原 著

聖徳栄養短期大学紀要No. 24 (1993)

Measurement of β-Glucobiose-Hydrolysis Activity and Comparison of Two β-Glucosidases

SATORU WATANABE, YOSHIMI USHIZAWA and TUNEO YASUI

Release of glucose(G1) from β-glucobiose(G2) by β-glucosidase action could be measured using G2 and "Glucose CII-Test Wakô" (Glucose Oxidase(GOD) Reagent). *Almond Emulsin* β-glucosidase hydrolyzed G2 to produce G1, and no transfer product was detected in the thin-layer chromatography. The relative rate liberating G1 from G2 by the enzyme was in the order of laminaribiose > sophorose > cellobiose > gentiobiose. The enzyme had higher hydrolysis activity for *p*-nitrophenyl β-D-glucoside(PNPG) than for G2, so that it could be called an "aryl β-glucosidase". However, *Streptomyces* β-glucosidase was different from the enzyme, and could be called a "transglucosidase". Absorption spectra of PNPG-hydrolysis system combined with the GOD reagent was also compared between the two β-glucosidases.

β-Glucosidase activity has usually been measured using a synthetic substrate, for example, *p*-nitrophenyl β-D-glucoside(PNPG). However, natural substrates are desirable to be used in order to study natural action, substrate specificity and function of enzymes.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) releases glucose(G1) from the substrate which has a structure of β-D-glucoside. Determination of G1 is a direct method for measurement of β-glucosidase activity which has no transfer activity. For determination of G1, "Glucose CII-Test Wakô" (GOD reagent) is one of the most effective reagent. The GOD reagent was applicable to assay for disaccharide-hydrolysis activity of glucosidase.[1,6]

In the present paper, the β-glucobiose (G2)-hydrolysis activity was measured using various G2s and the GOD reagent, and the actions of *Almond Emulsin* and *Streptomyces* β-glucosidase were compared.

MATERIALS AND METHODS

Chemicals.

p-Nitrophenyl β-D-glucoside(PNPG) was purchased from Sigma Chemical Co. (U.S.A.). Sophorose(So) was prepared from a partial acid hydrolyzate of stevioside.[2] Laminaribiose (La) and gentiobiose(Ge) were prepared from hydrolyzates of curdlan by the crude enzymes of *Streptomyces* sp. K27-4[3] and *Streptomyces* sp. W19-1[4], respectively. Curdlan was purchased from Wakô Pure Chemicals(Japan) and cellobiose(Ce) was from Nakalai Tesque Inc. (Japan). All other chemicals were commercially available products of analytical grade.

Two β-Glucosidases.

Almond Emulsin β-glucosidase was

Key words : β-Glucosidase, β-Glucobiose, GOD reagent

purchased from Sigma Chemical Co. (U.S.A.). The enzyme had been chromatographically purified.

For *Streptomyces* β-glucosidase, the crude enzyme of *Streptomyces* sp. W19-1 was prepared by the procedure described in a previous paper.[4] The medium for cultivation contained 1.5% curdlan, 0.7% peptone, 0.2% yeast extract, 0.5% corn steep liquor, 1.0% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. The cultivation was performed at 35-37℃ for 4 days on a reciprocal shaker (120 oscillations per min). The culture broth so obtained was filtered through Tôyô-Roshi No. 2, and the filtrate was used as *Streptomyces* β-glucosidase.

Measurement of β-Glucosidase Activity Using PNPG.

PNPG-hydrolyzing activity was assayed by spectrophotometric measurement of *p*-nitrophenol (PNP) released from PNPG. The assay mixture contained 0.4 ml of buffer solution, 0.5 ml of 2 mM PNPG and 0.1 ml of the enzyme solution. Acetate buffer solution (pH 5.0) was used for *Almond Emulsin* β-glucosidase, and McIlvaine buffer solution (pH 6.0) for *Streptomyces* β-glucosidase. The reactions were performed at 37℃ for *Almond Emulsin* and at 55℃ for *Streptomyces* β-glucosidase, respectively. After incubation for 10 min, the reactions were stopped by adding 1.0 ml of 0.2 M sodium carbonate. Within 2 hr, the absorbance at 400 nm was measured using Shimazu spectrophotometer UV-2200. One unit of PNPG-hydrolysis activity was defined as the amount releasing one μmol of PNP per min.

Measurement of β-Glucosidase Activity Using β-Glucobiose(G2) and the GOD Reagent.

β-Glucobiose (G2)-hydrolysis activity using G2 and the GOD reagent was measured by a modification of the method[6] described previously. That is, 100 μl of 1% G2 was mixed with 100 μl of the enzyme solution. After incubation for 30 min, the reaction mixture was heated at 100℃ for 5 min to inactivate the enzyme. Then, two hundreds μl of the reaction mixture was added into 5 ml of the GOD reagent and incubated at 37℃ for 10 min. Within 2 hr, the absorbance at 505 nm was measured.

Thin-Layer Chromatography(TLC).

TLC was done on a plate of Kiesel gel 60 (Merck Co. (Germany)) 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 140℃ for about 5 min after spraying with 30% sulfuric acid-methanol.

Spectrophotometric-Analysis of PNPG-Hydrolysis System Combined with the GOD Reagent.

Incubation of PNPG (0.5%, final conc.) with β-glucosidase was performed at 37℃ up to 3 hr. At intervals (0, 0.5, 1, 3 hr), the reaction mixtures were withdrawn and heated at 100℃ for 5 min to inactivate the enzyme. Then, each fifty μl of the treated sample was added into 5 ml of the GOD reagent and incubated at 37℃ for 10 min. Within 2 hr, absorption spectrum from 300 nm to 700 nm of each sample was measured using Shimazu spectrophotometer UV-2200.

RESULTS

Color Reaction of G2 against the GOD Reagent.

Two hundreds μl of G2 (1%) was added into 5 ml of the GOD reagent. After incubation at 37℃ for 10 min, absorbance at 505 nm was measured. Table 1 shows the reactivity of G2 against the GOD reagent compared to G1. The reagent did not virtually develop its color reaction with tested G2s. Sophorose (So) among tested G2s had the lowest reactivity

against the reagent.

Table 1 Reactivity of β-Glucobioses against Glucose CII-Test Wakô (GOD Reagent)

Sophorose	0.05
Laminaribiose	0.39
Cellobiose	0.09
Gentiobiose	0.65

Reactivity of glucose against the GOD reagent was set as 100.

Liberation of G1 from G2 by Almond Emulsin β-Glucosidase Action.

Hydrolysis of G2(0.5%, final conc.) by *Almond Emulsin* β-glucosidase(0.322 units) was performed at 37℃. Increase of absorbance was traced up to 3 hr as shown in Fig. 1. The relative rates liberating G1 from G2 were in the order of La>So>Ce>Ge. Each sample(0, 0.5, 1, 3 hr) was spotted on the silica gel plate, and then thin-layer chromatogram was obtained as shown in Fig. 2. The enzyme hydrolyzed G2 to produce G1, and no transfer product was detected chromatographically in this condition.

Liberation of Sugars from G2 by Streptomyces β-Glucosidase Action.

Incubation of G2(0.5%, final conc.) with *Streptomyces* β-glucosidase(0.089 units) was performed at 55℃. Increase of absorbance was traced up to 3 hr as shown in Fig. 3. The enzyme produced G1 and transfer products from G2[5]. However, increase of absorbance depended on mainly G1. Figure 3 shows that the relative rates of liberating G1 from G2 were in the order of La>So>Ge>Ce.

Spectrophotometric-Analysis of PNPG-Hydrolysis by Two β-Glucosidases.

Incubation of PNPG(0.5%, final conc.) with *Almond Emulsin* β-glucosidase(0.129 units) was performed at 37℃ up to 3 hr. Absorption spectrum of each sample(0, 0.5, 1, 3 hr) combined with the GOD reagent was measured as shown in Fig. 4. The

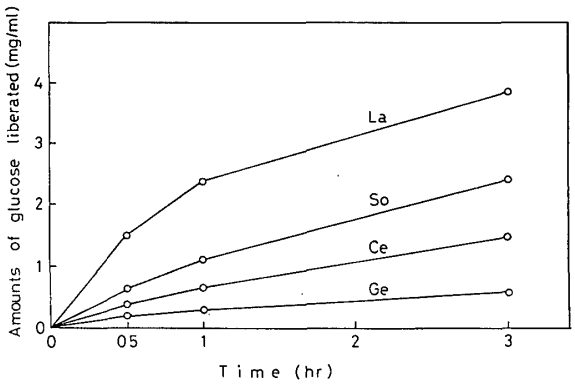

Fig. 1. Time Course for Glucose Liberation from β-Glucobiose by *Almond Emulsin* β-Glucosidase.

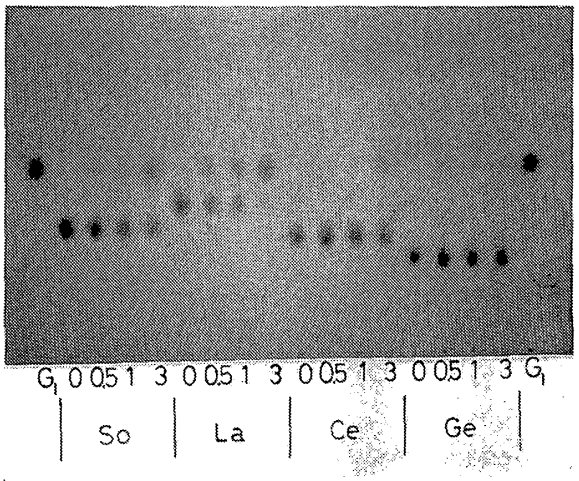

Fig. 2. Thin-Layer Chromatogram for the Hydrolyzates of β-Glucobioses by *Almond Emulsin* β-Glucosidase.
G1, authentic glucose; So, sophorose; La, laminaribiose; Ce, cellobiose; Ge, gentiobiose. Each number(0, 0.5, 1 and 3) corresponds to the incubation time (hr).

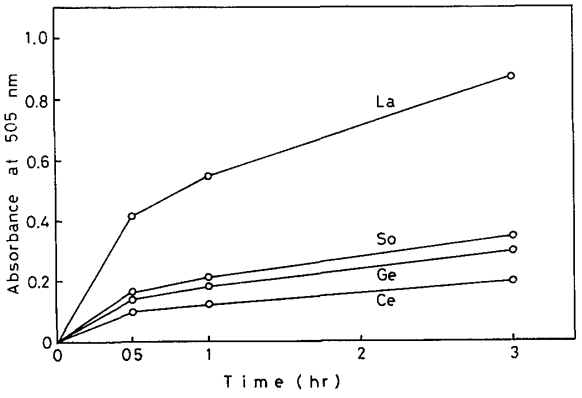

Fig. 3. Time Course for Sugar Liberation from β-Glucobiose by *Streptomyces* β-Glucosidase.

absorbance at 400 nm corresponded to the

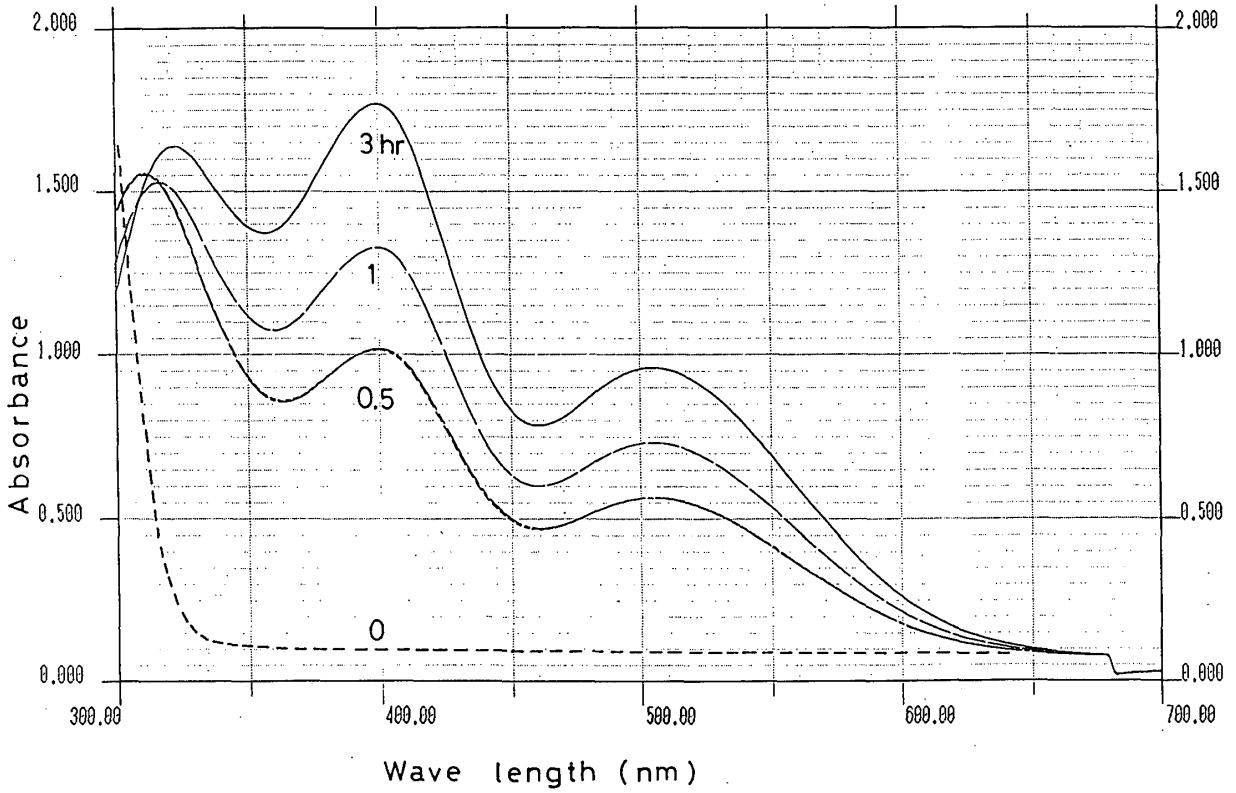

Fig. 4. Absorption Spectra of PNPG-Hydrolysis System Combined with the GOD Reagent (*Almond Emulsin* β-Glucosidase).

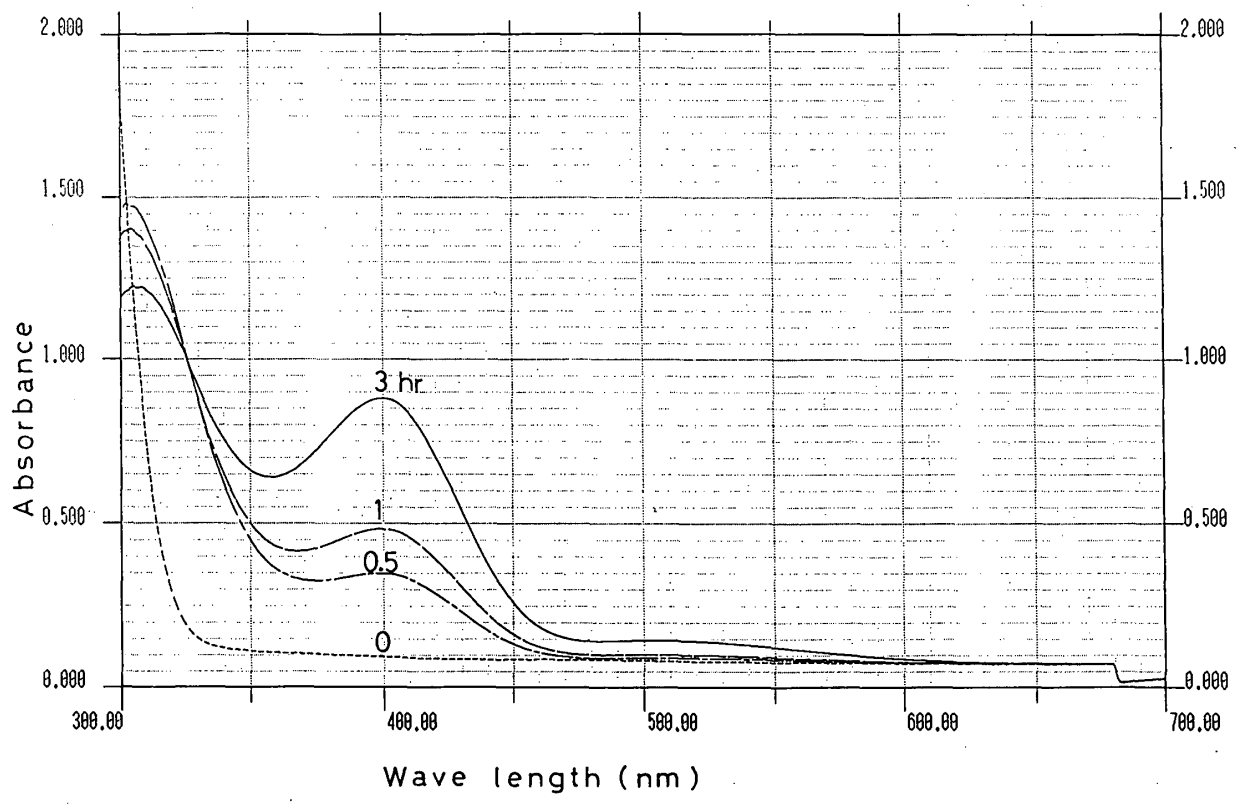

Fig. 5. Absorption Spectra of PNPG-Hydrolysis System Combined with the GOD Reagent (*Streptomyces* β-Glucosidase).

amount of *p*-nitrophenol(PNP), and that at 505 nm did to the amount of G1. The molar amount of PNP was equal to that of G1.

In case of *Streptomyces* β-glucosidase (0.089 units), absorption spectra were obtained as shown in Fig. 5. The molar amount of PNP was not equal to that of G1. *p*-Nitrophenol was released from PNPG by the enzyme, but G1 was hardly detected.

DISCUSSION

"Glucose CII-Test Wakô"(GOD reagent) is the most sensitive against glucose(G1) and did hardly react with β-glucobiose(G2). Therefore, G2-hydrolysis activity could be measured using G2 and the GOD reagent. At the aspect of color reaction against the blank, sophorose(So) was the best substrate among four G2s. Reactivity of G2 against the reagent was in the order of gentiobiose (Ge) > laminaribiose(La) > cellobiose(Ce) > sophorose(So). It is unknown why So is the lowest reactivity against the reagent among four G2s. However, La was the best substrate at the aspect of hydrolysis rate. It is interesting why hydrolysis of linkage 1-3 is the easiest.

The relative rate of liberating G1 from G2 by *Almond Emulsin* β-glucosidase was in the order of La>So>Ce>Ge. That by *Streptomyces* β-glucosidase was in the order of La>So>Ge>Ce. There are slight differences. The order of G2-hydrolysis may be applicable to the classification of β-glucosidases.

Comparison of the two β-glucosidases is summarized in Table 2. The Table also shows PNPG-hydrolysis and La-hydrolysis activity of the enzymes when G2 was hydrolyzed. *Almond Emulsin* β-glucosidase had higher hydrolysis activity for PNPG than for G2, so that the enzyme could be called an "aryl β-glucosidase". In contrast, *Streptomyces* β-glucosidase was reported as a transglucosidase.[5] Since this enzyme has a transfer activity, substrate concentration and enzyme concentration affect both the rates of hydrolysis and transfer reactions. However, the ratio of PNPG-hydrolysis activity to G2-hydrolysis activity seems to be applicable to the classification of β-glucosidases.

Spectrophotometric-analysis of PNPG-hydrolysis combined with the GOD reagent shows both amounts of PNP and G1. The action type of enzyme can be determined by comparison of the absorption spectrum. That is, *Almond Emulsin* β-glucosidase is a "Hydrolase" and *Streptomyces* β-glucosidase is a "Transglucosidase".

REFERENCES

1) Iizuka, Y., Kamiyama, Y. and Yasui, T. : *Biosci. Biotech. Biochem.*, **57**(4), 674 (1993).

2) Kusakabe, I., Kusama, S. and Murakami,

Table 2. Comparison between *Almond Emulsin* and *Streptomyces* β-Glucosidases.

Enzyme source	Reaction condition	PNPG-hydrolysis*	La-hydrolysis**	Relative rate	Transfer activity
Almond Emulsin	pH 5.0, 37℃	0.643 μmol	0.553 μmol	La>So>Ce>Ge	−
Streptomyces	pH 6.0, 55℃	0.178 μmol	0.618 μmol	La>So>Ge>Ce	+

* : amounts of PNP released from 1 mM PNPG by one ml of the corresponding enzyme per min.

** : amounts of G1 released from 0.5% La by one ml of the corresponding enzyme per min.

K.：*Agric. Biol. Chem.*, **51**(8), 2255(1987).
3) Kusama, S., Kusakabe, I., Zama, M., Murakami, K. and Yasui, T：*Agric. Biol. Chem.*, **48**, 1433(1984).
4) Kusama, S., Kusakabe, I. and Murakami, K.：*Agric. Biol. Chem.*, **49**, 2055(1985).
5) Kusama, S., Kusakabe, I. and Murakami, K.：*Agric. Biol. Chem.*, **50**, 2891(1986).
6) Watanabe, S., Ushizawa, Y. and Yasui, T.：*Memoirs of Seitoku Jr. College of Nutr.*, **23**, 8(1992).

和文要旨

β-グルコ2糖の分解活性の測定と二種のβ-グルコシダーゼの比較

－渡邉　悟，牛澤　良美，安井　恒男－

β-グルコ2糖（G2）とグルコースオキシダーゼ（GOD）試薬を用いることによって、β-グルコシダーゼによるG2からのグルコース（G1）の遊離が測定された。アーモンドエムルシンのβ-グルコシダーゼはG2を加水分解してG1を生成し、転移物の生成は見られなかった。G1の遊離速度はラミナリビオース＞ソホロース＞セロビオース＞ゲンチオビオースの順であった。本酵素はG2よりもp-ニトロフェニルβ-D-グルコシド（PNPG）に対し強い活性を有し、アリルβ-グルコシダーゼと分類されるのが妥当と思われる。一方、放線菌β-グルコシダーゼはアーモンドのものと異なり、トランスグルコシダーゼと呼ぶのが適当と思われる。PNPG分解とGOD試薬を組み合わせることで、上記二種の酵素を比較した。