Isolation, Characterization and a Application of Invertase from *Pseudozyma* sp. I-8

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Abstract

Resulting in the screening of invertase which can hydrolyze sucrose to glucose and fructose, a yeast strain I-8 was isolated as a invertase producer from digestive juice of *Nepenthes*. The yeast was identified as *Pseudozyma* sp. I-8 from its comparative 26S rDNA-D1/D2 gene sequence analysis.

The invertase was purified from the culture filtrate by $(NH_4)_2SO_4$ precipitation, CM-Toyopearl 650M, DEAE-Toyopearl 650M, Butyl-Toyopearl 650M and Toyopearl HW-55 chromatography. Nevertheless the purified enzyme was free from other enzyme activities, not shown a single band by SDS-PAGE.

The optimum temperature and pH were around 40℃ and 5.0, respectively. The enzyme was stable in a pH range from 3.0 to 8.0 and at below 50℃. The enzyme hydrolyzed sucrose completely and released glucose and sucrose without no reverse compound. Raffinose was also favorable substrate but it showed no activity towards cellbiose or maltooligosaccharides. From these results, the invertase produced by *Pseudozyma* sp. I-8 was recognized as a unique invertase.

The invertase was applied to bread making process. Addition of the invertase on bread making process presented excellent effects to improve the loaf volume and softness of bread.

INTRODUCTION

Enzymes are a type of protein in all living things and act as biological catalysts. They increase the rate of chemical reactions without undergoing any permanent change themselves.

Enzymes have been used since ancient times to make bread, cheese, beer and other fermented products, and to tenderize meat. Only recently, however, we have understood

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how they function. Nowadays, their functions as biological catalyst are very useful to human life and many industries. Using enzymes instead of "chemicals" we may produce fewer by-products, and the chemical reactions may be easier to control.

Most of industrial enzymes are manufactured by the fermentation with microorganisms. The bulk of enzymes used in industry are extracellular enzymes. Microbial enzymes are produced from high-yield strains by the fermentation under controlled conditions in submerged culture.

The biggest market for industrial enzymes is the use for washing detergent ingredients such as alkaline protease, cellulase and lipase. The second is the use for food processing. In the food processing field, enzymes are used for brewing, production of fruit juices, baking, hydrolyzed starch syrups and so on. The enzymes for food processing are required to be registered as food additives under the Ministry of Health, Labor and Welfare in Japan. There are 69 kinds of enzymes as food additive in Japan now.

Invertase (EC 3.2.1. 26 β-Fructofuranosidase) is an enzyme that removes the glucose and fructose from the sucrose. Althogh a number of invertase from cultures of various microorganisums including *Aspergillus*,[1] *Bacillus*,[2] *Sacchromyces*,[2)-4)] and *Fusarium*[2] so on, we have screened a invertase possessed unique properties for potential industrial application.

MATERIALS AND METHODS

Chemicals. The invertase from *Sacchromyces cerevisiae* were purchased from Sigma Chemical Co., Ltd. All other chemicals used were obtained from commercial sources.

Microorganisms. The strain I-8 used in this study was newly isolated from a digestive juice in pitcher of *Nepenthes bicalcarate* (Fig. 1) gathering from Higashiyama botanical garden in Nagoya city. Strain I-8 was identified as *Pseudozyma* sp. based on the 26S rDNA-D1/D2 analysis. The stock culture was maintained on agar slant (pH 6.0) containing agar (1.6%) yeast extract (2.0%), glucose (1.0%) and Polypepton S (2.0%).

Cultivation. Pieces of the colony *Pseudozyma* sp. I-8 were picked up from a agar slant and inoculated into 100 mL shaking flasks containing 20 mL of medium. The medium (pH 6.0) was consisted of 1% soluble starch, 1% glucose, 1% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1% yeast extract and 1% polypepton. After incubation at 25℃ for 20 hours, 40mL of the culture were inoculated into a 3 L jar fermentor containing 2 L of the medium described above. The cultivation was done at 25℃ for 72 h, with agitation at 350 rpm and aeration at 1.5 L per min.

Purification of a invertase. All operations were performed at 5℃.

Fig. 1 Pitcher of *Nepenthes bicalcarate*

Step 1. Ammonium sulfate fractionation. After 72 h cultivation, the cells grown were removed by centrifugation and 4380 ml of crude invertase solution were got by filtration. The filtrate was concentrated by ultrafiltration using AIV-1010 (Asahi Kasei Co.) with almost 100% recovery of invertase activity. The enzyme solution was re-filtered to remove insolubles. To 820 ml of the filtrate, solid $(NH_4)_2SO_4$ was added up to 80% saturation with stirring and left overnight at 5℃. The resulting precipitates were collected by filtration and dissolved in 20 mM phosphate buffer (pH 7.0). The enzyme solution was dialyzed against the same buffer.

Step 2. CM-Toyopearl 650M column chromatography. The enzyme solution in step 1 was applied to a CM-Toyopearl 650M column (2.2×16 cm) equilibrated with the same buffer. The column was eluted with the same buffer, and non-adsorbed active fractions (200 ml) were combined.

Step 3. DEAE-Toyopearl 650M column chromatography. The enzyme solution in step 2 was applied on a DEAE-Toyopearl 650M column (2.2×16 cm) equilibrated with the same buffer. The adsorbed enzyme was eluted in 3.5 mL fractions at a flow rate of 30 mL per h with a liner gradient of 0–500 mM NaCl in buffer, and the active fractions were collected. The enzyme solution equilibrated with 10 mM phosphate buffer (pH 7.0) containing 20% $(NH_4)_2SO_4$ by dialysis against the same buffer.

Step 4. Butyl-Toyopearl 650M column chromatography. The dialyzed enzyme solution in step 3 was applied on a Butyl-Toyopearl 650M column (2.2×16cm) equilibrated with the same buffer with 20% $(NH_4)_2SO_4$. After the column was washed with 50 mL of equilibration buffer, the adsorbed enzyme was eluted with 300 mL of a linear gradient of 20–0% $(NH_4)_2SO_4$ in the same buffer. The active fraction eluted was dialyzed against the 10 mM phosphate buffer containing 0.5 M NaCl.

Step 5. Toyopearl HW-55 column chromatography. Two mL of the active fractions obtained from step 4 was subjected to gel filtration on a Toyopearl HW-55

column (2.2×92cm) using the same buffer at a flow rate 20 mL per h. The active fractions were combined and dialyzed against a large volume of 10 mM phosphate buffer (pH 7.0). The dialyzate was stored at 4℃ and used for further experiments as the final enzyme preparation (Fig. 2).

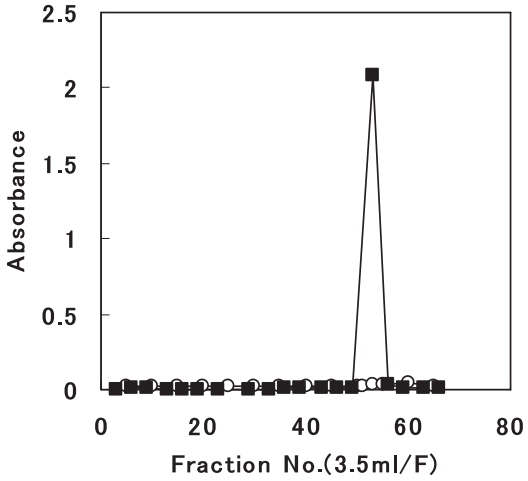

Fig. 2 Toyopearl HW-55 chromatography.
Symbols: ○, 280nm; ■, activity (660nm)

Enzyme assay. Sucrose (Kanto kagaku Co., Ltd) was used as substrate for invertase assay. The standard assay mixture for the enzyme contained 0.5 mL of 1% (w/v) substrate, 0.4 mL of 100 mM phosphate buffer (pH 6.0) and 0.1 mL of enzyme solution. After incubation for 30 min at 50℃, the reducing sugars released were estimated by the method of Somogyi-Nelson.[5] One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose per min under the above conditions.

Protein assay. The protein concentration was measured with Bio-Rad protein reagent (Bio-Rad Laboratories. Richmond, CA, US) using bovine serum albumin as the standard. The absorbance at 280 nm was used for monitoring protein in column effluent.

Glucose assay. Glucose was measured using the Glucose CII-test Wako (Wako Pure Chemical Industries Ltd.).

Electrophoresis. SDS-PAGE was done with the Compact PAGE AE-7305 (Atto Co., Ltd.) using C-PAGEL (Atto Co., Ltd.). To measure the molecular weight, *EZ* Standard AE-1440 (Atto Co., Ltd) was used as the standard proteins.

TLC. Thin layer chromatography was done using TLC plate (Silicagel 60, Sigma Co.). After development with the mixture of chloroform-methanol-water (30:20:4), the reagent consist of the mixture of diphenylamine-aniline-acetone-80%phosphoric

acid (2:2:100:15) was sprayed for appearance of sugar spots.

Baking.

Enzymes. The invertase, a fraction separated by the Butyl-Toyopearl 650M was used.

Chemicals. The flour named "Panmix" and Dry yeast were purchased from Nisshinfoods Inc. All other chemicals used were obtained from commercial sources.

Instruments. The automatic home baking instrument used was the type of SD-BT50 (Matsushita Electric Industrial Co., Ltd.).

Baking procedure. The baking procedure was performed according to maker manual. Dough ingredients consisted of flour mixture (252g), dry yeast (2.16g),water (160ml) and enzyme (40U). The bread making test was carried out three times by the same procedure.

Measurement of bread properties. The bread loaf volume was determined by canola seed displacement method. The crumb firmness was determined at one day after baking by measuring the compression force for bread slices (30×30×13 mm) using Texthurometer TDU (YAMADEN Co., Ltd.). The crumb firmness was defined with the relative rate (%) toward firmness of standard bread (no enzymes were added.). All loafs were stored at room temperature in plastic bags and sliced just before the measurement of crumb firmness.

RESULTS

Identification of an isolated yeast.

The strain, designated I-8, was subjected to a polyphasic taxonomic investigation. Comparative 26S rDNA-D1/D2 gene sequence analysis indicated that it formed a distinct phylogenetic lineage within the genus *Pseudozyma* together with several other species of the genus, e. g. *P. aphidis* and *P. rugulosa.* From these analysis, the yeast strain I-8 was identified genus *Pseudozyma* sp. and very closed to *P. aphidis.*

Purification of the enzyme.

The enzyme was purified by $(NH_4)_2SO_4$ fractionation and consecutive column chromatography. The results of purification are summarized in Table I. The final preparation of the invertase was purified 11.6-fold on the basis of the sucrose-hydrolyzing activity, with a yield of 1.0%. The specific activity toward sucrose was 216 U/mg of protein. The purified enzyme was used for subsequent characterization.

Properties of the purified enzyme.

The enzyme subjected purification procedure did not show a single band on SDS-PAGE. But the enzyme was free from all other enzymes activity. So, the molecular weight of the enzyme can not estimated by SDS-PAGE and Gel filtration.

Table I Purification of invertase.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield(%)
Culture filtlate	279.4	5204	18.6	100
CM-Toyopearl	19.2	868	45.2	16.7
DEAE-Toyopearl	5.4	118	37.2	3.8
Butyl-Toyopearl	1.0	62.8	65.7	1.2
Toyopearl-HW-55	0.3	53.4	216.0	1.0

Effect of pH and temperature on the activity of the enzyme.

The effect of pH and temperature on the activity of the partially purified enzyme was studied under the standard assay conditions. The enzyme showed maximum activity at pH 5.0 (Fig. 3) and at 40℃ (Fig. 4).

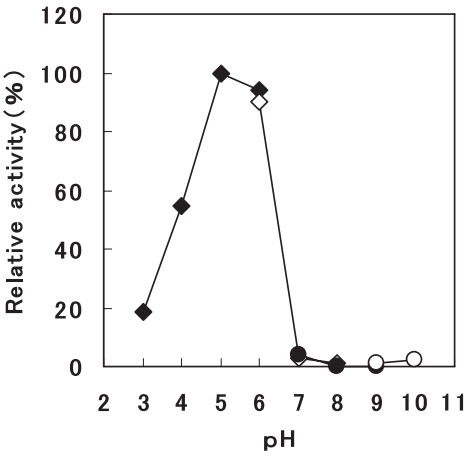

Fig. 3 Effect of pH on enzyme activity
McIlvaine buffer, pH3-7; Phosphate buffer, pH7-9; Tris buffer pH7-9; Atkins buffer, pH9-10.

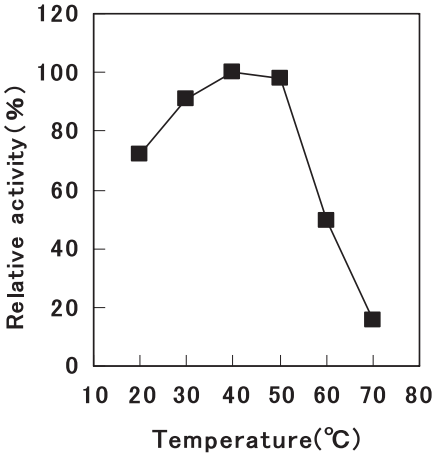

Fig. 4 Effect of temperature on enzyme activity

Stabilities of the purified enzyme toward pH and temperature.

Enzyme stability at different pH levels were measured by incubating the enzyme solution at 40℃ and several pH for 30 min. After cooling in an ice bath and 20-fold dilution with 100 mM McIlvaine buffer (pH 4.0), the residual activity was measured under the standard assay conditions. The enzyme was stable over a range of pH values between 3.0 and 8.0 (Fig. 5).

Thermostability was measured by incubating the enzyme solution at pH 4.0 and

various temperatures for 10 min. After cooling in an ice bath, the residual activity was measured under the standard assay conditions. The enzyme retained its original activity on heating below 50℃ (Fig. 6).

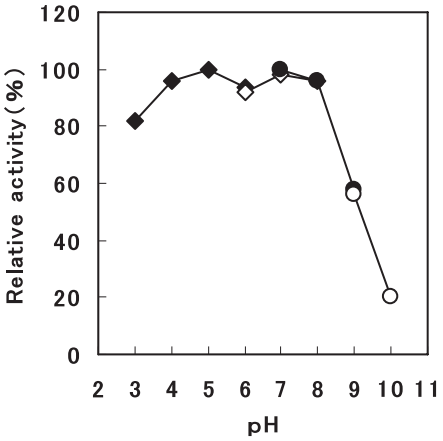

Fig. 5 pH stability of the enzyme

McIlvaine buffer, pH3-7; Phosphate buffer, pH7-9; Tris buffer pH7-9; Atkins buffer, pH9-10.

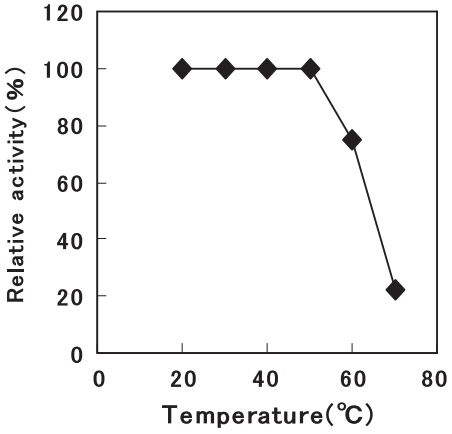

Fig. 6 Thermalstability on enzyme activity

Effect of metal ions and chemical reagents on the activity of the enzyme.

The enzyme activity was assayed with the standard assay method with 1 mM of metal ions or reagents. The enzymatic activity was inhibited by Ag^{+} (100%), Fe^{2+} (39%), sodium dodecylsulfate (89%) EDTA (16%) and *p*-chloromercuribenzoic acid (56%) respectively, but was not affected by monoiodoactic acid, and stimulated by Fe^{3+} (120%). No chemicals stimulated the activity of the enzyme (Table II).

Substrate specificity.

The partially purified enzyme was tested for its substrate specificity under the standard assay conditions with 1.0% substrate concentrations in the reaction mixture. The results are shown in Table III.

The invertase purified in this study exhibited significant sucrose-hydrolyzing activity. Raffinose was also the best substrates, whereas the relative activities toward other oligosaccharides such as maltose or lactose were not hydrolyzed. The products by hydrolysis reaction from sucrose were also detected with TLC (data not shown).

The time course on sucrose hydrolysis was observed by the method of TLC analysis (data not shown). The products from sucrose were glucose and fructose. The limits of hydrolysis of sucrose was almost 100%, but no other products were detected such as oligosaccharides.

Table II Effect of chemicals on invertase activity.

Chemical compound	Relative activity (%)
none	100
p-Chloromercuribenzoic acid	54
Sodium Dodecyl Sulfate	11
EDTA	84
Monoiodoacetic acid	101
$AlCl_3$	98
$CuCl_2$	103
$CaCl_2$	102
$ZnCl_2$	95
NaCl	90
$FeCl_3$	120
KCl	100
$MgCl_2$	96
$CoCl_2$	105
$FeCl_2$	61
AgCl	0

Table III Substrate specificity of the invertase.

Substrate	Relative activity (%)
Sucrose	100
Trehalose	0.5
Raffinose	93.9
Pullulan	1.0
Starch	0
Cellobiose	0
Lactose	0
Maltotriose	0
Isomaltose	0

Baking test with automatic bakery.

The results are shown in Fig. 7. The addition of the invertase showed significant effects on loaf volume. The softness was also improved by the addition of invertase (data not shown).

Enzyme(+)　　Enzyme(−)

Fig. 7 Comparison of loaf volume with or without invertase

DISCUSSION

Invertase (EC 3.2.1.26 β-Fructfranosidase) was partially purified and characterized from culture filtrate by a newly isolated yeast from a digestive juice in pitcher of *Nepenthes*. This strain, designated I-8, was identified to belong to a strain of *Pseudozyma*. Additionaly, the strain I-8 was strictly similar to *Pseudozyma aphidis* which was reported to isolate from a excretion of insect like aphid. *P. aphidis* was known to be a microorganisum in risk group 1. The enzymes for processing are required to be registered as food additives under the Ministry of Health, Labor and Welfare in japan. Therefore, the production of food enzymes must be carried out under the strict checks. The safety checks are also required on all microorganisms for enzyme production. From the view point, this strain I-8 is a good producor of food enzyme.

The invertase had a specific activity of 216 U/mg protein toward sucrose and released glucose and fructose, but no reverse compounds were not detected by TLC analysis. There are many invertases reported the production by fungus, yeast, bacteria, tomato fruit[6] and tobacco cell.[7] Those inverases were reported to produce oligosaccharides by reverse reacrion.

In general, reverse reaction of yeast invertase such as *Saccharomyces* and *Candida* is lower than that of *Aspergillus*.[2]

The enzyme was strongly inhibited by Ag^{2+} and sodium dodecylsulfate. And Ca^{2+} did not promote the activity. Regarding to substrate specificity, sucrose and raffinose were favorable substrates. Amylolitic substrates tested were not hydrolyzed under the standard conditions.

Today, enzymes are important in baking fields. Bread and baked products are the main nutritional sources in the world. A variety of hydrolytic enzymes are used in the baking industry to improve dough handling properties and enhance bread quality. They are amylase, protease, xylanase, lipase, glucose oxidase and so on. In the history of use of enzymes in the baking industry, both α-amylases[8] and proteases[9] have a long history of usage. Both enzymes can be used as dough softner that lead to improve machining properties, higher loaf volume and softer bread crumb.

The beneficial effects of using enzymes in the baking industry are also obtainable by using chemical agents such as potassium bromate, cystain, azodicarbonamide, etc. However, the food market today shows a clear trend toward more natural products, and this has clearly favored the use of enzymes. From these background, we investigated the use of invertase on baking processes.

Addition of invertase on bread making, we got the results of improvement of volume and softness. Invertase can provide glucose or fructose from added sugar, an important source of fermentable sugar, for gas production in longer processes. From the view point, invertase are another useful enzyme for baking. Invertase in baking is also expecting antistaling, i. e., improving the fresh keeping of breads.

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