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ORIGINAL PAPER

A very efficient bioconversion of soybean phytosterols mixtures to androstanes by mycobacteria

C. Pérez · A. Falero · H. Luu Duc · Y. Balcinde · B. R. Hung

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Abstract The production of several high value steroid drugs, used as progestational, adrenocortical, estrogenic and contraceptive agents, is mostly derived from 4-androstene-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD). Three Vietnamese phytosterols mixtures named VN-1, VN-2 and VN-3, isolated from soybean oil may be efficiently converted into these key compounds by mycobacterial cells. Their general phytosterol composition was 55.39, 70.55, 70.19% for VN-1, VN-2 and VN-3, respectively. Moreover, values of campesterol, β-sitosterol and stigmasterol were determined. After 120 h of shaking in suitable culture media and temperature, maximal yield conversion to ADD was higher than 70% and up to 64% to AD, for the various phytosterols mixtures assays. These results may be better when scaling-up such a procedure of phytosterols conversion.

Keywords Phytosterols · Conversion · Mycobacteria · AD · ADD

C. Pérez (✉) · A. Falero · Y. Balcinde · B. R. Hung
Department of Biotechnological Processes,
National Center for Science Research,
PO Box 6990, Havana, Cuba
e-mail: celso.perez@cnic.edu.cu

H. L. Duc
Laboratory of Steroid, Institute of Chemistry,
Vietnamese Academy of Science and Technology,
18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
e-mail: Lduchuy@netnam.vn

Introduction

The semi-synthesis of steroidal drugs has mostly taken advantage of the ability of microbial cells of making bioconversions to 17-ketosteroids in sterol metabolism. This strategy avoids the use of diosgenin and solasodin as traditional raw materials for manufacturing steroidal drugs [11]. *Dioscorea* and *Solanum* can only be cultivated every 4–5 years on agricultural land. Therefore, the price of diosgenin and solasodin in the world market becomes very high. For instance, phytosterols may be properly bioconverted in 4-androstene-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD) by means of mycobacterial mutants [25]. The production of several high value steroid drugs, used as progestational, adrenocortical, estrogenic, diuretic, anabolic and contraceptive agents, is mostly derived from these key compounds by chemical synthesis.

Phytosterols mixtures may be isolated from different inexpensive sources, mostly tall oil in paper industry or filter cake in sugarcane industry. In fact, various patents concerning isolation and purification of phytosterols mixtures have been reported [6, 12, 16, 23].

Another very important source of phytosterols is soybean, which constitutes a highly remarkable food additive in Asia and all over the World. Soybean oil has lately gained good acceptance in cuisine everywhere [19].

Paper and sugarcane as well as soybean industries are currently developing in Vietnam [10, 18, 22]. In fact, soybean has the third highest priority for crop research in Vietnam after rice and maize and is seen as a major feed source for an expanding livestock industry as well as a boost for human food supplies [1].

Increasing Vietnam's national production of soybeans from around 200,000 to a million tons a year by 2010 is a government goal, driven by projections in demand and being able to meet this domestically [2].

On the other hand, this country must import all steroidal drugs, so that it would be highly profitable to use by-products from these industries as raw materials and developing the technology necessary for synthesizing steroidal drugs.

Soybean phytosterols in Vietnam can be extracted and purified by an easy procedure, which renders a very good yield (H. Luu Duc, personal communication). The following results are concerned with the characterization of various samples of phytosterols mixtures and their bioconversion to the androstanes AD and ADD.

Materials and methods

Microorganisms

The strains of *Mycobacterium* sp., NRRL–B3683 and MB3683 were used throughout this work [13, 14]. They convert sterols mainly into ADD and AD, respectively.

Media

The NB medium [25], containing per l—8 g Nutrient Broth (OXOID), 1 g Yeast Extract (OXOID), 10 g Glycerol (Merck)—was used for growing mycobacterium cells. For avoiding aggregation of growing cells, 1 g Tween 80 (Merck) was also added, and for solid media, 20 g/l Agar Technical No. 3 (OXOID).

Phytosterols mixtures

These, designated as VN1, VN2 and VN3, were kindly supplied by Dr. Luu Duc Huy, from the Institute of Chemistry of the Vietnamese Academy of Science and Technology.

MS medium

This consists of various salts and molasses as reported [3].

Characterization of phytosterols mixtures

Samples of white-dust, purified phytosterols was used as the raw material for the study. The relative content of total phytosterols (%, w/w) in those mixtures as well as the relative composition (%, w/w) of each phytosterol was determined by gas chromatography on a wide bore 25 m × 0.53 mm internal diameter, BPX5 1.0 μm column. Samples were injected and monitored with a flame-ionization detector (FID) at 320°C. The column temperature was increased from 200 to 320°C to 5°C/min. The carrier gas was hydrogen at a flow rate of 10 ml/min. Cholesterol was used as an internal standard, and response factors of all sterols were assumed to be 1. Weight of sterols was calculated as follows [26]:

$$\text{Weight of sterol (mg)} = \left(\frac{A_{\text{sterol}}}{A_{\text{IS}}}\right)\left(\frac{W_{\text{IS}}}{\text{RRF}_{\text{sterol}}}\right)\left(\frac{1}{W_{\text{sample}}}\right)$$

where

A_{sterol}	the peak area of the sterol
A_{IS}	the peak area of the internal standard (IS)
W_{IS}	the weight of the internal standard (IS)
$\text{RRF}_{\text{sterol}}$	the relative response factor to cholesterol
W_{sample}	the weight of the sample.

Bioconversion protocol

Mycobacterial cells were grown in NB medium for 48 h, 30°C and 200 rpm of shaking. Cultures at 1.0 optical density (540 nm), 10 % (v/v) were transferred to 50 ml NB or MS media containing phytosterols mixture (1 mg/ml) as the substrate for bioconversion. The mixtures were suspended and water-bath heated in Tween 80, 1% (w/v), before adding to conversion media. After 5 days of incubation at the above temperature and shaking, cultures were autoclaved and submitted to further chemical analysis. Conversion of substrates into AD or ADD was estimated as follows:

$$\text{Conversion (\%)} = \left(\frac{\text{Weight AD(D)}}{\text{Weight of added substrate}}\right) \times \left(\frac{\text{MW substrate}}{\text{MW AD(D)}}\right) \times 100$$

MW: molecular weights.

Since β-sitosterol represents the greater molecular weight in phytosterols mixtures, it was taken for calculating conversion.

Chemical analysis of products

All cultures were extracted with ethyl acetate. Samples were spotted on Silica-Gel 60 (Merck) thin layer chromatography plates (TLC), with the fluorescence indicator F_{254} and developed under an elution mixture ethyl acetate: hexane (2:3, v/v). Chemical analysis were

carried out as before [17], by HPLC using an RP-8 column and methanol-water mixture (65:35, v/v) as mobile phase, delivered to 1.5 ml/min. Detection was done at 254 nm, using 17α-methyl testosterone as internal standard.

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was carried out by analysis of variance followed by Duncan's multiple-range test [4].

Results

Composition of phytosterols mixtures

The identification of every sterol in the mixture as well as further androstanes after bioconversion was carried out by TLC and spectral analysis.

As shown in Fig. 1, a chromatographic profile of the mixture was successfully performed.

Phytosterols content as well as relative composition of the various mixtures (VN-1, VN-2 and VN-3) is also shown in Table 1.

A similar total phytosterols content was noticed in VN-2 and VN-3 (70.55 and 70.19%, respectively), but different in VN-1 (55.39%). In contrast, the mixture VN-1 with the lowest content of total phytosterols shows the highest content of β-sitosterol (48.5%).

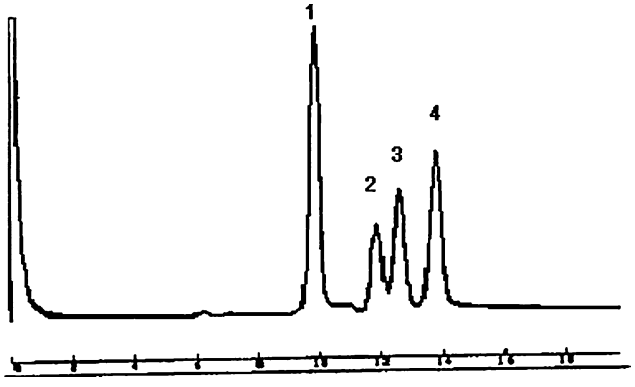

Fig. 1 A typical chromatogram showing separation of components of phytosterols mixture. The relative composition of phytosterols mixtures was determined by gas chromatography, using a wide bore 25 m × 0.53 mm internal diameter, BPX5 1.0 μm. Samples were monitored with a flame-ionization detector (FID). The corresponding peaks are the following: *1* cholesterol, used as internal standard for quantification, *2* campesterol, *3* stigmasterol and *4* β-sitosterol

Bioconversion of phytosterols mixtures

The results of every experiment were perfectly in agreement with the phenotypes of both strains. That is, B3683 produces mainly ADD, whilst MB3683 does to AD.

As noticed, all phytosterols mixtures can be bioconverted by mycobacterial cells into both androstanes (Table 2). Yields were remarkable, both for AD and ADD. Conversions were as high as 78% for ADD and 64% for AD.

It was observed that B3683 bioconversion to ADD was higher in VN-1 than in VN-2 or VN-3 (P 0.05). Meanwhile, MB3683 conversion to AD was higher in VN-1 and VN-2 than in VN-3 (P 0.05). A lower conversion to AD was also noticed in VN-3 (46.13%), which harbored greater stigmasterol content (42.85%) (Tables 1, 2).

Discussion

Phytosterols mixtures from various sources are mainly comprised by campesterol, stigmasterol and β-sitosterol, although sitostanol and stigmastanol can also be found in tall-oil phytosterols [12]. For sugarcane phytosterols, a relative general composition of (phytosterol weight to total weight of the mixture)—campesterol (25%), β-sitosterol (42%) and stigmasterol (33%)—has been reported [23].

In the case of soybean phytosterols, a composition of about 25% stigmasterol, about 70% β-sitosterol and about 5% miscellaneous phytosterols has been found [21]. Furthermore, according to a report from the Unilever Research Laboratories, the sterol composition in soybean oil was β-sitosterol (48%), stigmasterol (21%), campesterol (27%), and other sterols (5.9%) [15].

Phytosterols such as diosgenin (from *Dioscorea villosa*) and stigmasterol (from soybean seed oil) have been used as raw material for the semi-synthesis of steroidal drugs i.e. prednisolones and progesterone. Other phytosterols such as hecogenin and solasodine from *Agave* and *Solanum* species, respectively, have been used as well, but to a lesser extent. Furthermore, the utilization of, for instance, tall oil, sugarcane or soybean sterols as an economic source of intermediates for steroid manufacture has been long considered for supplementing or supplanting the commercial processes from diosgenin [24].

Several microbiological processes are now available for the conversion of phytosterols or 19-oxygenated phytosterols into androstanes [14].

Table 1 General composition of the various phytosterols mixtures

Phytosterol mixture	Total phytosterols (%, w/w) ($X \pm SD$)	Relative phytosterols content (%, w/w) ($X \pm SD$)		
		Campesterol	Stigmasterol	β-Sitosterol
VN-1	55.3 ± 2.1	25.3 ± 0.3[a]	26.2 ± 0.1[c]	48.50 ± 0.5[a]
VN-2	70.5 ± 1.1	22.77 ± 0.4[b]	32.7 ± 0.3[b]	44.53 ± 0.6[b]
VN-3	70.1 ± 0.9	17.89 ± 0.2[c]	42.8 ± 0.0[a]	39.26 ± 0.2[c]

Values were determined by Gas Chromatography (GC), using a wide bore BPX-5 (5 μm) and a 25 m column. Samples were monitored with a flame-ionization detector (FID). All figures represent the mean value of at least three independent determinations of samples ± the standard deviation ($X \pm SD$). Values not sharing a common letter (a, b, c) are significantly different at P 0.05 by Duncan's multiple-range test

Table 2 Conversion of phytosterols mixtures to androstanes

Strain	% Conversion to $X \pm SD$			
Phytosterol mixture	B3683		MB3683	
	ADD	AD	ADD	AD
VN-1	78.6 ± 1.1[a]	6.0 ± 0.0	2.3 ± 0.1	60.6 ± 0.2[a]
VN-2	74.3 ± 0.2[b]	1.9 ± 0.0	1.9 ± 0.1	64.8 ± 2.9[a]
VN-3	74.6 ± 0.4[b]	4.1 ± 0.2	1.0 ± 0.2	46.1 ± 1.9[b]

Mycobacterial cells were grown in NB medium for 48 h, 30°C and 200 rpm of shaking. Cultures at 1.0 optical density (540 nm), 10% (v/v) were transferred to 50 ml NB or MS media containing phytosterols mixture (1 mg/ml) as the substrate for bioconversion. Statistical analysis was done as before (Table 1)

Respect to the Vietnamese soybean phytosterols mixtures under study, they have shown variability, not only in general phytosterols content but also in composition of stigmasterol, campesterol and β-sitosterol (Table 1). These differences may apparently reflex differences in the processes of extraction and purification of every mixture. Moreover, the mixtures may contain some other phytosterols such as brassicasterol, Δ^5-avenasterol, Δ^7-stigmasterol and Δ^7-avenasterol as minor components [26], which are present as impurities.

On the other hand, results concerning the three phytosterols mixtures conversion to AD and ADD were remarkable. They were rather higher than those reported before [5, 14], or similar to cholesterol conversion to ADD in *Mycobacterium* sp. by adding cyclodextrins to culture media [5, 9]. The fact of direct-solubilizing the phytosterols mixtures in Tween-80 and re-suspending in culture media might explain these results. As it is known, insolubility of steroidal molecules in aqueous media is one of the most important factors just to hamper better yields in microbial bioconversion [5]. There are some reports concerning the use of sterols solubilizing agents i.e. tweens, vegetable oils, organic solvents or cyclodextrins in bioconversion media [7, 9, 14, 20].

The higher conversion to ADD when using VN-1 or conversion to AD in VN-1 and VN-2 might be associated to their higher β-sitosterol content (Table 2), despite the lower content of total phytosterols in VN-1 (Table 1). These results may reflex a better bio-accessibility to substrates of mycobacterial cells in the case of VN-1. Indeed, β-sitosterol was previously demonstrated to exhibit a better bioconversion of sugarcane phytosterols mixture to ADD by *Mycobacterium* sp. [8, 17], possibly by a depressing effect of C22=C23 double bond in the molecule of stigmasterol [14]. Nevertheless, this assumption must be rejected for explaining similar conversion to ADD in VN-2 and VN-3, despite of differences in stigmasterol content. In contrast, the effect seems to be correlated to lowering AD formation and might suggest that some kind of regulating mechanism in the enzyme 1,2 steroid dehydrogenase, mediated by stigmasterol, could be involved.

All the above results must be supported in future experiments and even correlated to extraction procedures and purification of soybean phytosterols mixtures.

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