 20°C for 24 hr, the remaining activities were determined. The pH ranges for stability of the both enzymes were observed to be pH 5.0 to 8.0 and pH 4.0 to 9.0, respectively.

Thermal stability and effect of temperature

The protein of the both enzymes solution pre-

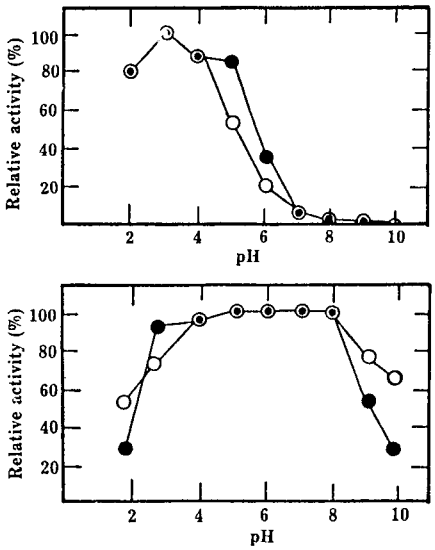

Fig. 7. Effect of pH stability for enzyme activity.
A; Optimal pH on internal and cell wall bound invertase B; Effect of pH on internal and cell wall bound invertase stability, ● – ●; internal invertase activity, ○ – ○; cell wall bound invertase activity.

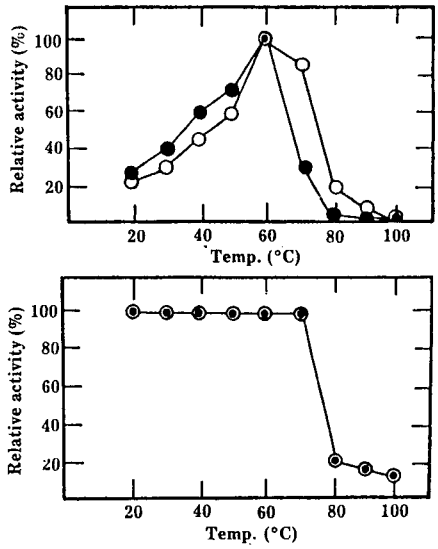

Fig. 8. Effect of temperature and thermal stability.
A; Effect of temperature on internal and cell wall bound invertase, B; thermal stability of internal and cell wall bound invertase, ●–●; internal invertase activity, ○–○; cell wall bound invertase activity.

pared in 0.1 M sodium acetate buffer, pH 4.0, were kept for 20 min at various temperatures and assayed residual activity. As seen in Fig. 8, the both enzymes are stable to 70°C, but drastically unstable above 80°C, identically. The maximum activities of the both enzymes were observed at 60°C in a same fashion.

Effects of inorganic salts and inhibitors

As shown in Table 2, the both enzymes are identically inhibited by $HgCl_2$, $AgNO_3$ and $MnSO_4$. Among the inhibitors tested, *p*-CMB, SDS, and NaCN strongly inhibited enzyme activity with 1.0 mM concentration.

Other characteristics of the internal invertase

The extinction coefficient ($E^{1\%}_{280\,nm}$) of the internal invertase was about 7.0. This enzyme was glycoprotein containing carbohydrate of about 41%.

Discussion

Since invertase was first isolated, many enzymes have been purified from a variety of sources and have been extensively studied. Although most of invertase preparations described in the past have had internal and intermural (cell wall) localization, a few microorganisms produced external invertase into the medium. But there in not a yet reported that the yeast invertase is liberated in medium, except *Saccharomyces cerevisie* which produces limited external invertase only when incubated at concentration of glucose lower than 1.0% (25, 37). Because synthesis of yeast external invertase depends on glucose concentration and perhaps on other hexoses too, the enzyme is synthesized only when the cells are incubated at concentration of hexose lower than one percent. The details for glucose (hexose) repression are unknown but it could take place at several levels; (a) by inhibition of DNA transcription into the invertase mRNA, (b) by inhibition of invertase mRNA translation into the enzymatic protein, (c) by inhibition of glycosylation and enzyme secretion, (d) by catabolite inhibition of the enzyme activity (25).

In our investigation, *Rh. glutinis* K-24 was found to liberate external invertase, and also produced internal and cell wall bound invertase. The enzyme activity in culture medium was increased gradually with cell growth. Accordingly, this enzyme was thought not to be detached from intermural space and not to be leaked by lysis of cells but to be secreted contitutively.

Although external invertase experiment datum in our laboratory has not been reported up to now, we compare the external enzyme with that of inter-

Table 2. Effects of inorganic salts and organic compounds on internal and cell wall bound invertase activity.

Reagents	Relative activity (%) 0.1 mM A	0.1 mM B	1.0 mM A	1.0 mM B
None	100	100	100	100
$Pb(NO_3)_2$	94	110	92	85
$NiCl_2$	103	122	96	74
$ZnSO_4$	113	98	79	81
$HgCl_2$	19	16	5	4
$AgNO_3$	36	28	36	3
$CoCl_2$	98	119	100	79
$FeCl_2$	100	119	91	104
$CaCl_2$	92	112	83	77
$MnSO_4$	90	53	40	35
EDTA	82	100	66	82
MIA	66	97	43	97
p-CMB	22	38	17	28
Phenol	98	103	85	92
NaCN	70	98	27	60
2-Mercapto-ethanol	70	83	70	83
SDS	75	75	79	34

A; Relative activity on internal invertase
B; Relative activity on cell wall bound invertase
EDTA; Ethylendiaminetetraacetic acid
MIA; Monoiodoacetic acid
p-CMB; *p*-chloromercuric benzoate
SDS; Sodium dodecylsulfate

nal and cell wall bound invertase in Table 3, in order to demonstrate some properties and the relative functions among three isozymes. Molecular weight of internal invertase was estimated to be 310,000, and consists of four identical subunits, which is the largest molecular size among them. And it is glycoprotein that has about 40% carbohydrate content, similar to internal invertases of other origin.

External invertase was best produced at 4% glucose concentration, without its catabolite repression and has no relationship with sucrose concentration for enzyme production in medium. The molecular weight of this enzyme was about 220,000 by gel filtration. SDS-polyacrylamide gel electrophoresis gave a monomeric molecular weight of about 110,000. Thus, the enzyme may have two equimolar subunits with a molecular weight of 110,000.

Table 3. Summary of properties of internal, cell wall bound, and external invertase.

Properties/ Isozymes	Internal	Cell wall bound	External*1
Molecular weight	310,000	67,000	220,000
K_m for sucrose(M)	4.3×10^{-3}	2.1×10^{-2}	1.0×10^{-2}
Optimum temp. for activity	60°C	60°C	60°C
Temp. stability	70°C	70°C	40°C
pH stability	5-8	4-9	3-9
Effects of inorganic salts	$HgCl_2$	$HgCl_2$	$HgCl_2$
	$AgNO_3$	$AgNO_3$	$AgNO_3$
	$MnSO_4$	$MnSO_4$	$MnSO_4$
Effects of organic compounds	SDS	SDS	SDS
	p-CMB	*p*-CMB	*p*-CMB
	MIA		MIA

*1; The datum have not been reported
SDS; Sodium dodecylsulfate
p-CMB; *p*-chloromercuric benzoate
MIA; Monoidoacetic acid

The molecular weight of cell wall bound invertase was calculated to be 67,000, the smallest molecular size among them. But the molecular weight of subunits was not evaluated due to its impurity. This enzyme was solubilized in reaction mixture only by the action of cell wall lytic enzyme. Thus, this enzyme may be strongly bound with glucomannan layer, core component of cell wall.

The physicochemical properties of pH, temperature, and stability *etc.* for each invertase isozyme activity are approximately similar. And also it has been shown that effects of various inhibitors for enzyme activity are far similar. In these results, three isozymes seem to have the similar enzymatic properties, but in view of molecular weight, these isozymes suggest to be different enzymes.

It is not yet to be solved why external invertase secretion is not repressed at 4% glucose concentration and why three isozymes are needed for cell growth. For exact demonstration, further investigations are needed.

요 약

세포내와 세포벽에 invertase가 존재하며 catabolite inhibition을 받지않고 세포외로 구성적으

로 invertase를 생산하는 *Rh. glutinis* K-24를 공시균주로 선택하여 세포내와 세포벽 invertase를 정제한 후 정제효소의 효소화학적 성질을 밝혔다.

세포내 invertase는 DEAE-Sephadex A-50에 의한 Batchment, DEAE-Sephadex A-50 column chromatography, gel filtration on Sephadex G-200, isoelectric focusing 등의 조작에 의해 Disc 전기영동상 단일한 효소단백질까지 정제되었다. 세포벽 invertase는 *B. pumilis*에 의해 생산되는 세포벽 용해효소를 작용시켜 invertase를 용출시킨 다음 DEAE-Sephadex A-50 column chromatography, gel filtration on Sephadex G-100의 조작에 의해 부분정제하였다. 세포내와 세포벽 invertase의 분자량은 각각 약 310,000과 67,000이었고 세포내 경우는 subunit 분자량이 약 70,000이었다. 세포내와 세포벽 효소의 반응 최적 pH와 온도는 모두 4.0와 60℃였으며 온도 안정성도 70℃까지 거의 비슷하게 나타났다. 그러나 Km값은 세포내 효소의 경우는 4.3×10^{-3} M이었고 세포벽 효소의 경우는 2.1×10^{-2} M이었다. 그외 inhibitor에 대한 효소의 저해양식도 양 효소에 비슷한 결과를 나타내었다.

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