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Purification and Characterization of the External Invertase Constitutively Produced by *Rhodotorula glutinis* K-24

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Rhodotorula glutinis K-24에 의해 구성적으로 생산되는 세포외 Invertase의 정제 및 특성

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***Rhodotorula glutinis* K-24 was found to produce external invertase in addition to internal and cell wall bound invertase. External invertase was purified to an electrophoretically homogeneous state and partitally characterized and was compared with internal and cell wall bound invertase of which procedures for purification and characterization were reported previously. The enzyme was purified by ethanol precipitation, column chromatographies on DEAE-Sephadex A-50 and SP-Sephadex C-50, and gel filtration on Sephadex G-100. The molecular weight and subunit molecular weight of external invertase were estimated to be 220,000 and 100,000, respectively. The isoelectric point of the enzyme was about pH 6.0. The optimum pH and temperature for enzyme activity were pH 4.0 and 60°C, respectively. The enzyme remained stable at the wide range, from pH 3.0 to 11.0 and stable up to 40°C, but was inactivated at temperatures above that. $HgCl_2$, $AgNO_3$, $MnSO_4$, SDS and p-CMB inhibited the enzyme activity. The K_m value of the enzyme for sucrose was 1.0×10^{-2} M. From these results, the three isozymes from *Rh. glutinis* K-24 seem to have the similar enzymatic properties, but to differ in molecular and subunit weights.**

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 an acceptor molecule. Generally, invertases are known as glycoproteins and the content of glycomoiety has been reported to vary somewhat according to the source and to the origin of the enzyme.

Many yeasts are known to produce internal and cell wall bound invertases, however not to produce external invertase. Only a small amount of invertase in the case of *Saccharomyces cerevisiae* was reported to be liberated into medium when the cells were incubated at concentration of glucose lower than 1.0%, which is 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 three kinds of invertase isozymes. It was found that the yeast produces not only the large amount of internal and cell wall bound invertase but also external invertase into medium (31). The invertase activity in culture medium significantly increased with the cell growth at high concentration of glucose. These facts clearly indicate that unlike other yeasts, *Rh. glutinis* is not subjected to catabolite repres-

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sion for the secretion of external invertase. From this point of view, *Rh. glutinis* is particularly interesting microorganism, being compared with other yeasts. Additionally, this yeast also contains a large amount 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 suggested that the enzyme might be covalently attached to a cell wall component.

To extend to understand the physiological functions of three invertase isozymes and to know the enzymatic similarities among the isozymes, the invertases with different localization were isolated from *Rh. glutinis* and characterized. We described the procedures for purification and some properties of internal and cell wall bound invertase in the previous paper (32). This paper describes the procedure for purification and characterization of external invertase which is produced into medium independent of concentration of glucose and is synthesized constitutively. Furthermore the external enzyme is compared to internal and cell wall bound invertase from *Rh. glutinis* K-24.

Materials and Methods

Strain and cultivation

Rhodotorula glutinis K-24 stocked in our laboratory was used as a strain which produced invertases.

In order to investigate the effect of glucose and sucrose on external invertase synthesis, *Rh. glutinis* K-24 was grown at 25°C for 48 hr in 500 m*l* shake flask containing 100 m*l* of each carbon source and basal medium which contained 1% polypeptone, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 4 ppm $MnSO_4 \cdot 6H_2O$, 4 ppm $FeSO_4 \cdot 7H_2O$ and 2 ppm thiamin (pH 5.5). The seed culture containing 7% glucose and basal medium was incubated at 25°C for 24 hr with continuous reciprocal shaking and then was inoculated at a concentration of 2% (v/v).

Preparation of enzyme solution

Cells were removed by centrifugation at 7,000 rpm for 20 min and the supernatant was used as crude enzyme solution.

Assay of invertase

Sucrose solution (2%) prepared in 0.1 M 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 (33). One unit of enzyme activity was defined as the amount of enzyme increasing one of optical density at 660 nm for 10 min.

Homogeneity of invertase

Disc gel electrophoresis was performed on a 5% polyacrylamide gel at 3 mM per gel for 7 hr at 4°C according to the method of Reisfel *et al.* (34). After the run, the gel was stained with 1% Amido Black 10 B and then diffusionally destained in 7% acetic acid.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by gel filtration on Sephadex G-200 equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl by method of Andrews *et al.* (35). The subunit weight of the enzyme was estimated by disc gel electrophoresis on a 10% polyacrylamide gel in presence of sodium dodecyl sulfate with 2-mercaptoethanol, as a described by Weber and Osborn (36). The standard proteins used for calibration were apoferritin (M.W. 443,000), alcohol dehydrogenase (M.W. 150,000), bovine serum albumin (M.W. 66,000), obalbumin (M.W. 45,000), and α-chymotrypsinogen (M.W. 25,700).

Determination of isoelectric point

The isoelectric point was determined by 5% polyacrylamide disc gel isoelectric focusing using 0.2% Ampholine (prepared by mixing pH 3.5-10 and pH 4-6 Ampholines in the ratios of 1:3). Two identifical gels (0.8×10 cm) were prepared, one for calibration of the pH gradient and the other for detection of the enzyme. Electrophoresis was carried out at constant voltage of 200 V per column for 5 hr.

Chemicals

Sephadex G-100, Sephadex G-200, DEAE-Sephadex, SP-Sephadex C-50 and protein standards for gel filtration and SDS-PAGE were obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

Results

Culture media and conditions for external inver-

Table 1. The proper conditions for external invertase produced by *Rh. glutinis* K-24.

Media	Glucose	4.00%
	Polypeptone	1.00%
	KH_2PO_4	0.50%
	$MgSO_4 \cdot 7H_2O$	0.05%
	$MnSO_4 \cdot 6H_2O$	4.00 ppm
	$FeSO_4 \cdot 7H_2O$	4.00 ppm
	Thiamin	2.00 ppm
Conditions	pH 5.5	
	Temperature	25°C
	Cultivation time	4 days

tase production

The effects of various concentrations of glucose and sucrose on the enzyme production were examined. Cell growth was the most excellent in the medium containing 7% glucose but enzyme production was the most effective in the medium containing 4% glucose and is nearly in proportion to cell growth and thus it is suggested that the enzyme was not derived from internal or intermural space but was produced into medium. *Rh. glutinis* was inoculated in a shake flask containing 100 m*l* of medium (Table 1). Fig. 1 shows the growth of cells, external invertase production, and the changes of broth pH during cultivation. The maximum production of the enzyme was obtained 4 days after incubation. The proper conditions for the enzyme production were summarized in Table 1.

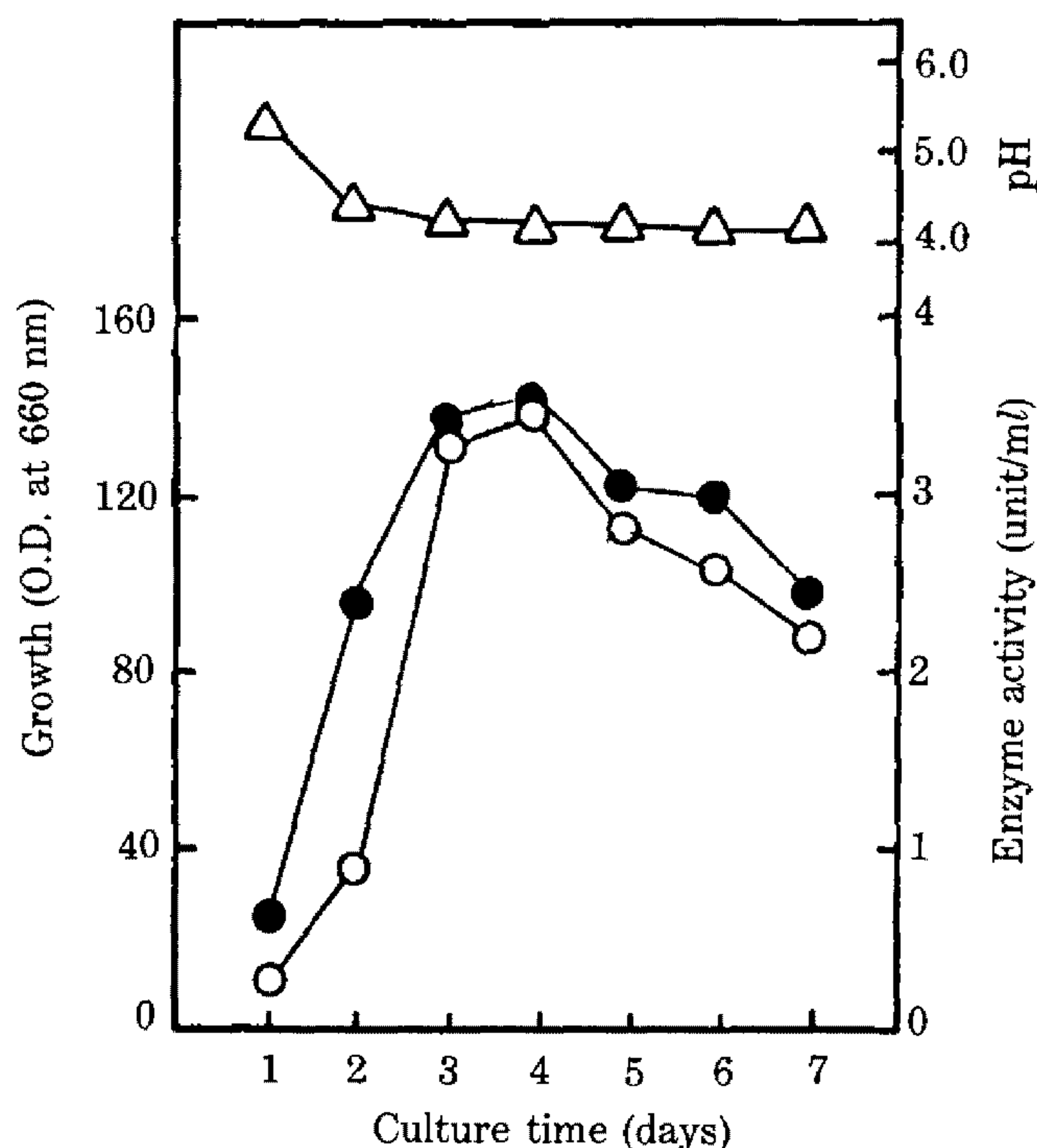

Fig. 1. Time course of growth, pH, and external invertase activity.

Rh. glutinis was aerobically cultured under the optimum condition indicated in the text. After the cultivation of the indicated time, the growth, pH, and enzyme activity in medium were detected.

-●-; growth, -△-; pH, -○-; enzyme activity.

Purification of external invertase

For the large-scale preparation of enzyme solution, the main culture was performed in 10 *l* fermentor. (B. Braun Melsurgan Co.) containing 6 *l* of proper medium and under the conditions given in Table 1. Cells were removed by centrifugation and the supernatant was used as crude enzyme solution. However the final pH of supernatant was pH 4.3 and therefore the broth was adjusted to pH 7.0 because of the possibility of inactivation of enzyme by the acid protease present in the broth.

Step I. Precipitation with ethanol

The crude enzyme solution was concentrated at 30°C using rotary evaporator to 1 *l* and then ethanol at −20°C was added to the enzyme solution to 45% saturation with continuous stirring, followed by standing the enzyme for 2 hr at 4°C. The resulting precipitate was collected by centrifugation, dissolved in a small amount of 0.01 M sodium phosphate buffer (pH 7.0) and then dialyzed against the same buffer at 4°C thoroughly. The precipitate formed during the dialysis was removed by centrifugation at 10,000 rpm for 10 min. After ethanol precipitation, the recovery yield of total activity exceeded 100%, which phenomenon was occasionally shown in purification procedure. It seems that because a certain compound present in the crude enzyme solution which affects enzyme assay system repressively is removed effectively through ethanol precipitation.

Step II. DEAE-Sephadex A-50 column chromatography

The enzyme solution obtained from the previous step was applied to a column of DEAE-Sephadex A-50 (2×24 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). After the column had been washed with the same buffer, a linear gradient of NaCl (0-0.4 M) in the buffer was applied in a total volume of 600 m*l*. As shown in Fig. 2, invertase activity was

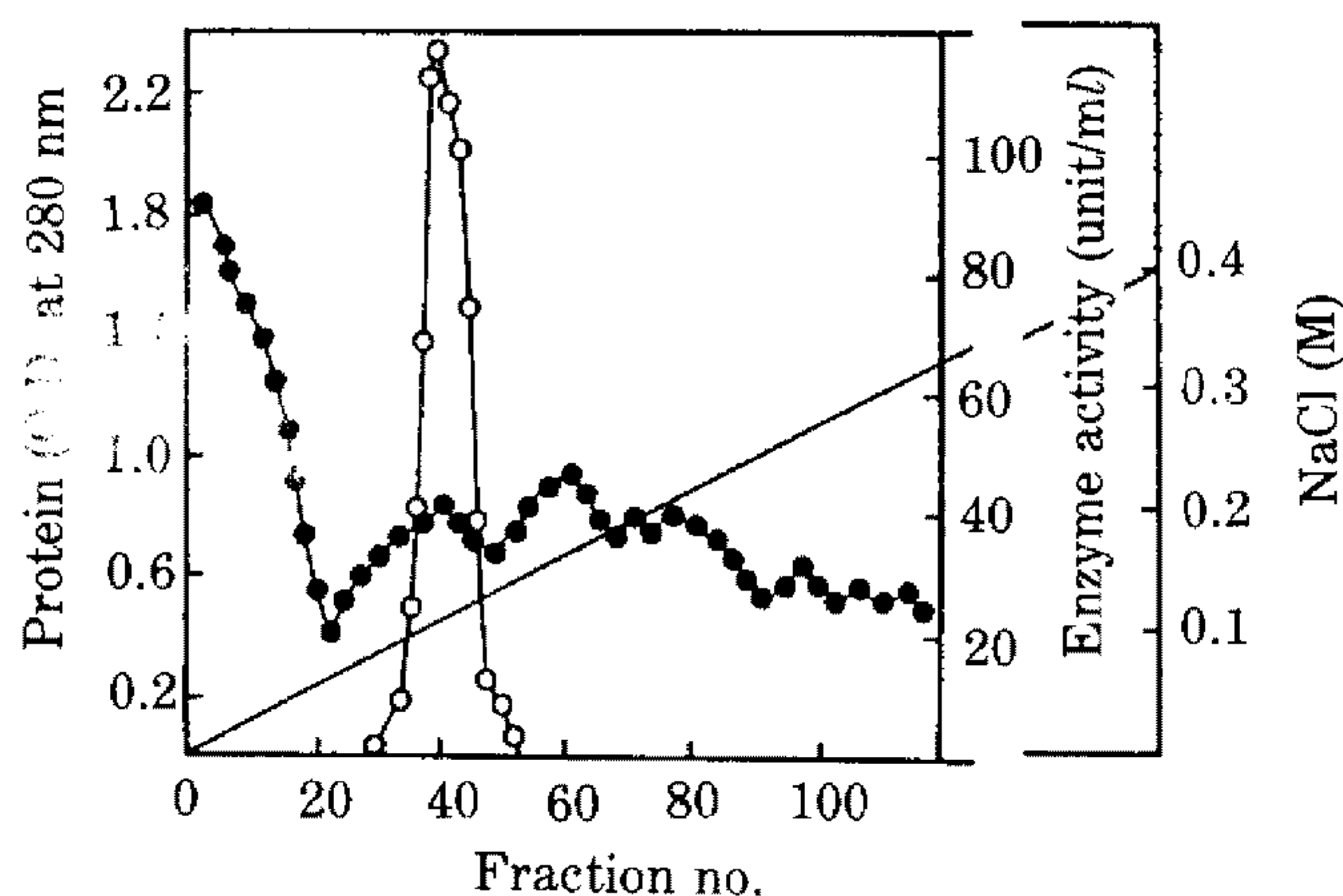

Fig. 2. Elution profile of ion exchange chromatography on DEAE-Sephadex A-50.
Enzyme solution was applied on a column of DEAE-Sephadex A-50 (2×24 cm) which had been equilibrated with phosphate buffer (pH 7.0, 0.01 M). The enzyme was eluted with a linear gradient of up to 0.4 M NaCl. Fraction volume was 4 ml.
-●-; absorbance at 280 nm, -○-; enzyme activity.

eluted at about 0.10 M of NaCl.

Step III. SP-Sephadex C-50 column chromatography

The pooled enzyme solution from DEAE-Sephadex chromatography was dialyzed in 0.01 M sodium lactate buffer (pH 3.0) with four changes and then loaded to a column of SP-Sephadex C-50 (2×15 cm) previously equilibrated with the same buffer. Proteins were eluted by this buffer and then by a linear gradient (0-0.4 M NaCl in sodium lactate buffer) with a total volume of 600 ml. Active portion was eluted as a single peak, there not being a constant ratio of protein to enzyme activity in each fraction. The enzyme active fractions were pooled and used for following procedure.

Step IV. Gel filtration on Sephadex G-100

The enzyme solution was concentrated by ultrafiltration through a CX-10 membrane (Millipore) and was applied to a Sephadex G-100 column (2.5×90 cm) previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Elution was done isocratically with the same buffer containing 0.1 M NaCl. Active fractions were eluted as a single peak, there being a constant ratio of protein to enzyme activity in each fraction. The active fractions (32 ml) were pooled and saved for further analysis.

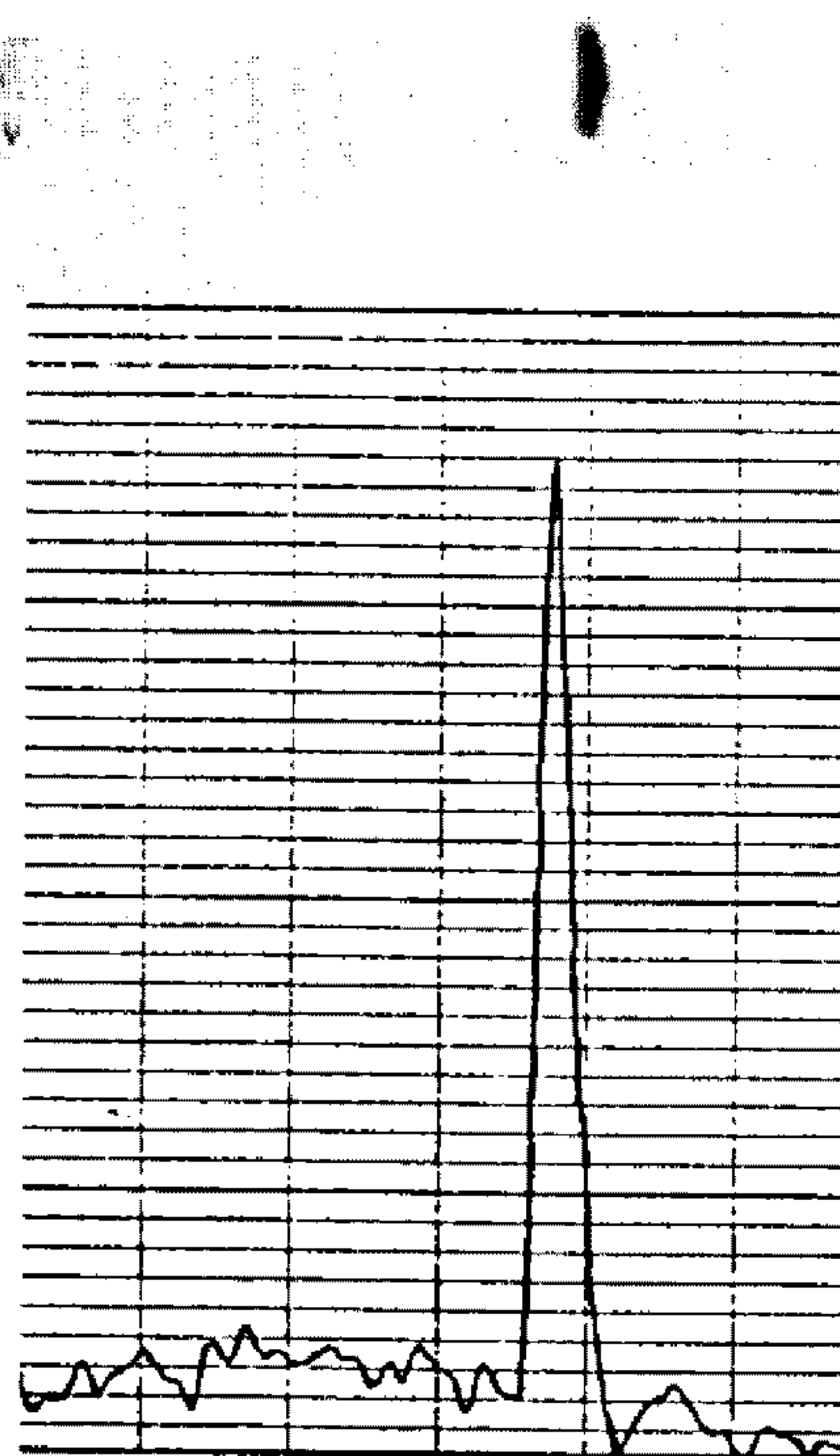

Fig. 3. Polyacrylamide disc gel electrophoresis and densitometric tracing of the purified enzyme.
Electrophoresis using about 50 ug of enzyme was carried out at pH 9.5 in 5% polyacrylamide gel at current of 3 mA per gel column.

Homogeneity of the purified enzyme

Homogeneity of the final purified enzyme was examined by polyacrylamide disc gel electrophoresis followed by densitometric tracing. As shown in Fig. 3, a single band on the gel and a single symmetrical peak in the tracing showed that the enzyme was electrophoretically homogeneous. The purification of the enzyme is summarized in Table 2. The yield was about 1.2 mg of purified enzyme with specific activity of 925.7 units/mg and about 9,100-fold purification from ethanol precipitation was achieved with a recovery of about 13%.

Properties of the purified invertase

Molecular weight: The molecular weight of invertase was estimated to be approximately 100,000 by comparison with those of marker proteins on SDS-polyacrylamide gel electrophoresis. On the other hand, the molecular weight of the enzyme was found to be about 220,000 by gel filtration on a Sephadex G-200 column. These results suggest that the native enzyme is a dimer consisting of two subunits of equal molecu-

Table 2. Purification of external invertase from *Rh. glutinis* K-24.

Purification step	Volume (ml)	Total protein (O.D.$_{280}$ × ml)	Total activity (units)	Specific activity (units/O.D.$_{280}$)	Yield (%)
Culture supernatant	6400	51200.0	5568.0	0.1	–
Ethanol precipitation	178	220.7	8544.0	38.7	100.0
DEAE-Sephadex A-50 column chromatography	62	42.8	4743.0	110.8	55.5
SP-Sephadex column chromatography	52	3.4	3102.0	912.4	36.3
Gel filtration on Sephadex G-100	32	1.2	1096.0	913.3	12.8

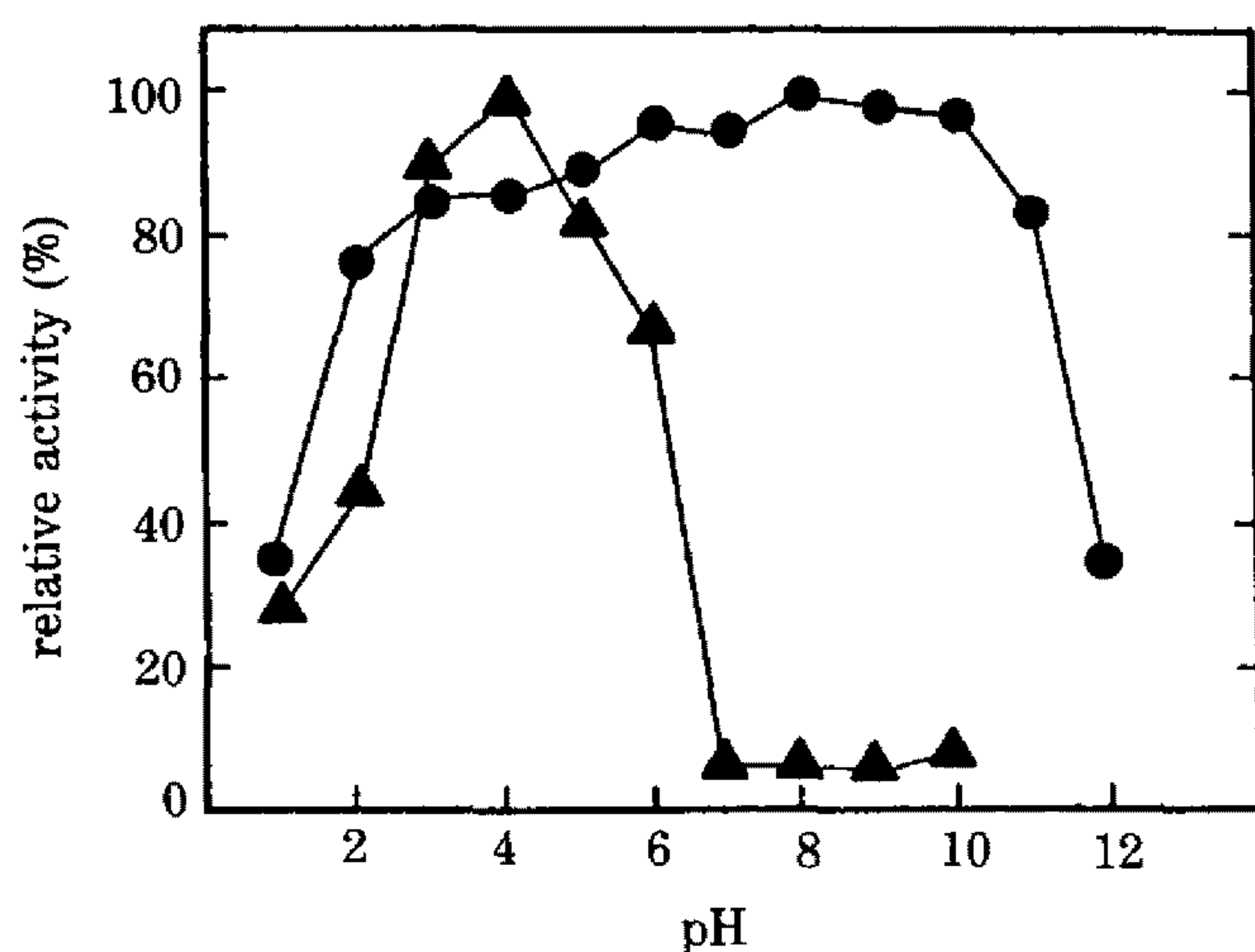

Fig. 4. Effect of pH for enzyme activity and stability.
Optimun pH; the relative activity was expressed as percent at various pH values compared to the enzyme activity at pH 4.0. Buffer systems used were sodium citrate, sodium acetate, sodium phosphate, glycine-NaOH, and Na_2CO_3-NaOH.
pH stability; the enzymes of various pH values were incubated for 24 hr at 20°C, and then the residual activity was assayed.
-▲-; optimal pH on invertase activity.
-●-; effect of pH on the stability of invertase.

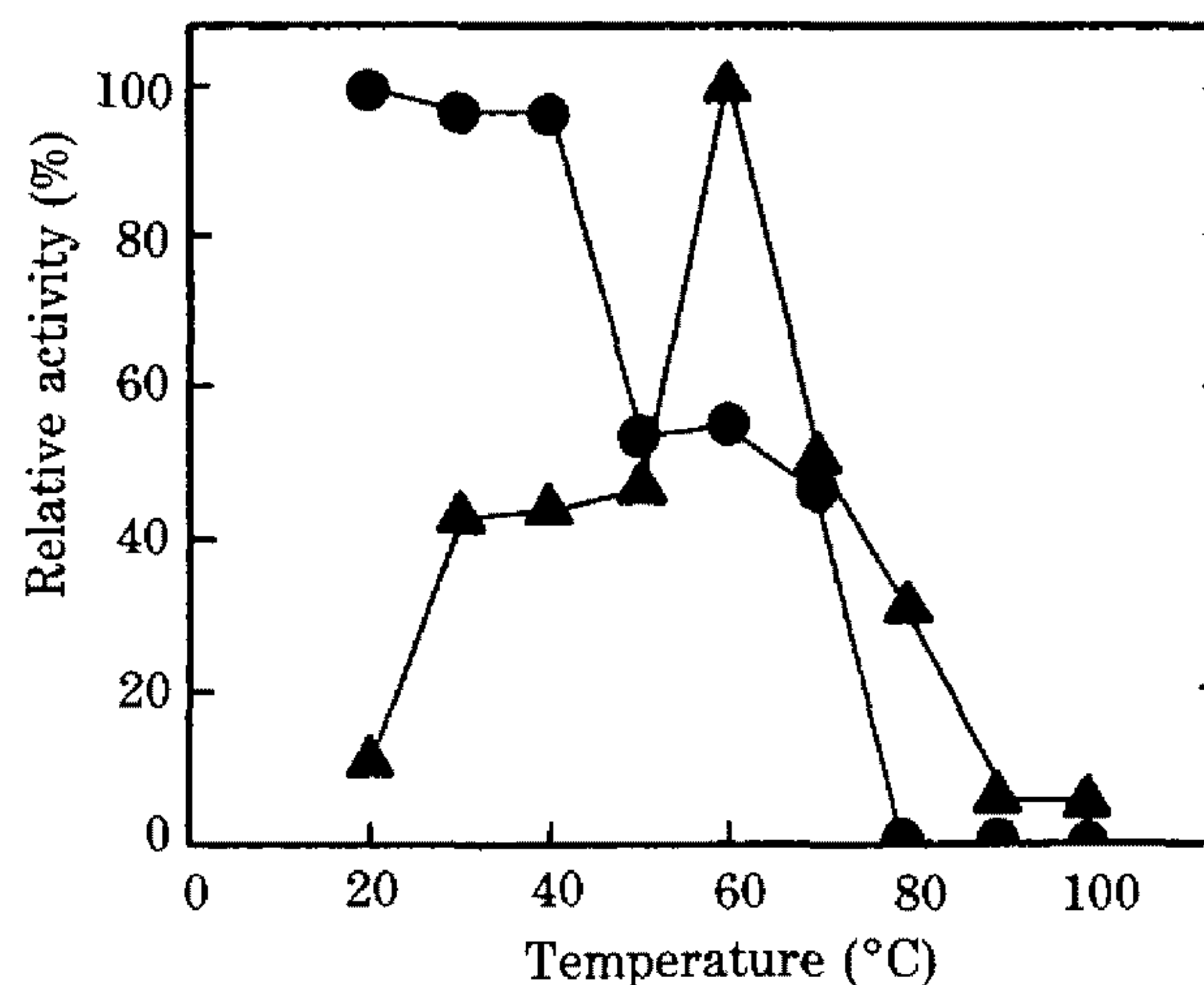

Fig. 5. Effect of temperature for thermal stability and enzyme activity.
The enzyme activities were assayed at various temperatures and at constant pH of 4.0 of 0.1 M sodium acetate buffer. The enzyme solution was incubated in sodium acetate buffer (pH 4.0) at the indicated temperatures for 20 min and then residual activity was measured.
-▲-; optimal temperature on invertase activity.
-●-; effect of temperature on invertase stability.

lar size.

Isoelectric point: The isoelectric point of the enzyme was determined to be about pH 6.0 by polyacrylamide disc gel isoelectric focusing.

Effect of pH and pH stability for enzyme activity: The enzyme assay was performed in the reaction mixture with various pHs at 30°C. The optimum pH for the enzyme activity was pH 4.0. The pH dependence of the enzyme stability was determined from the residual activity after 24 hr preincubation at various pHs and at constant temperature of 20°C. The purified enzyme remained comparatively stable at wide pH range, from pH 2.0 to pH 10.0. These results are shown in Fig. 4.

Effect of temperature and thermal stability for enzyme activity: The enzyme activities were assayed at various temperatures and at constant pH of 4.0 of 0.1 M sodium acetate buffer. The maximum activity of the enzyme was observed at 60°C. The thermostability of the enzyme at pH 4.0 was determined by means of 20 min precincubation of the enzyme at various temperature prior to the enzyme assay. The enzyme was stable up to 40°C but was inactivated at about 50°C by about 50% of total activity and was completely inactivated above 80°C. These results are shown in Fig. 5.

Table 3. Effect of inorganic salts and organic compounds on the external invertase activity.

Reagents	Relative activity (%) (0.1 mM)	(1.0 mM)
Pb $(NO_3)_2$	88	87
$NiCl_2$	108	108
$ZNSO_4$	100	89
$HgCl_2$	32	16
$AgNO_3$	42	32
$CoCl_2$	111	103
$FeCl_2$	103	102
$CaCl_2$	97	92
$MnSO_4$	83	25
EDTA	92	90
MIA	79	62
p-CMB	27	24
Phenol	97	92
SDS	73	18
NaCN	70	67
2-Mercaptoethanol	108	70
None	100	100

MIA; monoiodoacetic acid
p-CMB; p-chloromercuric benzoate
SDS; sodiumdodecyl sulfate
EDTA; ethylenediamentetracetic acid

Effects of inorganic salts and inhibitors: The effects of some inorganic salts and compounds known as enzyme inhibitors on the invertase activity were investigated. The enzyme was preincubated with each compound for 5 min at 20°C at final concentration of 0.1 mM and 1.0 mM before the addition of the substrate. The relative activity is represented as the percentage of the activity of a control. As shown in Table 3, the enzyme is inhibited by $HgCl_2$, $HgNO_2$, $AgNO_3$, $MnSO_4$. MIA, p-CMB, and SDS of 1.0 mM strongly inhibited enzyme activity.

Michaelis-Menten constant: The Michaelis-Menten constant for the sucrose hydrolyzing activity of the external invertase was determined from Lineweaver-Burk plot, as shown in Fig. 6. The K_m value for sucrose of the enzyme was calculated to be 1.0×10^{-2} M.

Discussion

Many invertases have been purified from a variety of sources and have been extensively studied since the enzyme was first isolated from an Yeast. Although most of invertase preparations described in the past have had internal and intermural (cell wall) localization, a few microorganisms such as *Aspergillus athecius* (23) and *Aspergillus oryzae* (37) produced external invertase into the medium. However, an external invertase from yeast has not so far been found except *Saccharomyces cerevisiae* (25) which produced limited external invertase only when it was incubated at the concentration of glucose lower than 1.0% (25, 38). It seems that the enzyme is synthesized only when the cells are incubated at concentration of hexose lower than one percent because synthesis of external invertase in yeasts depends on glucose concentration and perhaps on other hexose also. The details for glucose (hexose) repression are unknown, but it can 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).

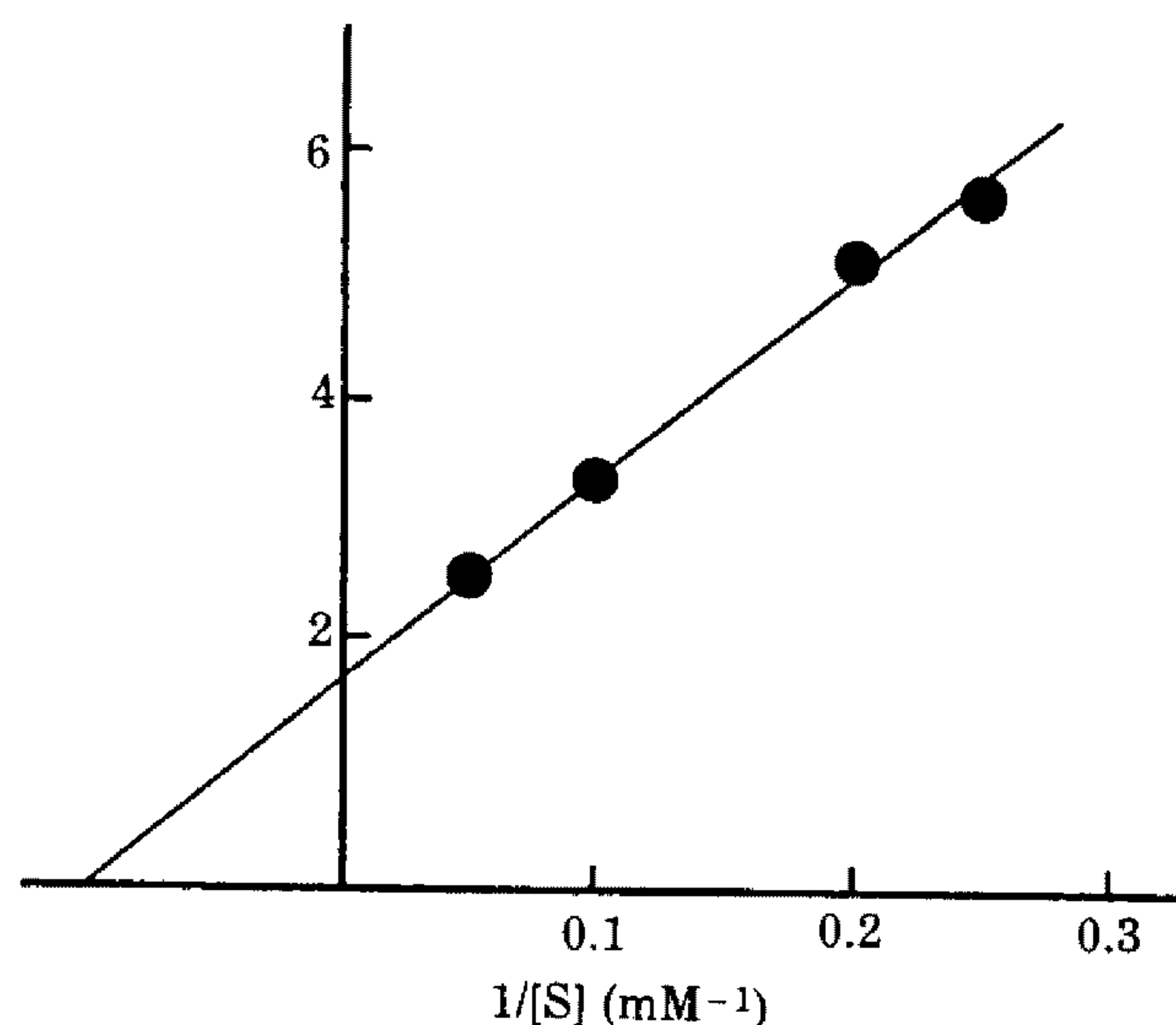

Fig. 6. Determination of K_m value for external invertase.
Reciprocal velocities were plotted against the reciprocal concentration of sucrose.

In our investigation, *Rh. glutinis* K-24 was found not only to produce internal and cell wall bound invertase but also to liberate external invertase at concentration of glucose higher than 1.0%, which means that the microorganism, unlike other yeasts, is not subjected to catabolite repression for the secretion of external invertase. The enzyme activity in culture medium

Table 4. Summary of properties of internal, cell wall bound, and external invertase.

Isozymes / Properties	Internal	Cell wall	External
Molecular weight	310,000	67,000	220,000
Subunit weight	70,000	–	100,000
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
Optimum pH for activity	3	3	4
pH stability	3-8	4-9	3-10
Effects of inorganic salts	$HgCl_2$ $AgNO_3$ $MnSO_4$	$HgCl_2$ $AgNO_3$ $MnSO_4$	$HgCl_2$ $AgNO_3$ $MnSO_4$
Effects of organic compounds	SDS p-CMB MIA	SDS p-CMB	SDS p-CMB MIA

SDS; sodium dodecylsulfate
p-CMB; p-chloromercuric benzoate
MIA; Monoiodoacetic acid
–; not determined

was increased gradually with cell growth. Therefore, this enzyme was throught not to be detached from intermural space and not to be leaked out by lysis of cells but to be secreted constitutively. It is likely that the three isozymes with different localization accomplish respective function in metabolism, however they are not understood.

In order to demonstrate some properties and the relative functions among three isozymes, we compare the external enzyme with internal and cell wall bound invertase, of which procedures for purification and characterization were already described in the previous paper (32). These results are summarized in Table 4. The molecular weight of internal invertase was estimated to be 310,000 which is the largest molecular weight among three isozymes, and the enzyme consists of four subunits of equal molecular size of 70,000. The enzyme is glycoprotein that has about 40% carbohydrate content, which is similar to invertases from other origins. The molecular weight of cell wall bound invertase was calculated to be 67,000, the smallest molecular size among them, but the subunit weight was not evaluated due to its impurity. This enzyme was solubilized from cell wall preparations 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 molecular weight of external invertase was about 220,000 by gel filtration. SDS-polyacrylamide gel electrophoresis gave a monomeric molecular weight of about 100,000. Thus, the enzyme may have two equimolar subunits with a molecular weight of 100,000. From the molecular weight point of view, three isozymes seem to be structurally different enzyme with different molecular weights and subunit weights. However, the physicochemical properties of pH, temperature, and stability etc. are approximately similar for three isozymes. And it is also shown that effects of various inhibitors on enzyme activity are much similar. From these results, the three isozymes seem to have the similar enzymatic properties except molecular weight and their localizations.

Effects of glucose and sucrose concentration on the external invertase synthesis were examined. The enzyme was best produced at 4% glucose concentration without its catabolite repression and was independent of sucrose, the substrate of the enzyme, for enzyme production in medium. It deserves special emphasis that unlike other yeasts such as *Sacch. cerevisiae, Rh. glutinis* external invertase is not repressed at high glucose concentration and is produced constitutively. In an attempt to demonstrate why three isozymes with different localization are needed for cell growth, further investigations on their functions are under study.

요 약

세포내 및 세포벽 뿐만 아니라, 세포외에도 invertase를 구성적으로 생산하는 효모 *Rh. glutinis* K-24로부터 세포외 invertase를 disc 전기 영동상으로 단일한 상태로까지 정제하였다. 정제 효소의 효소화학적 성질을 밝힌 후 이미 보고한 바 있는 세포내 및 세포벽 invertase와 그 개략적 성질을 비교 검토하였다.

Rh. glutinis K-24의 세포외 invertase 생산을 위한 배지조성은 4% glucose, 1% polypeptone, 0.5% KH_2PO_4, 0.05% $MgSO_4\cdot7H_2O$, 4 ppm $H_2SO_4\cdot6H_2O$, 4 ppm $FeSO_4\cdot7H_2O$, 2 ppm thiamin (pH 5.5)이었고, 25℃에서 72 hr 배양이 효과적이었다. 세포외 invertase는 crude enzyme solution을 감압농축하여 ethanol precipitation, DEAE-Sephadex, SP-Sephadex column

chromatography 그리고, gel filtration 등의 조작에 의해 정제하였다. 정제효소의 등전점은 약 6.0이었고 분자량은 220,000이었으며 2개의 동일한 subunit로 구성되어 있는 dimeric enzyme인 것으로 밝혀졌다. 세포외 invertase의 반응최적 pH와 온도는 각각 pH 4.0와 60℃이었으며 온도에 대해서는 40℃까지 안정하였다. pH 안정성은 pH 3.0-11.0까지로 비교적 넓은 범위에서 안정하였다. $HgCl_2$, $AgNO_3$, $MnSO_4$, p-CMB 및 SDS 등에 의해 효소활성은 저해받았으며 기질 sucrose에 대한 K_m값은 1.0×10^{-2} M이었다. 3종류의 invertase는 분자량과 그 국재성을 제외하고는 그외의 효소화학적 성질이 유사하였다.

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