Enhancing production and purity of 9-OH-AD from phytosterols by balancing metabolic flux of the side-chain degradation and 9-position hydroxylation in Mycobacterium neoaurum

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Abstract

9α-Hydroxyandroster-4-ene-3,17-dione (9-OH-AD) is a representative steroid drug intermediate that can be prepared by phytosterols (PS) biotransformation with mycobacteria in a resting cell-cyclodextrin system. In this study, over-expression of 17β-hydroxysteroid dehydrogenase (Hsd4A) was testified to enhance the side-chain degradation of PS and to reduce the incomplete degradation by-products. Meanwhile, the complete degradation product 4-androstene-3,17-dione (AD) was increased due to lack of 3-Ketosteroid 9α-Hydroxylase (KshA1) activities. To increase the production and purity of 9-OH-AD, the metabolic pathway of the side-chain degradation of PS and 9-position hydroxylation was modulated by balancing the over-expression of Hsd4A and KshA1 in mycobacteria and reducing the bioconversion rate via lowering the ratio of PS and cyclodextrin. The production and purity of 9-OH-AD in broth were improved from 22.18 g/L and 77.13% to 28.27 g/L and 87.84%, with a molar yield of 78.32%.

1 INTRODUCTION

9α-hydroxy-androst-4-ene-3,17-dione (9-OH-AD) is a C17 steroid intermediate that can be further synthesized into steroids, such as sex hormones and corticosteroids.[1,2] At present, steroid intermediates are usually prepared by microorganisms, mainly mycobacteria, to generate the required steroid intermediates by side chain degradation and nucleus oxidation of sterols.[3] Phytosterols (PS) are wastes separated from soybean processing and oil refining. It is used as the substrate to produce steroid intermediates to increase the added value of agricultural products. However, due to the low bioavailability of PS, the toxicity of steroids to cells, and the low viability of existing strains, its transformation efficiency is not ideal. Thus, how to improve the transformation efficiency of PS remains a research hotspot in the field of steroids.[4,5]

Currently, there are many approaches to improve the biosynthesis efficiency of steroid intermediates. The most common method to optimize the reaction system was to enhance the solubility of PS and thus improve the conversion efficiency. The resting cell-cyclodextrin system was established to solubilize PS and reduce the toxicity of PS to cells.[6,7] Gao et al. reported an increase in 9-OH-AD production by PS bioconversion through the resting cell-cyclodextrin system.[8] In addition, the use of genetic engineering technology to improve the production capacity of*mycobacteria* is also an effective way to enhance the biosynthesis efficiency of steroid intermediates. Yao et al. improved 9-OH-AD production by overexpressing *kshA* that facilitated the 9-position hydroxylation in *Mycobacterium neoaurum.* [9]Sun et al. increased the yield of 9-OH-AD in recombinant strains by 1.45-fold by overexpressing four key genes, *hsd, hsd4A* ,*kshA1* and *kshB* [10], that enhance the side-chain degradation. Chang et al. increased the production of AD from 3.2 g/L to 4.5 g/L through the overexpression of Hsd4A.[11] Liu reported that the yields of 9-OHPDC-M were remarkably improved by individually overexpressing the genes *hsd4A* and *kshA1.* [12]

However, there are still problems affecting the biosynthesis efficiency of steroid intermediates, especially the accumulation of by-products.[13] In the production of 9-OH-AD by*mycobacteria* , there are mainly two types of by-products accumulated, one is the incomplete degradation of the side chain, including the by-product 9,22-dihydroxy-23,24-bisnorster-4-en-3-one (DHBC) and 9,24-dihydroxycholester-4-en-3-one (DHC). The other is the complete degradation of side chain, such as androsten-4-ene-3,17-dione (AD), resulting in a lower purity and yield of the main product 9-OH-AD.

In this study, 9-OH-AD was prepared by the biotransformation of PS in the resting cell-cyclodextrin system with *Mycobacterium neoaurum* . Two key genes *kshA1* and *hsd4A* , which may be related to by-product accumulation, were selected to verify their functions in the degradation pathway of phytosterols. The genetically engineered strain facilitated for balancing metabolic flux of the side-chain degradation and 9-position hydroxylation in *Mycobacterium neoaurum* , which significantly improved the yield and purity of 9-OH-AD.

2 MATERIALS AND METHODS

2.1 Strains, media and reagents

All strains used in this study are listed in Table 1. The strains of*Mycobacterium* were cultured at 30 and 200 rpm in the MYC/01 seed medium containing glycerol 20 g/L, sodium citrate 2.8 g/L, NH_4Cl 2.7 g/L, K_2HPO_4[?]3 H_2O 0.5 g/L, $MgSO_4$[?]7 H_2O 0.5 g/L and ammonium ferric citrate 0.05 g/L for 2 days. Then the seeds were inoculated with 5% (v/v) into MYC/04 broth containing glucose 25 g/L , sodium citrate 2.8 g/L, corn steep powder 3 g/L, $(NH_4)_2HPO_4$ 5 g/L, starch 2 g/L, K_2HPO_4[?]3 H_2O 0.5 g/L, $MgSO_4$[?]7 H_2O 0.5 g/L, ammonium ferric citrate 0.05 g/L, PS 0.1 g/L and cultured at 30 and 200 rpm for 3 days. The initial pH of the medium was adjusted to 8.0.

The reagents described in the media components were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). PS (purity of 95%, the main components are stigmasterol, campesterol, and β-sitosterol) was obtained from Shanxi Sciphar Natural Products Co., Ltd. (Shangluo, China). The 9-OH-AD standard was purchased from Sigma (Shanghai, China). HP-β-CD was from Zhiyuan Biotechnology Co., Ltd. (Binzhou, China). The organic solvent was purchased from Bohr Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Construction of plasmids and recombinant strains

All plasmids used in this study are listed in Table 1 and the primers are listed in Table S1. The first is the linearization of the vector pMV261 and the amplification of the target genes. The co-expression genes *hsd4A -kshA1* and *kshA1 -hsd4A* were constructed and amplified by overlap PCR. Then the genes of interest obtained by PCR amplification were inserted into pMV261 through the in-fusion clone method to construct the recombinant plasmids. The recombinant plasmids were transferred into HK86 W via electroporation to construct recombinant *Mycobacterium* .

2.3 Preparation of resting cells and establishment of bioconversion system

Resting cells were prepared according to the method described by Gao et al.[14] The bioconversion of PS was performed in the reaction system of 10 mL phosphate buffer (20 mM, pH 8.0, PB), including PS 50 g/L, HP-β-CD 200 g/L, and resting cell 100 g/L in 250 mL flasks at 30 °C and 200 rpm.

2.4 Analytical methods

The sample from the PS bioconversion (100 μL) was extracted with ten times volume of ethyl acetate by shaking for 3 min. The mixture was centrifuged at 13,000 rpm for 3 min, and 100 μL of supernatant was withdrawn and evaporated, and then redissolved with five times volume of ethanol. Steroid metabolites were quantitatively analyzed by high performance liquid chromatography (Agilent Tech, USA) equipped with the Elit Hypersil ODS2-C18 (4.6 mm×250 mm, 5 μm) column using the mobile phase (methanol/water, 80:20, v/v) at a flow rate of 1 mL/min and the detection wavelength was 254 nm.

3 RESULTS AND DISCUSSION

3.1 KshA1 promotes the 9-position hydroxylation of the steroid nucleus

The engineered strains *Mycobacterium neoaurum* NwIB-HK86 W and HK86-K are 9-OH-AD production strains in the resting cell-cyclodextrin system. Under substrate feeding of 50 g/L and the mass ratio of PS and HP-β-CD (1:4, w/w), the 9-OH-AD yields of the two strains peaked at 22.18 g/L and 25.10 g/L, respectively, on the 4th day of reaction (Fig. 1). The yield of 9-OH-AD begins to decline after 4 days because it was degraded in the later stages of transformation and the production rate was less than degradation rate. The composition of the transformation products of the two strains is shown in Table 2, and the purity of the main product 9-OH-AD in broth was 77% and 78% respectively. Overexpression of kshA1 in mutant strains promoted the level of 9-position hydroxylation. The production of 9-OH-AD was increased in HK86-K, while large amounts of incomplete degradation by-products such as 9,22-dihydroxy-23,24-bisnorchol-4-en-3-one (DHBC) and 9,24-dihydroxychol-4-en-3-one (DHC) were also accumulated at the end of biotransformation. The excessive by-products will hamper the downstream process and reduce the yield of biotransformation.

3.2 Hsd4A enhances the complete degradation of steroid side chain

Hsd4A is a bifunctional enzyme with 17β-hydroxysteroid dehydrogenation and hydroxyacyl-CoA dehydrogenation activity. The transcription of*hsd4A* gene was highly upregulated along with other genes involved in sterol side chain degradation in *R. jostii* RHA1, so it is speculated that this gene is essential for sterol side chain degradation.[15,16] Zhao reported that Hsd4A is responsible for the dehydrogenation of 22-hydroxy-3-oxo-4-ene-24-carboxy-CoA in the second cycle of oxidation of the sterol side chain similar to fatty acids β-oxidation.[17] Furthermore, knock-out of*hsd4A* in *Mycobacterium* can accumulate the steroid intermediate HBC.[18] Hence, its function in sterol side chain degradation was investigated by overexpressing*hsd4A* in HK86 W.

The cell growth of HK86 A showed no significant difference from that of the original strain HK86 W. The yield and purity of 9-OH-AD were increased from 22.18 g/L and 77.13% to 25.16 g/L and 82.45% (Table 2). The principal component analysis showed that the proportion of complete degradation products (9-OH-AD and AD) was increased and the proportion of incomplete degradation products (DHBC and DHC) was decreased in HK86 A. Among them, the proportion of AD increased from 3.36% to 11.86% and DHBC decreased from 11.96% to 0.93%, respectively. These results indicated that the pathway of complete degradation of the side chain was enhanced in HK86 A and *hsd4A* was a key gene for complete degradation of the steroid side chain. However, further investigation found that the increase in AD proportion is much higher than that of 9-OH-AD. It was reported that the physiological substrates of KshAB are CoA thioester intermediates of sterol side chain degradation in *Mycobacteria* and the AD is not the favorable substrate of KshAB (Fig. 2). [19] It was deduced that the metabolic pathway of AD and 9-OH-AD is competitive and 9-position hydroxylase (KshA1) activity was limited when the pathway of side chain degradation was enhanced, leading to much more AD accumulation. To transfer the metabolic flux to the target product 9-OH-AD and decrease the generation of byproduct AD, an appropriate catabolic division of steroid was needed to balance the side-chain degradation and 9-position hydroxylation.

3.3 Effects of co-expression of *hsd4A* and *kshA1* on PS conversion to 9-OH-AD

To optimize the distribution of metabolic flux in PS biotransformation, KshA1 that promotes the 9-position hydroxylation of steroid nucleus and Hsd4A that enhances the complete degradation of steroid side chain were co-expressed in *Mycobacterium neoaurum* NwIB-HK86 W. Furthermore, it was reported that the distance of genes from promoters in the same operon affects their expression intensity.[20,21]The expression levels were higher for gene locations close to the origin of replication compared to locations close to the replication terminus. Based on these results, the mutants co-expressing endogenous genes*kshA1* and *hsd4A* were constructed in two patterns as shown in Fig. 3 and named *M. neoaurum* HK86 A-K and HK86 K-A. PS transformation using mutants HK86 A-K and HK86 K-A could balance the activity of KshA and Hsd4A. The PS transformation results using two mutants were shown in Table 3.

Compared with the PS transformation of strain HK86 A, HK86A-K showed no remarkable difference in the yield, purity of 9-OH-AD, and the proportion of other by-products, indicating that the augment of KshA

activity in the co-expression mutant did not contribute to improving the 9-position hydroxylation level. Considering the pivotal role of Hsd4A in enhancing the side-chain degradation of PS, it was speculated that the KshA activity did not match the Hsd4A activity in the co-expression mutant HK86 A-K.

The transformation results of HK86 K-A were significantly different from HK86 A-K (Table 3). Compared with HK86-A-K, the content of byproduct AD was significantly reduced from 11.73% to 3.61%, and the 9-position hydroxyl products, 9-OH-AD, DHBC, and DHC were increased correspondingly. The yield and purity of 9-OH-AD using HK86 K-A were further increased to 26.04 g/L and 85.61%, respectively, despite the slight decrease of total complete degradation products (AD and 9-OH-AD). These results indicated that the expression level of *kshA1* that was closer to the promoter was higher when co-expressed with *hsd4A* in the same operon. In mutant HK86 K-A, the decreased expression level of *hsd4A* could better balance the activities of Hsd4A and KshA1, then effectively increasing the proportion of 9-position hydroxyl products in metabolites and meanwhile enhancing the side-chain degradation to decrease the incomplete side-chain degradation byproducts.

3.4 Decreasing the PS conversion rate to balancethe metabolic flux of the side-chain degradation and 9-position hydroxylation

Deduced from the results abovementioned, the decreased PS conversion rate led to the metabolic flux decrease of the side-chain degradation and could better balance the reaction of 9-position hydroxylation. According to our previous work, the ratio of the PS and CD in the reaction system affects the PS conversion rate. The results of PS transformation in different ratios of the PS to CD were shown in Table 4. The PS conversion rate decreased as expected with the ratio of the PS to CD changed from 1:4 to 1:2 and the production of 9-OH-AD peaked at 26.04, 27.89, and 28.27 g/L respectively on the 4, 5, and 6th day of bioconversion. In the meantime, the purity of 9-OH-AD increased and the by-products decreased correspondently. The optimized result was obtained when the ratio of PS to CD was 1:2. If the ratio lowed to 1:1, the reaction rate was severely impeded and the nucleus degradation aggravated.

4 CONCLUSION

Mycobacterium could degrade the sidechain of PS to produce the steroidal intermediates 9-OH-AD. In this study, the production of 9-OH-AD from PS biotransformation was significantly improved by modulating the key gene expression of *kshA1* and *hsd4A* in mycobacterial mutants. The augment of gene *hsd4A* could effectively promote the side-chain degradation of PS and decrease the incomplete degradation products, DHBC, but increase the complete degradation product of 4-androstene-3,17-dione (AD) due to lack of 3-Ketosteroid 9α-Hydroxylase KshA1 activities. Therefore, co-expression of *kshA1* and *hsd4A* in an appropriate pattern in mycobacterial could optimize their expression level and achieve a better balance of the Hsd4A and KshA1 activity. Further decreasing the PS conversion rate could be best for the action of the Hsd4A and KshA1 activities to transfer the metabolic flux to the target product of 9-OH-AD and decrease the byproducts formation. Finally, the yield and purity of 9-OH-AD in PS transformation by HK86 K-A mutant were increased from 22.18 g/L and 77.13% to 28.27 g/L and 87.84%, respectively, with a molar yield of 78.32%.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

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Supporting Information

The primers used in this study (DOC).

Table 1 Strains and plasmids used in the study

Strains and plasmids	Description
Strains	
E. coli DH5α	General cloning host
Mycobacterium neoaurum NwIB-HK86 W	Starting strain, knocked of *kstD1*, *kstD2*, *kstD3*
HK86-K	*kshA1* over-expressed strain of HK86 W
HK86-A	*hsd4A* over-expressed strain of HK86 W
HK86 A-K	*hsd4A* and *kshA1* over-expressed strain of HK86 W
HK86 K-A	*kshA1* and *hsd4A* over-expressed strain of HK86 W
Plasmids	
pMV261	Shuttle vector of *Mycobacterium* and E. coli, carrying the heats shock hsp60 pr
pMV261-*hsd4A*	Recombinant pMV261, for over-expression of *hsd4A* in HK86 W
pMV261-*hsd4A- kshA1*	Recombinant pMV261, for over-expression of *hsd4A* and *kshA1* in HK86 W
pMV261-*kshA1-hsd4A*	Recombinant pMV261, for over-expression of *kshA1 and hsd4A* in HK86 W

Table 2 Results of PS transformation with *kshA1* or *hsd4A*over-expression mutants

Strains	9-OH-AD concentration(g/L)	Product distribution(Area%)[a]	Product distribution(Area%)[a]	Product distrib
		Complete degradation 9-OH-AD	Complete degradation AD	Incomplete deg DHBC
HK86-W	22.18±0.55	77.13±0.31	3.36±0.28	11.96±0.19
HK86-K	25.10±0.45	78.23±0.21	1.54±0.38	13.15±0.13
HK86-A	25.16±0.38	82.45±0.16	11.86±0.43	0.93±0.21

[a] The product allocation ratio is determined by normalization d (error bars for standard deviations, n=3).

Table 3 Results of PS transformation with *kshA1* and *hsd4A*co-expression mutants

Strains	9-OH-AD concentration(g/L)	Product distribution(Area%)[a]	Product distribution(Area%)[a]	Product dist
		Complete degradation 9-OH-AD	Complete degradation AD	Incomplete d DHBC
HK86 A-K	25.76±0.28	81.86±0.17	11.73±0.13	0.98±0.11
HK86 K-A	26.04±0.31	85.61±0.21	3.61±0.19	3.43±0.23

[a] The product allocation ratio is determined by normalization (error bars for standard deviations, n=3).

Table 4 Effects of PS and CD ratios on PS transformation with HK86 K-A

PS:CD w/w	Time days	9-OH-AD concentration(g/L)	Product distribution(Area%)[a]	Product distribution(Area%)[a]	Product distribution(Area%)[a]	Product distribution(Area
			Complete degradation 9-OH-AD	Complete degradation AD	Incomplete degradation DHBC	Incomplete degradation DHC
1:4	4	26.04±0.21	85.61±0.45	3.61±0.26	3.43±0.32	6.35±0.27
1:3	5	27.89±0.17	86.33±0.36	3.68±0.37	2.72±0.44	6.31±0.35
1:2	6	28.27±0.33	87.84±0.29	2.33±0.41	2.47±0.39	6.25±0.41

[a] The product allocation ratio is determined by normalization (error bars for standard deviations, n=3).

Figure legends

Fig. 1 Time profiles of 9-OH-AD production by *M. neoaurum*NwIB-HK86 W and HK86-K transforming phytosterols under cyclodextrin-resting cells system. The reaction system consist of 50 g/L of phytosterols, 100 g/L of wet biomass and 200 g/L of HP-β-CD in 250 mL shake flasks containing 10 mL of reaction solution for 5d (error bars for standard deviations, n = 3).

Fig. 2 Schematic diagram of metabolic pathway from phytosterols to 9-OH-AD.

Fig. 3 Schematic diagram of co-expression genes *hsd4A* and *kshA1* in the*Mycobacterium neoaurum* HK86 W strain. (a) Schematic of the gene overexpression in HK86 W, denoting the corresponding strains. (b) The schematic of co-expression plasmids.

Figure 1

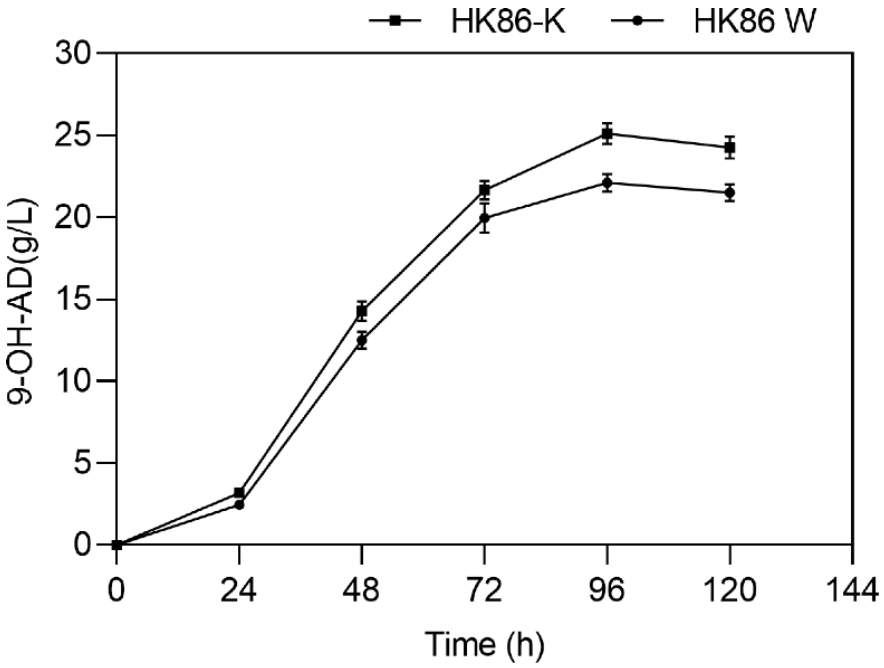

Figure 2

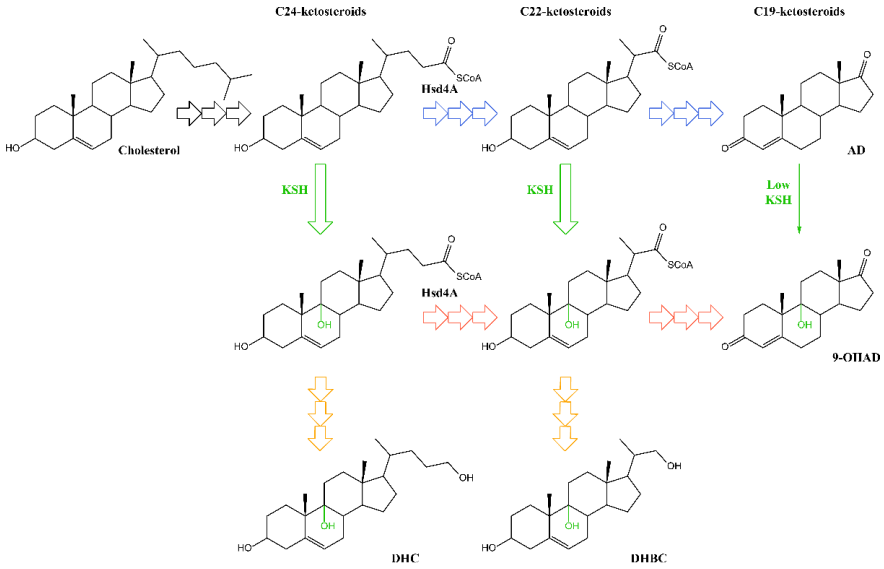

Figure 3