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Article

Purification and molecular characterization of β-galactosidase from yeast *Kluyveromyces lactis*

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β-Galactosidase from yeast *Kluyveromyces lactis* was purified to be pure using the gel filtration column chromatography and was examined for molecular weight, subunit composition, enzymatic properties (optimum and stable pH, optimum and stable temperature, effect of added metal ions), and steady-state kinetic parameters for the two substrates; lactose and oNPG.

Keywords:
β-galactosidase, yeast *Kluyveromyces lactis*, purification, molecular properties

β-Galactosidase (EC 3.2.1.32) has been purified from many kinds of organism and has been studied in the various fields including basic science and practically useful industries [1-5]. Purification of β-galactosidase from *Kluyveromyces lactis* strain Y1140 was reported for the characterization of its molecular constitution by Dickson R.C. *et al.* [6]. They have examined the induction of the enzyme β-galactosidase in *K. lactis*, characterized the physical and molecular properties of the induced β-galactosidase [6].

β-Galactosidase-catalyzed reactions are studied in the various experimental conditions, such as pH, temperature, ionic strength, high concentration, and presence of organic reagents, of which environmental factors are essentially important in the regulation of the enzyme reactions [7-12]. In the intracellular organelles and cytoplasm, biological functions of the proteins and enzymes are of great interest in understanding their mechanisms. However, the function and mechanism of proteins/enzymes in the membrane and cytoplasm are further difficult for investigation in

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this stage [13-15].

Kinetic analysis is sometimes very effective for the characterization and/or understanding of the enzyme-catalyzed reaction and substrate specificity. In fact, the steady-state kinetic parameters, the Michaelis constant Km, and the molar activity k_0, have been determined for glucoamylase from *Rhizopus niveus* using various substrates in the presence of urea, polyethylene glycol, or aceto-nitrile, and applied to the evaluation of the mechanism on the enzyme-catalyzed reactions and the subsite structure [11,12,16,17].

Under the specified conditions, it is very possible that a glycohydrolase catalyzes a synthesis reaction (condensation, transglycosylation) to produce saccharides having higher degree of polymerization than that of substrate. We are interesting in enzymatic properties of β-galactosidase, which is considered to locate at membrane of the yeast *K. lactis* cells.

In this paper, β-galactosidase was purified from yeast *K. lactis*, and the molecular and enzymatic properties were examined for the successive investigation on the reaction mechanism.

EXPERIMENTAL

Materials

A commercial material of the enzyme β-galactosidase from yeast *K. lactis* was purchased from Amano Enzymes Co. Ltd., Nagoya. Lactose, glucose, galactose, o-nitorophenyl-β-D-galactopyranoside (oNPG), o-nitro phenol (oNP), glycerol, sodium-hydroxide, -carbonate, -chloride, -dihydrogen phosphate, and –hydrogen phosphate, potassium-chloride, and -dihydrogen phosphate, and magnesium sulphate, all in guaranteed grade were obtained from Nacarai Tesque Inc., Kyoto, and were used without further purification. Bovine serum albumin was used of a product from Sigma Chemical Co. A glucose determination-kit, Glucose C-II Test Wako, was a product of Wako Pure Chemical Industries, Ltd., Osaka. Superdex 200 for the column chromatography was purchased from Pharmacia Biotech. Japan Co., Tokyo.

Enzyme assay

β-Galactosidase-catalyzed hydrolysis reaction was carried out in a 20mM phosphate buffer pH 7.0 at 25.0°C with lactose as a substrate to obtain the reaction time-curves. In this experiment, phosphate buffer contains 10mM KCl and 1 mM $MgSO_4$. Reactions were initiated by addition of the enzyme solution to the substrate solution, and aliquots (usually 100μl) were taken out at appropriate time intervals and mixed with a 50mM NaOH solution (usually 100μl) to stop the enzyme-catalyzed reaction. Amount of glucose released from a substrate lactose by the catalyzed reaction was measured using a routine procedure with glucose assay-kit, Glucose C-II Test Wako (GOD) [18], which was used after 4-fold dilution in the 20 mM phosphate buffer pH 7.0.

The β-galactosidase-catalyzed hydr-

olysis for another substrate oNPG was measured spectrophotometrically in 20mM phosphate buffer pH 7.0 at 25ºC. Reactions were initiated by addition of the enzyme solution to the substrate oNPG solution, and aliquots (100µl) were removed at appropriate time intervals and mixed with a 100mM Na_2CO_3 solution (2ml) to stop the catalyzed reaction. Based on the absorbance at 420nm, the amount of oNP released from the substrate oNPG by the enzyme-catalyzed reaction was measured to obtain the time-curves.

One unit of enzyme activity was defined to release 1 µmol of oNP per min for the substrate oNPG or 1µmol of glucose per min for the substrate lactose. Concentrations of the enzyme were determined spectrophotometrically using the Bradford method with the Coomassie reagent, a product of PIERCE Co. and bovine serum albumin as a standard protein sample.

Purification of the enzyme preparation

A commertial product of ß-galactosidase from yeast *K. lactis*, of Amano Enzymes Co. Nagoya, was purified using a gel filtration chromatography with Superdex 200. A 50cm long 1.5cm diameter column (bed volume 85ml) was equilibrated with a 20mM phosphate buffer pH 7.0 before use. The sample containing ß-galactosidase was added on to the column and eluted with 1 - 1.5 bed volume of a 20mM phosphate buffer, of which flow rate is 0.38ml per min and fractions of 2ml volume were collected in fraction tubes. All purification procedures were conducted at 4ºC unless noted otherwise.

Each fraction was estimated for the protein concentration by measurement of absorbance at 280nm using a Shimadzu UV-1600 spectrophotometer and for the lactose hydrolysis enzyme-assay according to the method described above and the specific activity was evaluated for the purified ß-galactosidase. Glycerol was added to the purified enzyme preparation to keep the stability.

RESULTS AND DISCUSSION

Purification of ß-galactosidase

The enzyme sample of ß-galactosidase (6.2mg in 600µl), was added to the Superdex column and eluted with a 20 mM phosphate buffer containing 10 mM KCl and 1 mM $MgSO_4$. The result is shown in Fig. 1, clearly indicating that the first peak in the two peaks has the lactose-hydrolysis enzymr-catalyzed activity. Fractions containing the enzymatic activity were pooled and concentrated by ultrafiltration. After the ultrafiltration, protein concentrations and hydrolysis enzymatic activity were determined to evaluate the specific activity as follows: The specific activity of the enzyme preparation showed about 2-fold increase and the yield of total activity was 72%. The purified enzyme was stored in 20 mM phosphate buffer pH 7.0 containing 20% glycerol.

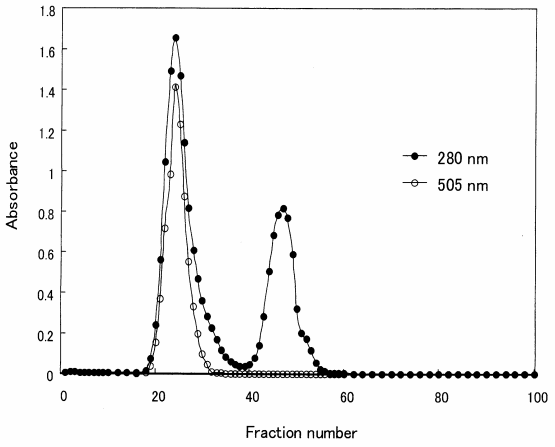

Fig. 1. Superdex chromatogram for purification of β-galactosidase
Column φ 1.5 X 50cm, 20mM phosphate buffer pH 7.0 containing 10mM KCl and 1mM $MgSO_4$, fraction 2ml, flow rate 0.38ml/min, 4℃, ●; protein, ○; enzyme activity.

Physicochemical and molecular properties of the purified ß-galactosidase preparation

The molecular weight of the purified ß-galactosidase preparation was determined by gel filtration technique: standard proteins used were: ferritin from horse spleen (MW440,000), catalase from bovine liver (232,000), ß-glucosidase from *Aspergillus niger* (137,000), and bovine serum albumin (66,000). Molecular weight of the purifiedß-galactosidase preparation was analyzed to be 240,000.

Subunit composition of the purified ß-galactosidase preparation was examined by SDS-PAGE technique with a 10% polyacrylamide gel where standard proteins were: rabbit muscle myosin (MW212,000), bovine plasma α_2-macroglobulin (170,000), ß-galactosidase from *E. coli* (116,000), human transferring (76,000), and bovine liver glutamic dehydrogenase (53,000). Molecular weight of a subunit protein in ß-galactosidase was estimated to be 120,000; thus, ß-galactosidase obtained from *K. lactis* was concluded to be a dimmer.

ß-Galactosidase catalyzed reaction in the presence of metal ions

The effect of metal ions in the enzyme-catalyzed reaction as analyzed by using 30mM lactose as a substrate in 20mM phosphate buffer pH 7.0 at 25°C. The enzyme-catalyzed activity in addition of KCl at concentration of 50 - 200mM was found to be 1.5 fold larger than that in the reference (no addition), whereas the activity in the addition of 0.3 - 5mM $MgSO_4$ was 3.3 fold larger than that in the reference. Based on these results, both 10mM KCl and 1mM $MgSO_4$ were chosen to be presence in 20 mM phosphate buffer pH 7.0 when the ß-galactosidase-catalyzed reaction was examined.

pH optimum and pH stability for the ß-galactosidase-catalyzed reactions

The ß-galactosidase-catalyzed reactions were observed with 30mM lactose as the substrate in 20mM phosphate buffer containing 10mM KCl and 1mM $MgSO_4$ at the pH range of 5.5 to 9.0 at 25°C. The experimental results are shown in Fig. 2, where the enzymatic reactions dropped at below pH 6.5 and at above pH 7.0, suggesting that the optimum pH of the enzyme-catalyzed reaction is 7.0. Thus, the enzyme-

catalyzed reactions were carried out at pH 7.0 in this study.

The pH stability for the β-galactosidase-catalyzed reactions was examined by measuring the remaining activity after 30min incubation at 25°C with 30 mM lactose as the substrate in 20mM phosphate buffer pH 5.5 - 9.0. The experimental results are illustrated in Fig. 2, suggesting that the enzyme is stable in a pH range of 7.0 to 8.5.

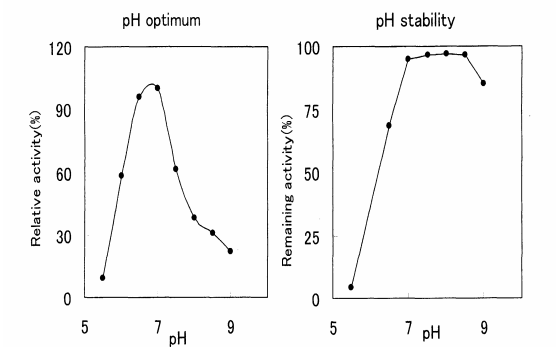

Fig. 2. Effect of pH on the β-galactosidase-catalyzed reaction and stability of the enzyme
$[E]_0$; 3.2nM, 20mM phosphate buffer containing 10mM KCl and 1mM $MgSO_4$,25°C, stability; 25°C for 30min

Temperature-optimum and -stability for the β-galactosidase-catalyzed reactions

The effect of temperature on the β-galactosidase-catalyzed reactions was analyzed at 5 - 80°C with 30mM lactose as the substrate in 20mM phosphate buffer pH 7 containing 10mM KCl and 1 mM $MgSO_4$. The experimental results are shown in Fig. 3, suggesting that the temperature optimum is 50°C.

The temperature stability of the β-galactosidase-catalyzed reactions was analyzed by measuring the remaining activity after 30min incubation at 5 - 50°C and pH 7.0 with 30mM lactose as the substrate. The experimental results are illustrated in Fig. 3, where the enzyme activity dropped off rapidly above 35°C, suggesting that the stable temperature range is 5 - 35°C.

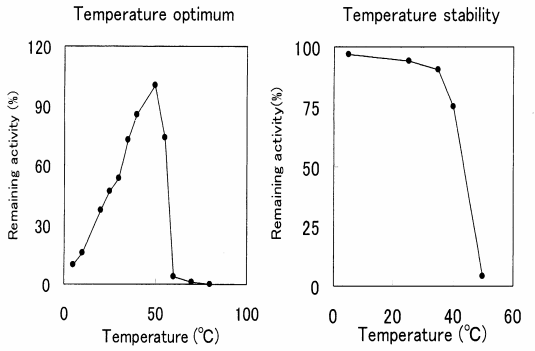

Fig. 3. Effect of temprature on the β-galactosidase-catalyzed reaction and stability of the enzyme
$[E]_0$; 3.2nM, 20mM phosphate buffer pH 7.0 containing 10mM KCl and 1mM $MgSO_4$, stability; pH 7.0 for 30min

Steady-state kinetic properties of the β-galactosidase-catalyzed reactions

Based on the initial velocity, kinetic parameters including the Michaelis constant Km and molar activity k_0 were evaluated for lactose or oNPG as a substrate, where concentrations were selected at the range from 2.4 to 57mM for lactose and from 0.38 to 3.8mM for oNPG. Some typical examples of the reaction-time courses are shown in Figs. 4 and 5 for the substrate lactose and oNPG, respectively. The experimental data were treated by a software, Biograph, Kyoto Soft Co., [19].

From slopes of the reaction time-courses, the initial velocity (v) was

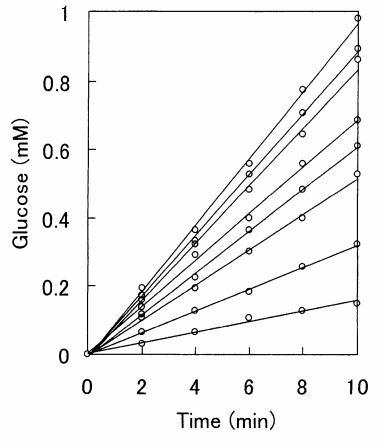

Fig. 4. ß-Galactosidase-catalyzed reaction for a substrate lactose
$[E]_0$; 5.9nM, pH 7.0, 25°C.

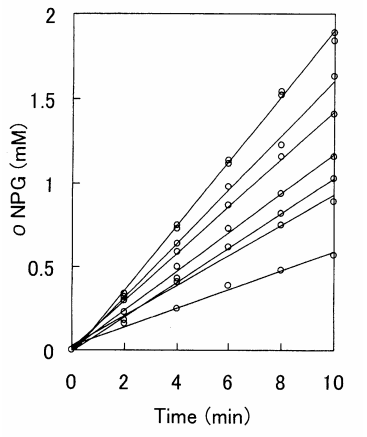

Fig. 5. ß-Galactosidase-catalyzed reaction for a substrate oNPG
$[E]_0$; 1.6nM, pH 7.1, 25°C.

Table 1. Kinetic parameters on the ß-galactosidase-catalyzed reaction for lactose and oNPG

	Km (mM)	k_0 (sec^{-1})	k_0/Km ($mM^{-1}sec^{-1}$)
Lactose	13.6 ± 0.7	19 ± 2.0	1.4
*o*NPG	5.3 ± 0.2	249 ± 7.3	46.9

$[E]_0$; 1.6nM, 20mM phosphate buffer pH 7.0, 25°C.

obtained at different concentrations of substrate $[S]_0$. As shown, that the plots are excellently fitted to the Michaelis-Menten fashion. Thus, the kinetic parameters evaluated by using the steady-state methods are summarized in Table 1, where the weighted mean values were obtained on the basis of the experiments carried out more than three times.
With kinetic parameters Km and k_0, k_0/Km values were calculated as shown in Table 1, suggesting that oPNG is a better substrate for the ß-galactoseidase -catalyzed reaction than lactose.

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