Cellulase-Catalyzed Transglucosylation of Acetaminophen and Acyclovir: Preparative Enzymatic Synthesis of β-glucose Conjugate

Takashi Mizuma,[1] Satoshi Masubuchi,[1] and Shoji Awazu[1,2]

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INTRODUCTION

It has been reported that conjugation of glucose or galactose allow drugs a new route of intestinal absorption by way of Na^+/glucose transport system (1–4), and that phenyl β-o-glucose conjugates were transported fastest by the system among monosaccharide conjugates such as β-glucose, α-galactose, and β-galactose conjugates. In order to perform the strategy using this transport system for improvement of poorly absorbable drugs, synthesis of β-o-glucose conjugates of drugs is necessary. Chemical synthesis of glycoside by the reaction with activated glucose such as acetobromoglucose in a highly hydrophobic solvent such as chloroform is generally known (5). However, it is difficult to control α- or β-anomer specific formation. Furthermore, since the objective drug is poorly absorbable because of high hydrophilicity it requires several steps for derivatization to synthesize glycoside chemically in a hydrophobic solvent. Enzymatic synthesis of β-D-glucose conjugate (1-O-phenylcarboxyl-β-D-glucose esters) has been reported (6). However, the enzyme is not available in market, and must be purified from oak leaves with low yield. Furthermore, the glucose donor was uridine-5′-diphospho-glucose (UDP-glucose), which was available commercially but expensive for preparative scale. Therefore, in this report, we studied enzymatic synthesis of β-o-glucose conjugate by commercially available, cellulase (EC 3.2.1.4.) which is well known to be active on β-glucoside bond of polysaccharide. Furthermore, we optimized the condition of the reaction for preparative synthesis. Acetaminophen (APAP) was first selected as a model phenolic compound, since APAP β-glucoside, which was synthesized from commercially available glucoside derivative, was absorbed by Na^+/glucose cotransporter in intestine (4). Furthermore, this method was applied to the poorly absorbable antiviral drug, acyclovir (ACV), which has an alcoholic hydroxy group.

[1] Department of Biopharmaceutics and Drug Rational Research Center School of Pharmacy, Tokyo University of Pharmacy and Life Science (formerly Tokyo College of Pharmacy), 1432-1 Horinouchi, Hachioji 192-03, Tokyo.

[2] To whom correspondence should be addressed. (e-mail: awazu@ps.toyaku.ac.jp)

MATERIALS AND METHOD

ACV, p-nitrophenyl β-D-glucoside (p-NPβglc), gentiobiose, cellulase (*Aspergillus niger, Trichoderma reesei, Trichoderma viride*, and *Penicillium funiculosum*), α-glucosidase (from yeast), and β-glucosidase (from almonds) were purchased from Sigma Chemical Co. (St. Louis, USA). Cellobiose was from Nacalai tesque (Kyoto, Japan). Acetaminophen (APAP) and methanol (HPLC grade) were obtained from Wako Chemicals (Osaka, Japan).

Transfer of β-Glucose from p-nitrophenyl β-glucoside to APAP

APAP, p-NPβglc, and cellulase (*A. niger*) were dissolved in 0.05 M acetate buffer (pH 5.0). One hundred microliters of reaction mixture was periodically sampled and mixed with 0.1 ml of 10% perchloric acid for the following HPLC assay.

Transfer of β-Glucose from Cellobiose to APAP

APAP, cellobiose, and cellulase (*A. niger*) were dissolved in 0.05M acetate buffer (pH 5.0) or 1–10% dimethylsulfoxide (DMSO) in 0.05M acetate buffer (pH 5.0). One hundred microliters of reaction mixture was periodically sampled and mixed with 0.1 ml of 10% perchloric acid for the following HPLC assay.

Transfer of β-Glucose from Cellobiose or Gentiobiose to ACV

ACV, cellobiose (or gentiobiose), and cellulase (*A. niger, P. funiculosum, T. reesei, T. viride*) were dissolved in 0.05M acetate buffer (pH 5.0) or 1–70% DMSO or 20% N,N-dimethylformamide (DMF) in 0.05M acetate buffer (pH 5.0). One hundred microliters of reaction mixture was sampled and mixed with 0.1 ml of 10% perchloric acid for the following HPLC assay.

Preparative Transglucosylation of ACV

Cellobiose (15 g) was added to 35 ml acetate buffer (pH 5.5) and stirred. ACV (225.2 mg) was dissolved in DMSO (15 ml) to be mixed with the cellobiose solution in acetate buffer. Transglucosylation reaction was started by adding cellulase (5000 units/ml final concentration) to the mixture with stirring at 37°C. Periodically, a portion (0.1 ml) of the reaction mixture was sampled to check transglucosylation for the optimal reaction time (around 10% of peak area of initial ACV) by HPLC assay.

Purification of ACVβglc

Perchloric acid (70%, 3.85 ml) was added to the reaction mixture (50 ml) to stop the transglucosylation. One hundred and ten milliliters of purified water was added to the resultant mixture. The mixture was divided into 4 glass tubes, and centrifuged at 3000 rpm for 30 min. The supernatant was filtered through glass fiber membrane (0.4 μm pore size). Into each tube where precipitate remained, forty milliliters of purified water and one second milliliter of perchloric acid (70%) were added and mixed. After centrifuge of the mixture at 3000 rpm

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for 20 min, the same procedures were performed again. Supernatant in each tube was collected. Sodium bicarbonate was added to the collected supernatant to bring pH to around 5.5. The resultant solution was concentrated to around 50 ml by evaporation under reduced pressure at 40°C, and was applied to Sephadex G-10 column (9 cm I.D., 70 cm length). ACVβglc was eluted with water at a flow rate of 4.0 ml/min. Each fraction (15 ml/tube) was checked by HPLC assay. Fractions containing ACVβglc were collected and concentrated by the evaporation described above. Three milliliters of the concentrated ACVβglc fraction were applied to the preparative HPLC using an ODS column (21.5 mm I. D., 300 mm length). ACVβglc was eluted with mobile phase (2% methanol, 0.05% trifluoroacetic acid in water) at a flow rate of 4.0 ml/min. Each fraction (10 ml/tube) was checked by HPLC assay. Fractions containing ACVβglc were collected and concentrated by the evaporation described above. Finally, ACVβglc was obtained as yellow powder after freeze-drying. Recovery of ACVβglc by this procedure was about 75%.

β-Glucosidase Treatment

Glucoside was incubated in 0.05M sodium phosphate buffer (pH 6.8) in the presence of α-glucosidase (10 units/ml) or in 0.05M sodium acetate buffer (pH 5.0) in the presence of β-glucosidase (10 units/ml) for 2 hr at 37°C. One hundred microliters of reaction mixture was sampled and mixed with 0.1 ml of 10% perchloric acid for the following HPLC assay.

HPLC Assay

The perchloric acid-treated sample was bench-top centrifuged at 12000 rpm for 5 min. The resultant supernatant was applied to the HPLC. The HPLC system consisted of a pump (655A-11, Hitachi Ltd., Tokyo, Japan), a UV detector (655A, Hitachi Ltd., Tokyo, Japan), and an integrator (D-2500, Hitachi Ltd., Tokyo, Japan). The UV detector and a flow rate were set at 250 nm (for APAPβglc) or 254 nm (for ACVβglc) and 1.5 ml/min, respectively. An ODS column (80 TM, 6 mm i.d. × 15 cm length, Tosoh Corp., Japan) was used. The mobile phase consisted of 6% methanol and 0.05% phosphoric acid in water for the assay of APAPβglc. For the assay of ACVβglc, mobile phase consisted of 4% methanol and 0.05% phosphoric acid in water.

RESULTS AND DISCUSSION

Transfer of β-Glucose from p-nitrophenyl β-glucoside to APAP in the Presence of Cellulase

An HPLC chromatogram of the reaction mixture showed a new peak eluted earlier than APAP. The retention time of the new peak was identical to APAP β-glucoside, which was obtained by chemical synthesis (4). The compound of the new peak was hydrolyzed by β-glucosidase to form a compound having the retention time of HPLC identical to that of APAP, as opposed to that of α-glucosidase (data not shown). Although the concentration of APAP β-glucoside increased with incubation time for a while, it also decreased (Fig. 1a). At the time (Tmax), which was required to reach maximum concentration (Cmax) of APAPβglc, p-NP β-glucoside (glucose donor) was less than one third of the initial concentration (Fig. 1b). These results indicate that APAP glucoside was also substrate for cellulase, and that there was an optimal incubation time for glucoside synthesis. The higher concentration of glucose donor (p-nitrophenyl glucoside) was, the longer Tmax (time for maximum formation) was (Fig. 1a). Furthermore, the higher concentration of glucose donor was, the higher Cmax (concentration of maximum formation) was (Fig. 1a). This showed that glucose donor concentration affected Cmax as well as Tmax. The higher cellulase concentration was, the faster the degradation rate of glucose donor as well as the formation rate of APAPβglc was, and thereby Tmax was shortened by the increase of cellulase concentration (data not shown). But Cmax was not changed under this condition (data not shown).

Transfer of β-glucose from Cellobiose to APAP

Transfer of β-glucose from cellobiose to APAP by cellulase was also observed (data not shown). The higher APAP concentration as well as cellobiose concentration was, the more Cmax and Tmax of APAPβglc formation were. Initial rate of APAPβglc formation was not saturated under the APAP concentration (0.4–16 mM) (data not shown). However, the initial rate of APAPβglc formation in the presence of 80 mM cellobiose were lower than that of 16 mM cellobiose. This indicates the substrate inhibition of transglucosylation by cellobiose. The effect of concentrations of cellulase and cellobiose

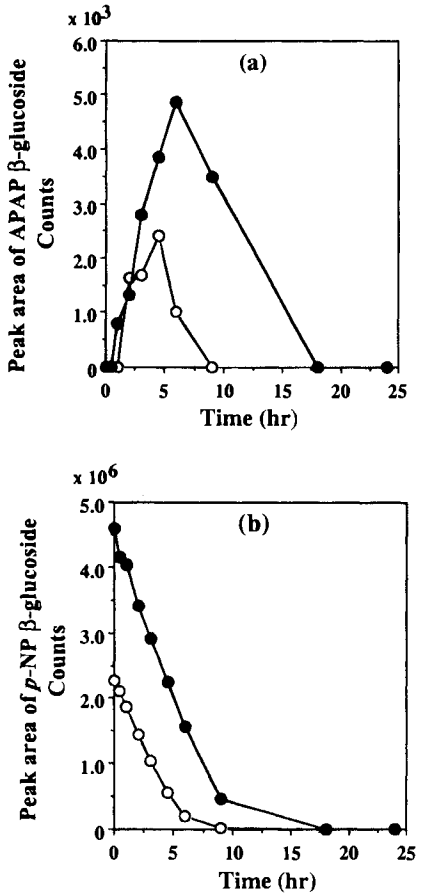

Fig. 1. Time course of APAPβglc formation (a) and hydrolysis of p-NPβglc (b). Reaction conditions. APAP, 4 mM; p-NPβglc, 8 mM (○) and 16 mM (●); cellulase (*A. niger*), 0.33 units/ml.

on the formation of APAPβglc were shown in Figs. 2a–c. The increase of cellulase concentration decreased Tmax but not Cmax in the presence of 32 and 48 mM cellobiose (Fig. 2a and b). In the presence of 80 mM cellobiose, on the other hand, Cmax (or Tmax) was not observed within 8 hr, indicating that glucose donor, cellobiose, was enough for the transglucosylation under this condition. Effect of DMSO as solvent on the formation of APAPβglc was shown in Fig. 2d. Even in 10% DMSO, the APAPβglc formation was around two fifths that in the absence of DMSO. This indicates that DMSO can be used as solvent to dissolve drug.

Transfer of β-glucose from Cellobiose or Gentiobiose to ACV

Transglucosylation of ACV (4 mM) in the presence of cellobiose (5 to 200 mM) and cellulase (2 units/ml) occurred to form ACV β-glucoside (data not shown). Mass spectra of ACVβglc by ESI spectrometry showed molecular ion peaks, $[M + H]^+$ at 388.0, $[M + Na]^+$ at 409.5 and $[M + K]^+$ at 426.4. 1H-NMR (500 MHz) spectra were obtained in DMSO-d_6 as follows. Acyclovir: 1H-NMR (Me_2SO-d_6): 3.45 (s, 4H, OCH_2CH_2O), 4.65 (s, 1H, OH), 5.33 (s, 2H, NCH_2O), 6.48 (br s, 2H, NH_2), 7.80 (s, 1H, CH), 10.61 (s, 1H, NH). Acyclovir β-glucoside: 1H-NMR (Me_2SO-d_6): 5.35 (s, 2H, NCH_2O), 6.54 (br s, 2H, NH_2), 7.65 (s, 1H, CH), 10.69 (br s, 1H, NH). ACVβglc was hydrolyzed by β-glucosidase to ACV, but not by α-glucosidase. These indicate the formation of ACVβglc. ACV and purified ACVβglc were determined by HPLC under the same condition as in transglucosylation reaction. The ratio of peak area of ACVβglc to that of ACV at equal concentration in HPLC assay indicated that the ratio of molecular extinction coefficient of ACVβglc to that of ACV at 254nm was 0.505.

The higher cellobiose concentration was, the more Cmax and Tmax of ACVβglc were, as observed in the formation of APAPβglc from p-NPβglc and cellobiose. Relationship between initial rate of ACVβglc formation and cellobiose concentration showed the substate inhibition of cellobiose at more than 20 mM as well (data not shown). Formation activities of ACVβglc by cellulase from *P. funiculosum*, *T. reesei*, and *T. viride* were compared with that from *A. niger* (Fig. 3a). Forma-

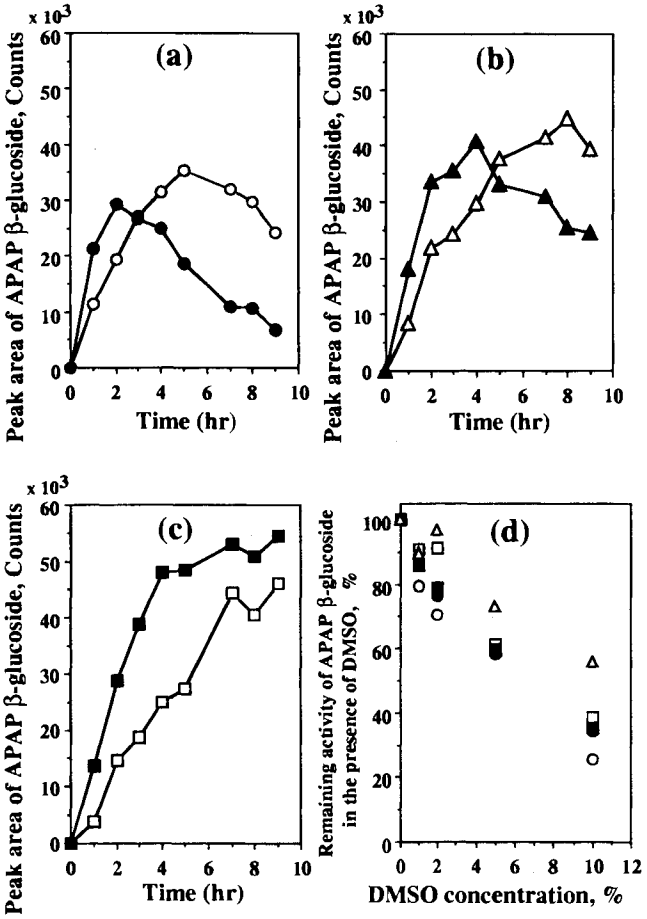

Fig. 2. Effect of cellobiose, cellulase and DMSO on formation of APAPβglc. Reaction conditions (a–c): cellobiose, 32 mM (a), 48 mM (b), 80 mM (c); cellulase (*A. niger*) open symbols 0.67 units/ml, closed symbols 1.67 units/ml; APAP, 4mM. Reaction condition (d): cellobiose, 80 mM; cellulase (*A. niger*), 0.66 units/ml, APAP, 16 mM; incubation time (○) 12 hr, (●) 15 hr, (□) 18 hr, (■) 21 hr, (△) 26 hr. DMSO, 0–10% in 0.05 M sodium acetate buffer (pH. 5.0).

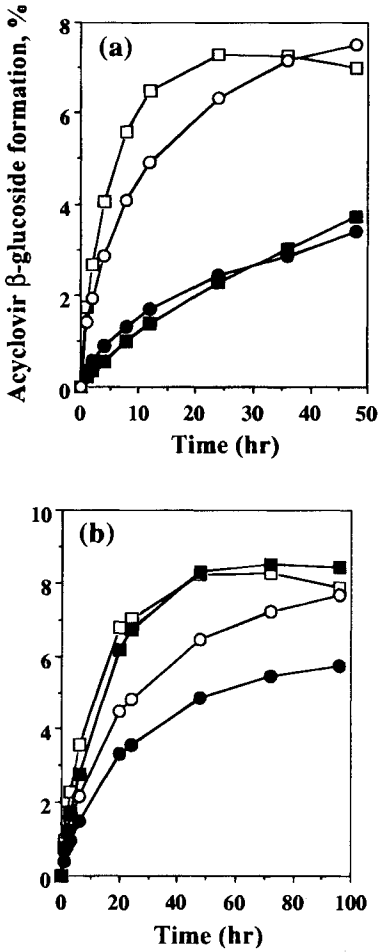

Fig. 3. Effect of cellulase origin (a) and gentiobiose (b) on the formation of ACVβglc. Reaction conditions: (a) (□) *Aspergillus niger*, (■) *Trichoderma reesei*, (●) *Trichoderma viride* and (○) *Penicillium funiculosum*. ACV 4 mM. Cellobiose 200 mM. Cellulase 20 units/ml. (b) Cellobiose (open symbols) or gentiobiose (closed symbols), 200 mM. Cellulase 10 units/m (circle) *Penicillium funiculosum*, (triangle) *Aspergillus niger*. ACV 4 mM.

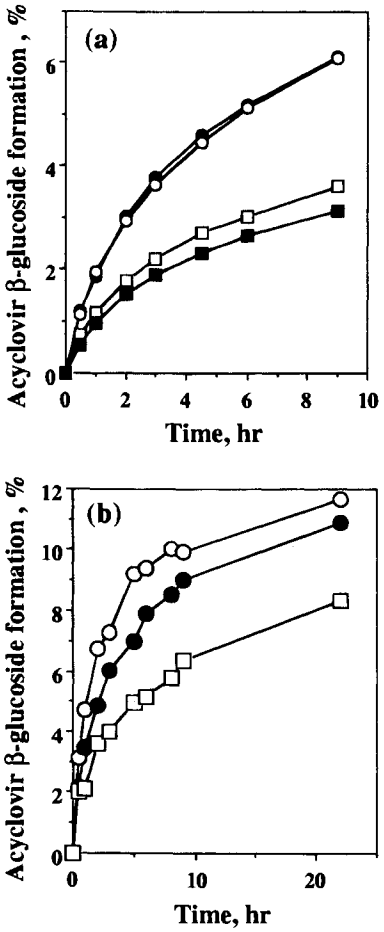

Fig. 4. Effect of DMSO and DMF on the formation of ACVβglc. Reaction conditions: (a) (●) control, (○) 1% DMSO, (□) 20% DMSO, (■) 20% DMF. ACV 12 mM, cellobiose 500 mM, cellulase (*P. funiculosum*) 50 units/ml. (b) (○) 16 mM ACV in 20% DMSO, (●) 20 mM ACV in 30% DMSO, (□) 24 mM ACV in 40% DMSO Cellobiose 1000 mM, cellulase (*P. funiculosum*) 1000 units/ml.

tion activity of ACVβglc by cellulase from *P. funiculosum* was as much as from *A. niger*, whereas the activity of *T. reesei* or *T. viride* was less than that of *A. niger*. Effect of structure of glucose donor on the transglucosylation was studied by comparing cellobiose with gentiobiose (Fig. 3b). Any significant preference of gentiobiose (β1-6 bond) to cellobiose (β1-4 bond) as β-glucose donor was not observed when cellulase from *A. niger* and *P. funiculosum* were used.

Effect of DMSO on the ACVβglc formation by cellulase from *A. niger* and *P. funiculosum* were studied. Transglucosylation activity of cellulase from *P. funiculosum* were observed even in DMSO mixed with acetate buffer (Fig. 4a), and these activities were almost the same as in that from *A. niger* (data not shown). ACVβglc formation was examined at saturable concentration of ACV in various concentration of DMSO in acetate buffer (Fig. 4b). Although the percentage of formed ACVβglc was decreased by the increase of DMSO concentration, the order of the amount of formed ACVβglc was 30% > 40% > 20% DMSO in acetate buffer. The increase of cellulase concentration to 5000 units/ml in the preparative scale resulted in the shorter Tmax (5–6 hr) but equivalent yield (around 10% by HPLC peak area) of ACVβglc (data not shown). The kinetic pattern of the ACVβglc formation in the preparative scale was compatible with those of formation of APAPβglc and ACVβglc as described above. The yield of ACVβglc was estimated to be around 20% from the ratio of molecular extinction coefficient of ACVβglc to ACV (0.505), which was described above.

These results indicated that cellulase could catalyze the transfer of β-glucose from cellobiose or phenyl β-glucoside to phenolic or alcoholic compounds, forming phenolic O-glucoside (APAPβglc) and alcoholic O-glucoside (ACVβglc) even in reaction medium containing DMSO.

In summary, this study indicated that cellulase, which is available on the market, could catalyze the transfer of β-glucose from cellobiose to acyclovir possessing an alcoholic OH group as well as acetaminophen possessing a phenolic OH group. This β-anomer specific transglucosylation also occurred in p-NPβglc or gentiobiose in addition to cellobiose. The transglucosylation activity of cellulase remained even in DMSO/buffer solution, which could make higher a concentration of drug than buffer solution, resulting in higher yield of β-glucose conjugate.

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