Preparation, Characterization, and Biotransformation of the Inclusion Complex of Phytosterols and Hydroxypropyl-β-cyclodextrin by *Mycobacterium neoaurum*

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The inclusion complex of hydroxypropyl-β-cyclodextrin (HBβCD) and phytosterols (PSs) was prepared and characterized by thermogravimetric analysis (TGA) and infrared (IR) spectroscopy. Biotransformation of the inclusion complex of phytosterols and hydroxypropyl-β-cyclodextrin (PSs-HBβCD) by *Mycobacterium neoaurum* to 1,4-androstadiene-3,17-dione and 4-androstene-3,17-dione [AD(D)] was studied. The TGA and IR results indicated that the thermal stability of PSs was improved in the complex with HBβCD. Biotransformation improved the solubility of PSs in the aqueous medium a lot because the AD(D) production was increased remarkably compared with the control, but growth of the bacteria was inhibited in the presence of HBβCD. The optimal inclusion ratio, ultrasonic treating time, dosage, and time of addition of PSs-HBβCD complexe were found to be 2:1, 10 min, 1.5 g/30 ml medium, and 48 h after incubation, respectively. This inclusion technique not only increased the availability of the substrates for the microorganisms, but also the capability of these microorganisms to produce AD(D) from PSs.

Key words: Phytosterol, Hydroxypropyl-β-cyclodextrin, *Mycobacterium neoaurum*

Introduction

1,4-Androstadiene-3,17-dione (androstadienedione, ADD) and 4-androstene-3,17-dione (androstenedione, AD) are major compounds in the synthesis of certain steroid drugs. Many of these steroids are essential substances (Fernandes *et al.*, 2003). ADD and AD [AD(D)] are intermediates in the synthesis of several corticoids (cortisol, prednisolone, *etc.*), androgens (testosterone and its derivatives), gestagens (acetomepregenol, 17α-hydroxyprogesterone esters, *etc.*), diuretics (canrenone and spironolactone), and anabolic compounds (boldenone and methandrostenolone) (Rodina *et al.*, 2008). Currently, AD(D) are mainly obtained from plant sterols, *i.e.* phytosterols (PSs), such as cholesterol, sitosterol, and stigmasterol, by biotransformation with microorganisms such as *Mycobacterium neoaurum*, which is regarded the most promising strain to biotransform PSs into AD(D) by side-chain cleavage of the substrate molecules (Rodina *et al.*, 2008).

The reaction, however, is not homogeneous as both the substrate (*e.g.* cholesterol, sitosterol, and stigmasterol) and products [AD(D)] have low solubilities in the aqueous medium in which biotransformation occurs. The use of an organic-aqueous two-liquids phase system provides an effective and well-established approach to overcome such drawback, since the organic pool allows the solubilization of high amounts of lipophilic substrates and products (Léon *et al.*, 1998; Schmid *et al.*, 2001; Wendhausen *et al.*, 2005). Cyclodextrins have been widely known to increase the solubility in the aqueous phase of various lipophilic substrates. One of the pharmaceutically important cyclodextrin derivatives is 2-hydroxypropyl-β-cyclodextrin (HBβCD) which is a powerful solubilizer of several drugs (Manosroi *et al.*, 2005). The cyclodextrin molecule has a torus shape, with the hydrophilic region outside and the hydrophobic region inside the cavity. By complexation with HBβCD, the guest molecule which is included within the HBβCD cavity is entirely or partially surrounded by the HBβCD molecule. The outer surface of HBβCD contributes to the hydrophilic property of a guest mole-

cule. Thus, the solubility of the hydrophobic guest compound is subsequently increased. Complexation with HBβCD and permethyl-β-cyclodextrin (PMβCD) has been shown to provide the most efficient solubilization of P with the highest apparent stability constants (Malika *et al.*, 2006). It is very important that the complex has low influence on cell growth and does not denature proteins or interfere with any enzymatic reactions (Hesselink *et al.*, 1989; Manosroi *et al.*, 2008).

Some studies have reported that complexation of steroids with certain types of β-cylodextrins (β-CD), using the inclusion technique, could result in increased solubility of steroids and a positive effect on the production rate (Calderini and Pessine, 2008; Manosroi *et al.*, 2005, 2008; Wang *et al.*, 2009), but only few focused on the thermal stability of the complexes. The aims of the present investigation were to study the effects of complexation on thermal stability, solubility, and biotransformation capability of PSs and HBβCD complexes. These results can be applied to other precursors with poor aqueous solubility in the biotransformation processes.

Material and Methods

Maintenance and preculture of bacteria

Mycobacterium neoaurum, described by Rodina *et al*. (2008), was maintained on potato dextrose agar slants at room temperature. A preculture of *M. neoaurum* was grown in medium composed as follows (g/l): glucose (20), yeast extract (5), anhydrous magnesium sulfate (2), potassium dihydrogen phosphate (3), and disodium hydrogen phosphate (1) in 1 l tap water (pH 7.0).

Chemicals

A mixture of phytosterols (PSs), containing 46.52% β-sitosterol, 25.76% campesterol, 14.17% brassicasterol, and 5.26% stigmasterol, served as a transformation substrate (purchased from Hubei Hengshuo Chemical Co., Ltd, Wuhan, Hubei Province, P. R. China). 4-Androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) standards were obtained form Sigma (St. Louis, USA).

Preparation of PSs-HBβCD complexes

The PSs-2-hydroxypropyl-β-cyclodextrin (PSs-HBβCD) complex was prepared by co-precipitation. HBβCD (3.5 g) was dissolved in distilled water (50 ml) at 70 °C for 1 h. PSs dissolved in acetic anhydride were slowly added to the saturated HBβCD solution with continuous agitation. The final molar ratios of PSs to HBβCD were adjusted to 1:2 and 1:3.5. The vessel containing 40 ml PSs and HBβCD solution was sealed and stirred continuously for 6 h, 4 ml ethanol was added dropwise to regulate the solubility of the hydrophobic solute in HBβCD solution. The final solution was then treated ultrasonically for 5, 10 and 15 min and stored overnight at 4 °C. The precipitated PSs-HBβCD complex was recovered by filtration and washed with absolute ethanol to remove unencapsulated PSs. This residue was dried in a vacuum oven at 70 °C for 48 h. The final powder was stored at 4 °C in an airtight bottle.

Biotransformation trials

After 2 d of cultivation, 3 ml of preculture were added to the biotransformation medium (30 ml in a 250-ml flask) which contained the following (g/l): glucose (20), yeast extract (2), ammonium nitrate (2), anhydrous magnesium sulfate (2), potassium dihydrogen phosphate (3), and disodium hydrogen phosphate (1) in 1 l tap water (pH 7.0). To investigate the effects of the time of addition of the PSs-HBβCD complex on growth and AD(D) production, 24, 48, 72, and 96 h after inoculation with the seed medium, 1.5 g PSs-HBβCD complex were added to the broth for biotransformation. To study the effects of dosage of the PSs-HBβCD complex on growth and AD(D) production, 0.9, 1.5, and 2.1 g/30 ml of PSs-HBβCD complex were added to the biotransformation media (30 ml in a 250-ml flask) after 2 d of cultivation, respectively. The biotransformation was carried out at 28 °C and 220 rpm. The biotransformation media collected at day 5 were analysed by HPLC. Each experiment was performed twice at the same time to check the reproducibility.

Analytical methods

The infrared (IR) spectra of different samples, *i.e.* PSs, PSs-HBβCD complex, and the physical mixture of PSs and HBβCD (PSs and HBβCD simply mixed together), were obtained using a 470 Fourier transform infrared spectrometer (Nicolet Co., Madison, USA). Samples were prepared by mixing 5 mg of material with 150 mg of KBr (Sinopharm Chemical Reagent Co., Ltd, Shang-

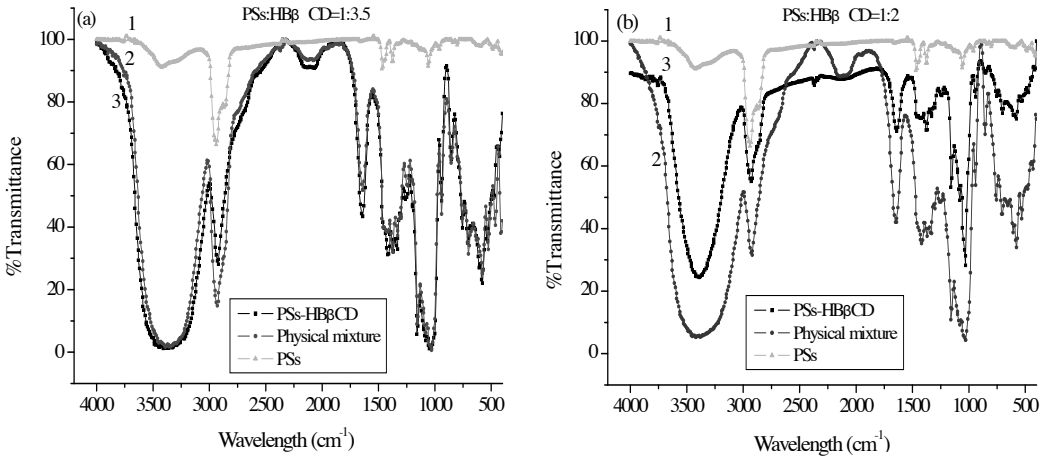

Fig. 1. FT-IR spectra of PSs (1) and PSs-HBβCD complex (3), and the physical mixture of PSs and HBβCD (2). (a) PSs:HBβCD=1:3.5. (b) PSs:HBβCD=1:2.

hai, China) and pressing them into tablet form. All IR spectra were recorded over 4000–400 cm^{-1} with a resolution of 0.2/cm. Thermogravimetric analysis (TGA) measurements of different samples were carried out in a Netzsch STA 4493F3 thermal analyzer (Netzsch Co., Selb, Germany) with 20–25 mg of sample under a nitrogen flow of 40 ml/min at a heating rate of 20 °C/min from 20 to 600 °C.

At the 5th day of the incubation period, 5 ml culture broth were taken from the flask and centrifuged; 3 ml supernatant were extracted with 6 ml ethyl acetate by vigorous mixing for 10 min in a separatory funnel. The organic layer was evaporated and dried. The residue was then dissolved in 1.0 ml mobile phase. A 100 μl sample diluted with mobile phase were filtered through a 0.45-μm nylon syringe filter and transferred to a sampling vial. Samples (20 μl) were analysed by HPLC with equipment consisting of a solvent delivery system (L-7100; Hitachi, Tokyo, Japan) and a variable wavelength UV-Vis detector (L 7420; Hitachi) set to 244 nm, equipped with a sample injector (7725i; Rheodyne, Cotati, USA) fitted with a 20-μl sample loop. An Eclipse XDB-C18 column (250 mm × 4.6 mm i.d., 5 μm particle diameter, 250 Å average pore size; Agilent, Technologies, Santa Clara, USA) with a guard column holder and distilled water/methanol (35:65, v/v) as the mobile phase at a flow rate of 1.0 ml min^{-1} were used. The retention times of the biotransformation products were compared to those of AD and ADD, at 11.465 and 7.811 min, respectively. Calculations of the obtained weight of AD(D) were done by means of a calibration curve of authentic reference samples using the area under the curve of biotransformation products in each chromatogram.

Results and Discussion

IR spectroscopy of PSs and PSs-HBβCD complex and the physical mixture of PSs and HBβCD

Fig. 1 shows the IR spectra of PSs, PSs-HBβCD complex, and the physical mixture of PSs and HBβCD in the region 4000–400 cm^{-1}. The absorption band at about 3420 cm^{-1} could be assigned to the -OH stretching vibration. The absorption bands at about 2940 and 1380 cm^{-1} could be attributed to -CH stretching. In addition, the absorption band at 1470 cm^{-1} for the -CH vibration of PSs in HBβCD shifted to lower wave numbers in the PSs-HBβCD inclusion complex, which might be caused by the formation of hydrogen bonds between PSs and the molecule of HBβCD.

From the IR spectra of the three samples, it could be found that the molar ratios of PSs:HBβCD (1:2 and 1:3.5) differentially affect the transmittance. Fig. 1a reveals that the IR spec-

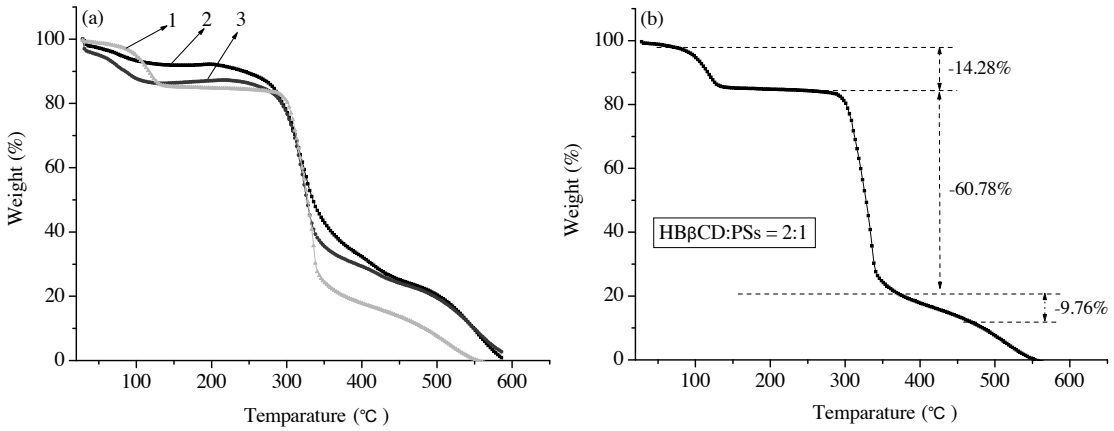

Fig. 2. (a) TGA profiles of PSs-HB*β*CD complex (1), HB*β*CD (2), and the physical mixture of PSs and HB*β*CD (3). (b) Weight loss ratio of PSs-HB*β*CD complex upon heating.

tra of the PSs-HB*β*CD complex and the physical mixture of PSs and HB*β*CD (PSs:HB*β*CD = 1:3.5) nearly overlap, but when PSs:HB*β*CD is 1:2, the transmittances of the PSs-HB*β*CD complex and of the physical mixture of PSs and HB*β*CD are clearly separated (Fig. 1b).

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed at a heating rate of 20 °C/min in a nitrogen atmosphere. Fig. 2a presents the TGA profiles of HB*β*CD, the PSs-HB*β*CD complex, and the physical mixture of PSs and HB*β*CD. It can be seen that all three samples demonstrate two regions of major weight loss. Moreover, the respective first regions are almost at the same temperature range, *i.e.* 50–100 °C. This first region can be attributed to the loss of absorbed water from the samples. The respective second regions are related to the decomposition of the samples.

Based on the initial decomposing temperature, it can be seen clearly that the PSs-HB*β*CD complex is the most stable of the three samples. That is to say, the initial decomposing temperature for the PSs-HB*β*CD complex is 275 °C, and those of HB*β*CD and the physical mixture are almost identical with 250 °C. This suggests that some bonds are formed between the PSs and HB*β*CD which lead to the formation of the PSs-HB*β*CD complex and thus increase the initial decomposing temperature by about 25 °C. These findings are consistent with the literature, according to which the thermal stability of guest molecules was improved when they were enclosed in HB*β*CD (Calderini and Pessine, 2008; Tsai *et al.*, 2010; Wu *et al.*, 2010). Additionally, the weight loss ratio of HB*β*CD to PSs was 60.78 to 14.28, as shown in Fig. 2b, which was close to the molar ratio (PSs-HB*β*CD = 2:1) during preparation of the PSs-HB*β*CD complex.

Effects of ratio and ultrasonication time of the PSs-HBβCD complexe on growth and AD(D) production

Low solubility of the substrates in the media often leads to poor mass transfer of substrates and products to and from cells, which then becomes the rate-limiting step in the biotransformation process. PSs have a low solubility in water, but after complexation the solubility of PSs increases significantly (Hesselink *et al.*, 1989; Manosroi *et al.*, 2008; Wang *et al.*, 2009). The effects of ratio and ultrasonication time of the PSs-HB*β*CD complexe on cell growth and AD(D) production are shown in Fig. 3. Compared with the control, cell growth was inhibited in the presence of HB*β*CD in all the cases (Fig. 3a). Fig. 3b shows that the time of ultrasonic treatment of the PSs-HB*β*CD complexe significantly affects the AD(D) production, a 10-min treatment being optimal.

When the ratios of PSs:HB*β*CD were 1:2 or 1:3.5, the yields of AD(D) production by *M. neoaurum* were 225.6 and 199.3 mg/l, respectively, which was 73.1% and 53.1% higher in comparison to the yields obtained with the free PSs, respectively. This shows that complexation with HB*β*CD substantially enhanced the conversion of PSs by

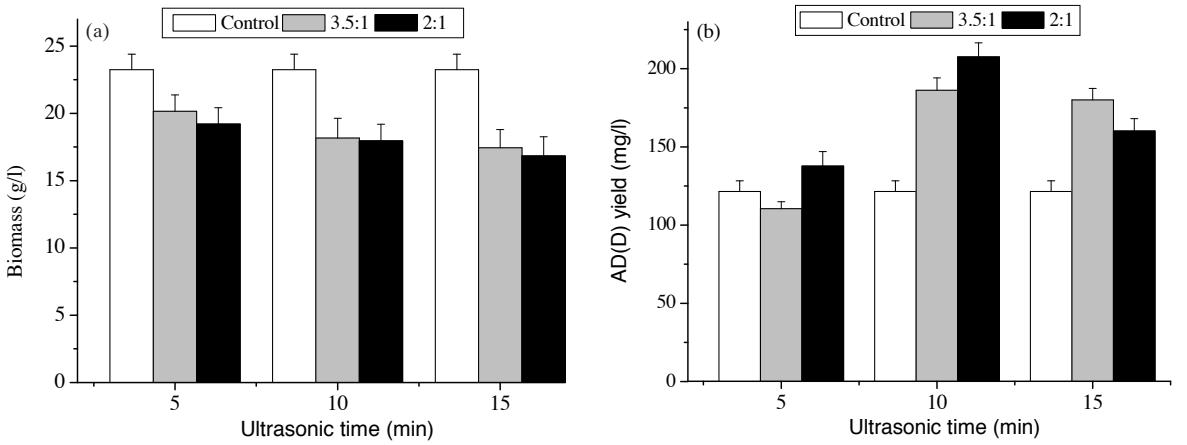

Fig. 3. Effects of ratio and ultrasonic treatment time of the PSs-HBβCD complexe on (a) growth and (b) AD(D) production by *M. neoaurum.*

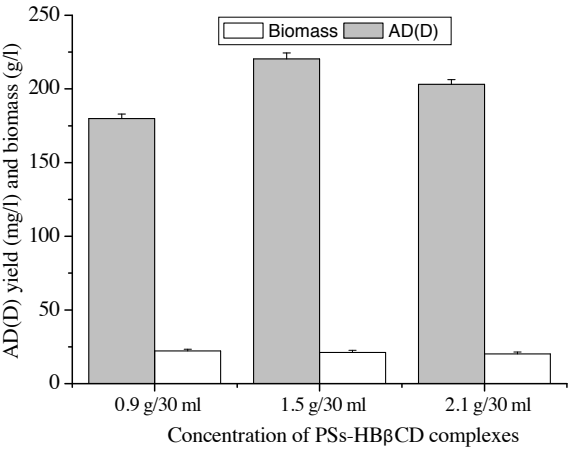

Fig. 4. Effects of the dosage of the PSs-HBβCD complexe (ratio = 1:2) on growth and AD(D) production by *M. neoaurum.*

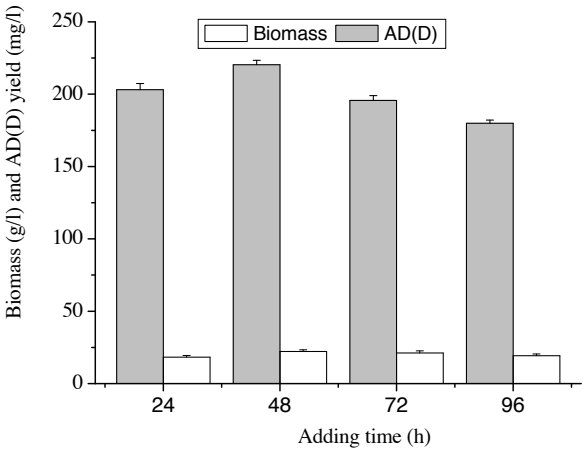

Fig. 5. Effects of adding time of the PSs-HBβCD complexe (ratio = 1:2) on growth and AD(D) production by *M. neoaurum.*

M. neoaurum in an aqueous fermentation system to a mixture of AD and ADD. The optimal ratio of HBβCD and the PSs was 2:1, which increased the specific side-chain cleavage activity with some negative influence on cell growth (Wang *et al.*, 2009). When the ratio of HBβCD and PSs was 2:1, the AD(D) yield was 72.2% compared to the control after 10 min of ultrasonic treatment of the PSs-HBβCD complexe. So the optimal ratio of PSs:HBβCD was 1:2.

Effects of dosages of the PSs-HBβCD complexe on growth and AD(D) production

Effects of the dosages of PSs-HBβCD complexe on growth and AD(D) production were studied. According to Figs. 3 and 4, the maximum yield of AD(D) production by *M. neoaurum* was 132.2 mg/l at 15 g/l of free PSs, whereas the PSs-HBβCD complex gave 179.9, 220.2, and 203.1 mg/l at dosages of 0.9, 1.5, and 2.1 g/30 ml PSs-HBβCD (ratio of PSs:HBβCD was 1:2) in a 250-ml flask, respectively. The PSs-HBβCD complex gave higher yields than the free PSs of about 36.1%, 66.6%, and 53.9%, respectively, and the optimal dosage of the PSs-HBβCD complex was 1.5 g/30 ml.

Effects of the time of addition of the PSs-HBβCD complexe on growth and AD(D) production

As depicted in Fig. 5, the optimal time of addition of the PSs-HBβCD complexe was at 48 h after incubation, but at 96 h it was disadvantageous both to growth and biotransformation. The highest AD(D) production was 220.3 mg/l, *i.e.* an enhancement of 66.6% over the control. The results indicate that the biotransformation of PSs into AD(D) was highest in the logarithmic growth phase at 48 h after incubation.

Conclusion

The inclusion complex of HPβCD and PSs was synthesized and characterized by TGA and IR spectroscopy, which indicated that the thermal stability of PSs was improved when it was included in HPβCD. The biotransformation showed that the solubility of PSs in the aqueous medium was improved greatly because the AD(D) production was increased remarkably compared with the control. The optimal inclusion ratio, ultrasonic treating time, dosage, and time of addition of PSs-HBβCD complexe were found to be 2:1, 10 min, 1.5 g/30 ml medium, and 48 h after incubation, which caused that the AD(D) products were 73.1%, 72.2%, 66.6%, and 66.6% higher than in the controls, respectively.

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