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Note

Enzymatic transformation of de-*O*-acetyl-lanatoside C into digoxin ☆

Žika Lepojević *, Branislav Pekić

Department of Pharmaceutical Engineering, Faculty of Technology, University of Novi Sad, Boulevard Cara Lazara 1, 21000 Novi Sad, Serbia Montenegro

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Partial enzymatic hydrolysis of primary cardioglycosides represents a method of choice for preparation of the corresponding secondary glycosides. However, the reported procedures [1] have a serious disadvantage due to the low solubility of the starting primary glycosides in the aqueous medium. This problem was recently overcome in our laboratory in the case of the enzymatic transformation of de-*O*-acetyl-lanatoside A (DLA) into digitoxin (D_x) in cyclodextrin solutions [2].

We now report a novel enzymatic conversion of de-*O*-acetyl-lanatoside C (DLC) into the clinically useful [3,4] secondary form of digoxin (D_{gx}), by using barley seed β-D-glucosidase (EC 3.2.1.21) in solutions of cyclodextrins (CDs).

1. Experimental

Enzyme preparation. — Partly purified β-D-glucosidase isolated from barley seed [5]; specific activity, 1.0×10^{-2} μkat mg^{-1} (substrate: de-*O*-acetyl-lanatoside A in γ-CD solution; 0.1 M citrate buffer, pH 5.0; temperature, 55°C).

Chemicals. — De-*O*-acetyl-lanatoside C was obtained by methanolysis [6] of lanatoside C, isolated from the lanatoside complex ABC by liquid–liquid extraction [7]. Digoxin used as reference was purchased from Aldrich (Milwaukee, Wisconsin), cyclomaltooctaose (γ-cyclodextrin, γ-CD) from Fluka (Buchs, Switzerland), and cyclo-

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* Corresponding author.

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maltoheptaose (β-cyclodextrin, β-CD) from Chinoin (Budapest, Hungary). All other chemicals were of analytical reagent grade.

Semipreparative purification of de-O-acetyl-lanatoside C. — The product of methanolysis of lanatoside C was subjected to chromatographic purification (silica gel for TLC; column: 4 × 30 cm; solvent system: 4 : 1 CH_2Cl_2–EtOH). The purity of DLC thus obtained was not satisfactory, so that further purification was carried out by HPLC (column: Micro Pak MCH-10, 8 mm i.d. × 30 cm; mobile phase, 1 : 3 MeCN–water).

Determination of solubility of DLC in aqueous solutions of β- and γ-cyclodextrins. — The weighed CD sample (20–200 mg) was transferred to a 5-mL serum bottle, and bidistilled water (1.0 mL) was added to dissolve the CD. Then, solid DLC was added in excess, the bottle closed with a rubber stopper and with a crimped aluminium seal, and the system cyclodextrin–de-*O*-acetyl-lanatoside C was equilibrated by shaking in a thermostat at 25 ± 0.5°C during 48 h. Determination of solubility of de-*O*-acetyl-lanatoside C in CD solutions was carried out for each particular concentration of CD in separate bottles. After 48 h the bottle content was centrifuged (3000 rpm) for 5 min. The clear supernatant solution was filtered through a 0.5 μm Millipore membrane filter, and the content of DLC in the solution was determined by HPLC using the Micro Pak column MCH-5 (Varian). As the mobile phase, 1 : 3 MeCN–water was used and Dgx as the external standard.

Activity of the enzyme preparation. — The incubation mixture (1.0 mL) contained the following components: DLC solution (0.36 mL, 3 mg mL^{-1}) prepared in an aqueous solution of CD (5 mg mL^{-1}), 50 mM citrate buffer (pH 5.0, 0.6 mL), and the enzyme preparation in the buffer (40 μL) with protein concentration of 0.33 mg mL^{-1}, and was kept at 45°C. The reaction was stopped after 30 min by heating the incubation mixture on a boiling water bath for 2 min. After cooling, 0.30 mL of 95% EtOH was added and the mixture filtered using a 0.5-μm membrane filter. The HPLC determination of the D_{gx} obtained was carried out using the mobile phase MeCN–citrate (45 : 55, v/v), buffer pH 5.0, and D_{gx} solution (0.02%) in the mobile phase as external standard.

Preparation of digoxin from de-O-acetyl-lanatoside C [8]. — A solution of γ-CD (6.5 g; 5.01 mmol) and de-*O*-acetyl-lanatoside C (3.5 g; 3.71 mmol) in distilled water (50 mL) was stirred at 25°C for 48 h, then diluted with citrate buffer (pH 5.0; 30 mL; 50 mM) and warmed up to 45°C. A solution of the enzyme (20 mL; 50 mg mL^{-1} in citrate buffer) was added and the resulting mixture was incubated at 45°C for 45 min. The enzyme was inactivated by heating the vessel on a steam bath for 15 min. After cooling to room temperature, the mixture was filtered and stirred with toluene (0.5 mL) for 20 min. The resulting suspension was extracted with a mixture of $CHCl_3$ and 2-propanol (4 : 1, 3 × 40 mL). The combined extracts were dried (Na_2SO_4) and evaporated to yield chromatographically pure digoxin (2.6 g; 89.7%).

2. Results and discussion

The first task of the present study was to determine the dependence of DLC solubility on β- and γ-CD concentration. The results presented in Fig. 1 show that the increase in CD concentration is accompanied by an increase in the solubility of DLC. With γ-CD,

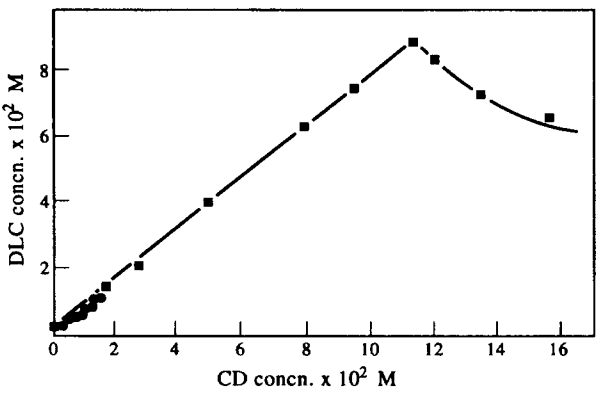


Fig. 1. Phase solubility diagrams of de-*O*-acetyl-lanatoside C–CD systems in water at 25°C: ●, β-CD system; ■, γ-CD system.

this increase in DLC solubility is observed at a concentration of γ-CD of 0.115 M, followed by a decrease in DLC concentration in the solution (precipitation of the solid complex). The relationship between the solubility of DLC and the concentration of γ-CD has a course similar to that of DLA [2], except for a much higher solubility observed in the present case (8.9×10^{-2} M). The solubility isotherm for DLC in the β-CD solution differs from the analogous curve for DLC solubility in γ-CD, and also differs from the isotherm describing the solubility of DLA in β-CD solution [2], and its course is completely linear — the solubility of A_L-type [9].

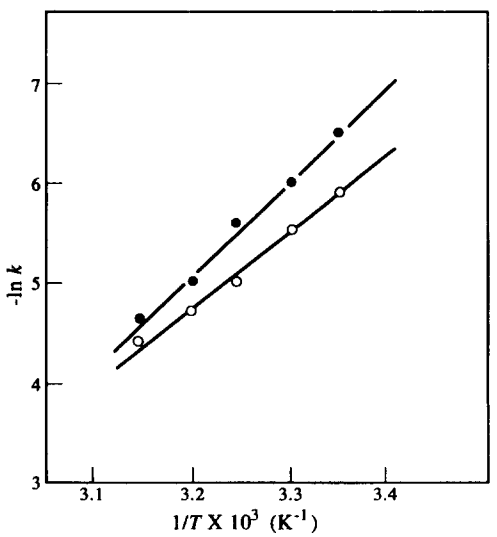


Fig. 2. Temperature dependency of enzyme reaction: ●, DLC–β-CD system; ○, DLC–γ-CD system.

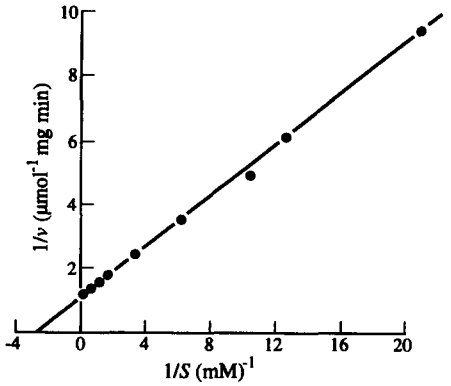
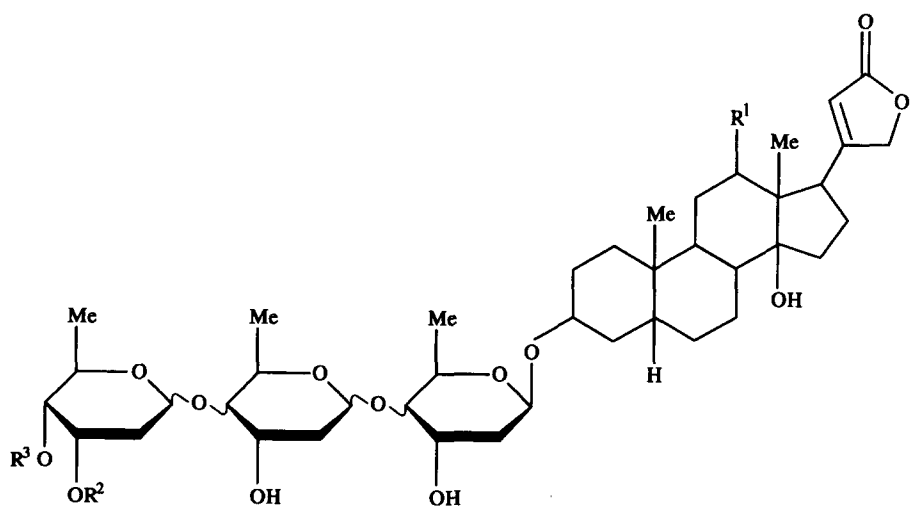


Fig. 3. Lineweaver–Burk plot for K_m determination. Substrate: DLC in γ-CD solution. Conditions: pH 5.0; 45°C; 30 min.

These data suggest that the DLC solubility in γ-CD increases 417 times compared to that in water, whereas the increase in solubility of DLC in β-CD was only 60 times.

The investigations of temperature and pH on activity of β-D-glucosidase in transforming DLC into D_{gx} in solutions of β- and γ-CD showed that maximum activity is obtained at 45°C and pH 5.0.



Lanatoside A: R^1 = H; R^2 = Ac; R^3 = β-D-glucopyranosyl
Lanatoside C: R^1 = OH; R^2 = Ac; R^3 = β-D-glucopyranosyl
De-*O*-acetyl-lanatoside A: $R^1 = R^2$ = H; R^3 = β-D-glucopyranosyl
De-*O*-acetyl-lanatoside C; R^1 = OH; R^2 = H; R^3 = β-D-glucopyranosyl
Digitoxin: $R^1 = R^2 = R^3$ = H
Digoxin: R^1 = OH; $R^2 = R^3$ = H

The activation energy for the transformation of DLC in the solutions of β- and γ-CD was determined in the range 25–45°C with a step of 5°C. On the basis of the Arrhenius plot (Fig. 2) we calculated the energies of activation and parameters of linear regression for (a) DLC in β-CD solution: $\ln k = 23.7 - 9011\ 1/T$; $E_a = 74.92$ kJ mol^{-1}, and (b) DLC in γ-CD solution: $\ln k = 19.1 - 7462\ 1/T$; $E_a = 62.04$ kJ mol^{-1}. The calculated activation energy for the transformation of DLC by the action of β-D-glucosidase in a γ-CD solution is lower than that for the reaction in β-CD solution. These values are lower than the activation energy obtained [2] for transformation of DLA to D_x. The determination of kinetic parameters for the enzymatic transformation of DLC into D_{gx} gave for β-D-glucosidase: $K_m = 2.35 \times 10^{-4}$ M and $V_{max} = 0.76$ μmol mg^{-1} min^{-1} for DLC in β-CD, and $K_m = 4.09 \times 10^{-4}$ M and $V_{max} = 1.01$ μmol mg^{-1} min^{-1} in γ-CD solution (Fig. 3).

On the basis of the above investigations it can be concluded that the substantial increase in the substrate solubility in CD allows the enzymatic transformation of DLC by β-D-glucosidase to be used for obtaining D_{gx} on a relatively large scale.

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