原 著

Measurement of β-Mannosidase Activity Using β-1, 4-Mannobiose

SATORU WATANABE, YOSHIMI USHIZAWA and TSUNEO YASUI

"Glucose CII-Test Wakô" (Glucose Oxidase (GOD) Reagent) reacts glucose well, and is sensitive against xylose and mannose (M_1).[3] Using this reagent and β-1, 4-mannobiose (M_2), *Aspergillus niger* β-mannosidase activily could be measured. The enzyme hydrolyzed M_2 to produce M_1, and no transfer product was detected in the thin-layer chromatography. Optimal pH of the enzyme using M_2 was measured to be the same around pH 3-4 as using *p*-nitrophenyl β-D-mannopyranoside (PNPM). The enzyme activity measured by the developed method seemed to be identical with the activity using PNPM as substrate. This result indicate that β-mannosidase activity can be measured by the developed method using M_2 instead of PNPM.

β-Mannosidase activity has been measured using PNPM which is a synthetic substrate. However, natural substrates are desirable to be used in order to solve natural action, substrate specificity and function of enzymes. Iizuka *et al.*[3] confirmed that "Glucose CII-Test Wakô"(Glucose Oxidase(GOD) reagent) was slightly reactive against xylose, mannose(M_1) or other sugars. They developed the method for measurement of β-xylosidase activity using β-1, 4-xylobiose and the GOD reagent. In the present paper, the method for measurement of β-mannosidase activity using β-1,4-mannobiose(M_2) and the GOD reagent was developed, and *Asp. niger* β-mannosidase activity was measured by the developed method.

MATERIALS AND METHODS

Chemicals.

Mannose(M_1) and "Glucose CII-Test Wakô" were purchased from Wakô Pure Chemical Co. β-1, 4-Mannobiose(M_2) was prepared from a hydrolyzate of copra galactommannan.[7] *p*-Nitrophenyl β-D-mannoside(PNPM) and guar gum were from Sigma Chemical Co. All other chemicals were of analytical grade.

Measurement of β-Mannosidase Activity Using PNPM.

β-Mannosidase(EC3. 2. 1. 25) activity was measured using PNPM(1mM) as substrate. The amount of *p*-nitrophenol released was determined spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of enzyme releasing one μmol of *p*-nitrophenol per min under the incubation conditions of 60℃, at pH 4.0(McIlvaine buffer) for 10 min. For stopping the reaction, 0.2 M Na_2CO_3 was added.[9]

Key Words: β-Mannosidase, β-1,4-Mannobiose, Mannose.

Production of Asp. niger β-Mannosidase.

For the production of *Asp. niger* β-mannosidase, guar gum was used as a carbon source. The medium was composed of 1.0% guar gum, 0.9% peptone, 0.1% yeast extract, 1.0% potassium phosphate (monobasic), 0.05% magnesium sulfate and 0.5% corn steep liquor.[9] One hundred ml of the medium was placed in a 500-ml shaking flask and sterilized at 120℃ for 12 min in an autoclave. After cooling, *Asp. niger* was inoculated to the flask.

The cultivation was carried out at 35-37℃ on a reciprocal shaker for 5 days. The mycelium was filtered off through a Buchner funnel with a Tôyô-Roshi No. 2 filter paper, and the filtrate was used as the β-mannosidase solution.

Measuremnet of Optimal pH for the β-Mannosidase.

Asp. niger β-mannosidase activity was measured at several pHs (2-8, McIlvaine buffer) using PNPM (1 mM) and M_2 (50mM). A reaction mixture containing McIlvaine buffer (pH 2-8) was incubated for 10 min at 60℃ for PNPM and 37℃ for M_2, respectively. In the case of PNPM, 0.2 M Na_2CO_3 was added to the reaction mixture in order to stop the reaction. In the case of M_2, the reaction was stopped by heating at 100℃ for 5 min, and then 200 μl of the reaction mixture was withdrawn for the determination of the amount of M_1.

Thin-Layer Chromatography (TLC).

TLC was done on a plate of Kiesel gel 60 (Merck Co.) using a solvent system of chloroform-methanol-water (90 : 65 : 15, v/v) for about 2 hr at room temperature. The sugars on the plate were visualized by heating to 120℃ for about 10 min after spraying with 30% sulfuric acid-ethanol.

RESULTS

Calibration Curve for Mannose (M_1).

"Glucose C II-Test Wakô" reacted only with M_1 but not with M_2, so we could measure the amount of M_1 using this reagent. Two hundred μl sample was added into 5 ml of the reagent. After incubation at 37℃ for 10 min, the absorbance at 505 nm was measured. Figure 1 shows the calibration curve for M_1. Up to 40 mg/ml M_1, a straight proportional line was obtained against the absorbance at 505 nm.

Mesurement of β-Mannosidase Activity Using β-1,4-Mannobiose (M_2).

Since the GOD reagent does not react with M_2, *Asp. niger* β-mannosidase activity can be measured by determining the amount of M_1 using the GOD method combined with M_2 hydrolysis system. This developed reaction system is as follows. One hundred μl of 100 mM M_2 is mixed with 100 μl of the enzyme solution (2.0 units/ml). After incubation (37℃, 10min), the reaction mixture is heated at 100℃ for 5 min to inactivate the enzyme. Then, two hundreds μl of the reaction mixture is added into 5 ml of the GOD reagent and incubated

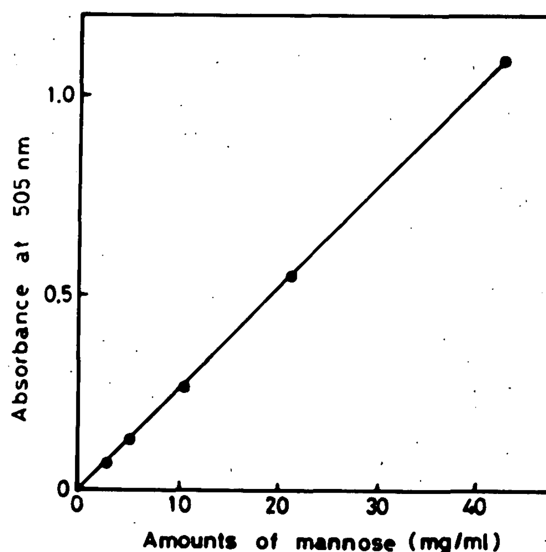

Fig. 1. Calibration Curve for Mannose.

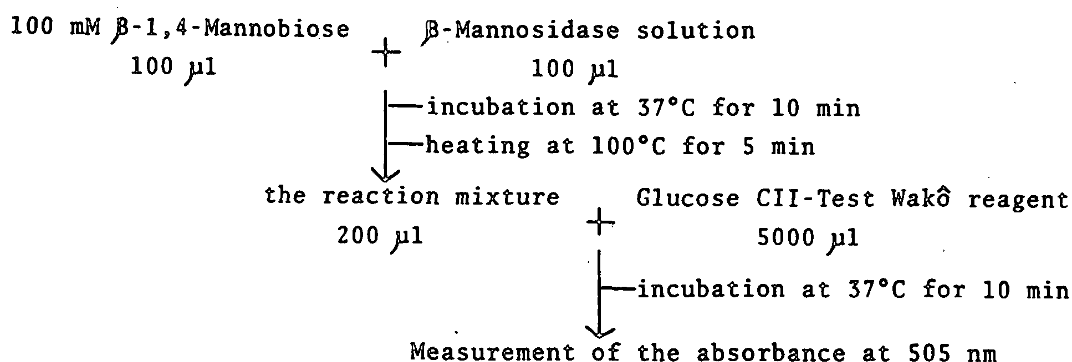

Fig. 2. Flow Sheet for Measurement of β-Mannosidase Activity.

at 37℃ for 10 min. Within 2 hr, the absorbance at 505 nm is measured. Figure 2 shows the flow sheet of the new method for measurement of β-mannosidase activity.

Time Course of the Hydrolysis of M_2 by Asp. niger β-mannosidase.

The hydrolysis of M_2 was carried out with 2.0 units/ml of *Asp. niger* β-mannosidase as shown in Fig. 2. Time course of the reaction was traced up to 3 hr as shown in Fig. 3. Each sample (0, 0.5, 1, 3 hr) was spotted on the silica gel plate, and then thin-layer chromatography was done as shown in Fig. 4. The enzyme hydrolyzed M_2 to produce M_1, and no transfer product was detected chromatographically. Using PNPM (1%) as substrate, the enzyme hydrolyzed PNPM to produce *p*-nitrophenol and M_1, and transfer product was not detected either as shown in Fig. 5.

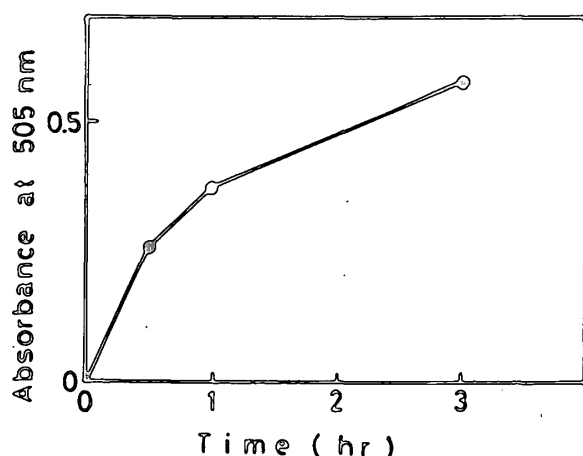

Fig. 3. Time Course of the Hydrolysis of M_2 by *Asp. niger* β-Mannosidase.

Optimal pH for Asp. niger β-Mannosidase.

The optimal pH of the enzyme activity was found to be around pH 3-4 in the

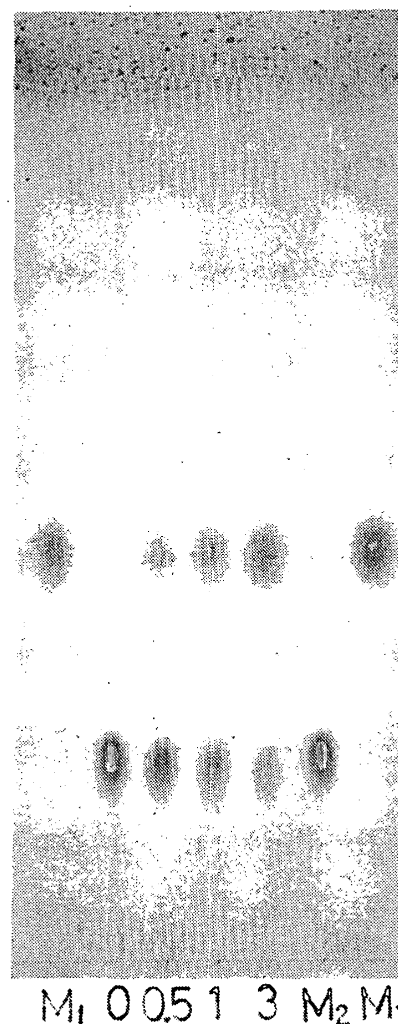

Fig. 4. Thin-Layer Chromatogram of the Hydrolyzates of M_2 by *Asp. niger* β-Mannosidase.

M_1, authentic mannose ; M_2 authentic mannobiose ; 0, 0.5, 1 and 3 correspond to the sample of corresponding time in Fig. 3.

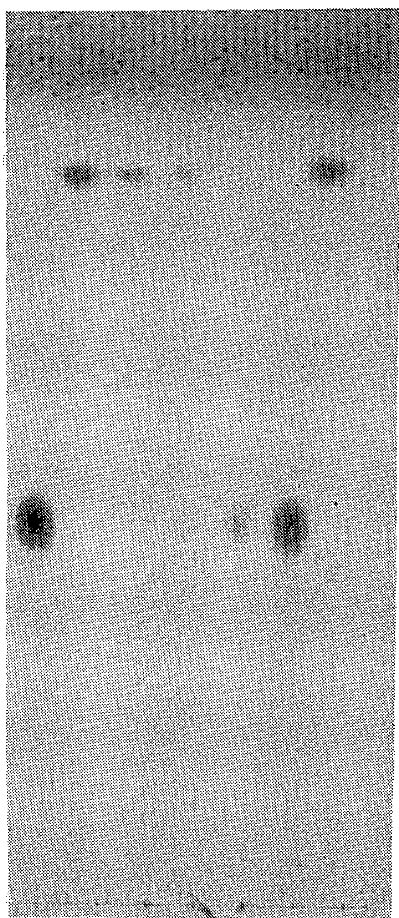

Fig. 5. Thin-Layer Chromatogram of the Hydrolyzates of PNPM by *Asp. niger* β-Mannosidase.

M_1, authentic mannose ; P, authentic PNPM ; 0, 0.5, 1 and 3 correspond to the sample of corresponding time for the β-mannosidase action on PNPM (1 %).

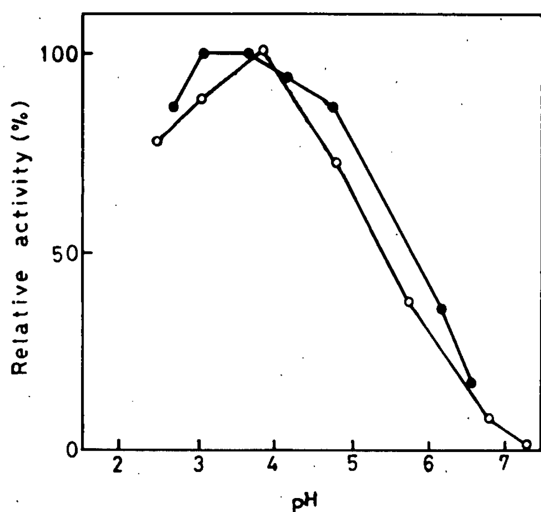

Fig. 6. Optimal pH for β-mannosidase from *Asp. niger*.

Conditions of the reactions are described in **MATERIALS AND METHODS**. ○——○, PNPM ; ●——●, M_2.

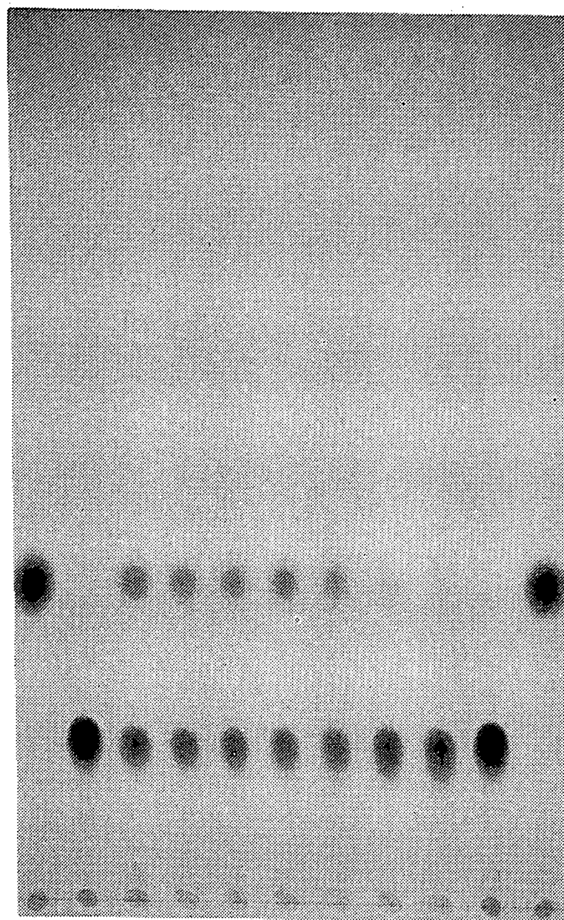

Fig. 7. Thin-Layer Chromatogram of the Hydrolyzates of M_2 by *Asp. niger* β-Mannosidase on Several pHs.

M_1, authentic mannose ; M_2 authentic mannobiose.

both substrates (M_2 and PNPM) as shown in Fig. 6. Figure 7 shows thin-layer chromatogram of the hydrolyzates of M_2 by *Asp. niger* β-mannosidase on several pHs. The enzyme hydrolyzed M_2 to produce M_1 and no transfer product was detected under pH 2-8.

DISCUSSION

"Glucose CII-Test Wakô" (GOD reagent) is one of the most effective reagent for the determination of free glucose especially in serum or urine. However, it was known that GOD and the GOD reagent were slightly reactive against xylose, M_1 or other sugars.[6)]

Using the GOD reagent, some glycosidase activity can be measured, if the GOD reagent

is exclusively reactive against the component or liberated monosaccharides but not reactive against disaccharide substrate in an assay system containing the disaccharide as substrate.[8,10]

Iizuka *et al.*[3] confirmed that the GOD reagent did react considerably with xylose but not with xylobiose. According to this fact, they deviced a method for the determination of β-xylosidase activity using the GOD reagent and the xylobiose substrate. In the present paper, since the GOD reagent reacts with M_1 but not with M_2, the GOD reagent could be applied for the determination of *Asp. niger* β-mannosidase activity.

Asp. niger β-mannosidase hydrolyzed M_2 and PNPM without any transfer reaction in this experiment (as shown in Fig. 4, 5 and 7). However, some reports[4,5] described some transmannosidation reaction of *Asp. niger* β-mannosidase. If there is some transmannosidation reaction, the GOD reagent should be examined whether it reacts transfer products or not.

Optimal pH of the enzyme was around pH 3-4 which corresponds to the value in some literature[1,2,4] using PNPM as substrate. This value is the same in the case using M_2 as substate. This indicates that the enzyme activity measured by using M_2 seemed to be identical with the activity using PNPM.

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β-1,4-マンノビオースを用いたβ-マンノシダーゼ活性測定法

—渡邊　悟、牛澤　良美、安井　恒男—

グルコースCⅡ—テストワコー（グルコースオキシダーゼ試薬）は、グルコースはもちろん、キシロースやマンノースにも少し反応する。当試薬とβ-1,4-マンノビオース（M_2）を用いて、アスペルギルス・ニガーのβ-マンノシダーゼ活性が測定できた。当酵素はM_2を加水分解し、マンノースを生成し、転移物は見られなかった。M_2を用いた場合の至適pHは、*p*-ニトロフェニルβ-D-マンノピラノシド（PNPM）を用いた場合と同様で3～4の値を示した。PNPMを用いた場合の酵素活性はM_2を用いた場合と同等と思われ、人工基質PNPMの代わりに天然基質M_2を用いてβ-マンノシダーゼ活性が測定できることがわかった。