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The influence of host–guest inclusion complex formation on the biotransformation of cortisone acetate Δ^1-dehydrogenation

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ABSTRACT

An intensive and systematic investigation had been carried out on the Δ^1-dehydrogenation of cortisone acetate (CA) to prednisone acetate (PA) by *Arthrobacter simplex* TCCC 11037 in the presence of native and modified β-cyclodextrins (β-CDs). The biotransformation was improved through the formation of the host–guest inclusion complex between CA and CDs in aqueous solution. The inclusion complexes of CDs with CA were investigated by means of phase solubility, 2D NMR spectroscopy and differential scanning calorimetry (DSC). The structural difference of CDs resulted in the stoichiometric differences between the complexes, the RM-β-CD-CA, SBE-β-CD-CA, HP-β-CD-CA complexes were 1:1 whereas β-CD-CA gave both 1:1 and 2:1 complexes, of which the 2:1 complex decreased the soluble CA concentration and inhibited the dissociation of β-CD-CA in aqueous solution. The increase in solubility of CA was in the order of RM-β-CD > SBE-β-CD > HP-β-CD > β-CD. RM-β-CD-CA, SBE-β-CD-CA and HP-β-CD-CA exhibited the higher biotransformation rate in comparison with native β-CD. And the solubilization of CDs for CA in aqueous medium plays a key role in the biotransformation process. The article focuses on the various factors influencing the substrate water solubility, complex stability and biotransformation of CA through the addition of CDs in order to solve many problems associated with the process of drug delivery and biotransformation of different novel steroids.

1. Introduction

Steroidal drugs are an important class of compounds that are widely used in health and fertility management. In the synthesis of various steroidal drugs and drug intermediates certain reaction steps are often carried out by means of microorganisms. However, there are numerous steroids which are poorly soluble in water, and it is considered as the rate-limiting step of steroid biotransformation [1]. In order to increase substrate solubility, biotransformation in organic media has been developed as an alternative. However, the high toxicity of organic solvents to cells which results in the low tolerance of cells to organic solvents, is still a limiting step in the process [2–5]. So the key issue was put on finding the medium which not only could improve the solubility of steroids but also possess a preferable biocompatibility.

Cyclodextrins (CDs) are cyclic molecules consisting of 6, 7 or 8 glucopyranose units forming α-1,4-glucose units, usually referred to as α-, β-, γ-CD, respectively. The CDs have the ability to form inclusion complexes with a variety of molecules that fit inside the CD cavity. The guest molecule is surrounded or encapsulated by CD in the inclusion complex, as a result, shows the advantageous changes in its chemical and physical properties [6]. In particular, solubility, stability and bioavailability can be improved, and these changes have been intensively applied in facilitating and enhancing microbe mediated transformations of hydrophobic organic compounds [7]. CD-mediated steroids bioconversions were first introduced by Udvardy who demonstrated that biotransformations were intensified by adding CDs to the reaction mixture of the steroid substrate [8]. Consequently, CDs had been intensively used to form host–guest complex to increase the reaction rate and degree of conversion in many steroid biotransformations [9–13]. Bar systematically investigated steroid biotransformation by *Rhodococcus erythropolis* in the presence of natural and chemically modified CDs, they found that the biotransformation was strongly affected by the mode of the addition of the natural CDs [14–16]. Zarzycki demonstrated the influence of the temperature on the association constant of host–guest complexes formed by CDs [17–19], which help to select appropriate experimental condition. Furthermore, mathematical model was also proposed to study the influence of β-CD on the kinetics of steroid biotransformation, in the model framework the formation of complexes

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leads to an increase of the maximal reaction rate and a decrease of substrate inhibition [10,20,21]. We had used HP-β-CD in order to enhance cortisone acetate (CA) bioavailability in aqueous transformation media and consequently to increase the rate of steroid Δ^1-dehydrogenation. This work not only provided a systematic study on the influences of HP-β-CD on the CA Δ^1-dehydrogenation reaction, but also suggested an applicable and effective method for enhancing enzyme-catalyzed steroids biotransformation [22].

Unfortunately, the comprehensive understanding of the effect of CDs on bioconversion through the formation of host–guest inclusion complex is rather limited, so the mechanism of the CD-promoted biotransformation is still uncomprehending.

In the present study, effects of β-CD and its derivatives on the biotransformation of steroid Δ^1-dehydrogenation were studied using the Δ^1-dehydrogenation of CA to prednisone acetate (PA) by *Arthrobacter simplex* TCCC 11037 as a model process. The formation of inclusion complexes of CDs with CA was investigated by phase solubility, 2D NMR, DSC. This study was aimed to investigate the influence of the guest–host molecular complexation on the biotransformation of CA from the view point of supramolecular. The results should be significant in the study of the biotransformation of steroids, as well as in the application of CDs in drug delivery.

2. Materials and methods

2.1. Materials

CA and PA were purchased from Sigma–Aldrich Co., β-cyclodextrin (β-CD, Mw: 1135), hydroxypropyl-β-cyclodextrin (HP-β-CD, Mw: 1522, average degree of substitution: 6.5), sulfobutyl-ether-β-cyclodextrin (SBE-β-CD, Mw: 2295, average degree of substitution: 7.3) and randomly methylated-β-CD (RM-β-CD, Mw: 1310, average degree of substitution: 5.2) were obtained from Wacker Biochem. Corp. (Munich, Germany). The buffer used throughout was 50 mM phosphate, pH 7.2. All other reagents were of BDH or HPLC reagent grade.

2.2. Bacterial strain and medium

Arthrobacter simplex TCCC 11037 stored in our laboratory was maintained at 4 °C on slant, consisting of glucose 10 g L^{-1}, yeast extract 10 g L^{-1} and agar 20 g L^{-1} (pH 7.2). Seed medium consists of glucose 10 g L^{-1}, corn slurry 10 g L^{-1}, peptone 5 g L^{-1} and KH_2PO_4 2.5 g L^{-1} (pH 7.2).

2.3. Cultivation and preparation of biocatalyst

The *Arthrobacter simplex* cells were prepared in two consecutive cultivation steps (18 h for seed culture and 27 h for cell cultivation) in shake flasks. The whole *Arthrobacter simplex* cells were grown in 500 mL shake flasks containing 100 mL culture media using 5% (v/v) of seed culture on a rotary shaker (180 rpm) at 34 °C. 0.01% (w/v) CA used as an inductor of steroid dehydrogenase was added into cell cultivation medium after 6 h growth. The cells were centrifuged at 6000 rpm for 10 min, washed two times with 50 mM KH_2PO_4–NaOH buffer (pH 7.2). And then the washed cells were resuspended in the same buffer, kept cell concentrations at 2.00 mg dry weight mL^{-1}.

2.4. Biotransformation of CA

30 mL cell suspension was added into a 250 mL shake flask and pre-incubated at 34 °C, 180 rpm for 15 min to keep temperature equilibration. Then a described CA and CDs were added, and to give a final CA of 10 g L^{-1}, β-CD of 1, 10, 20 mM, its derivatives of 1, 10, 30, 60, 100 mM. Then the flasks were incubated on a rotary shaker at 34 °C and 180 rpm for 25 h. During incubation, 100 μL samples were drawn at various time intervals, and then analyzed by HPLC. All experiments were made with 2 replicates at least three times.

2.5. Phase-solubility studies

Solubility measurements were determined according to the modification of method of Higuchi and Connors [23]. Excess amounts of CA was added to aqueous solution with various concentrations of CDs, ranging from 1 to 100 mM for HP-β-CD, SBE-β-CD and RM-β-CD, and 0.1–22 mM for β-CD. The suspensions were shaken at 34 °C for 2 days. After equilibration, the suspensions were filtered through 0.45 μm membrane filters, appropriately diluted with the mobile phase and the total concentration of the CA in the filtrate was analyzed by HPLC. An apparent stability constant ($K_{1:1}$) was calculated from the initial straight line portion of the phase-solubility diagrams.

2.6. Solubility and dissolution rate measurements of steroid

In 250 mL shake flasks, bioconversion medium (phosphate buffer) was added to a final volume of 30 mL. After adding CA or its equivalent in CDs physical mixture to 10 g L^{-1}, the shake flasks were incubated under condition identical to those employed for bioconversion. The flasks were tightly sealed to avoid evaporation. Intermittently, a 5 mL aliquot of the slurry was withdrawn first 1 min and then each 10 min for a period of 80 min, and filtered through a 0.45 μm filter. The filtrate was assayed by HPLC, the absorbance values were expressed as a percentage of the highest absorbance measured.

2.7. Partition coefficient (octanol:water) determination

Partition coefficients of CA were determined between octanol and water using the shake-flask method. Aqueous solutions of CA or complexes (CA: β-CD, CA: HP-β-CD, CA: SBE-β-CD and CA: RM-β-CD) were prepared containing CA 30 mg L^{-1}. Equal volume of octanol and each CA solution were shaken for 30 min. After allowing to stand for 5 min, the supernatant was removed, and the residue was centrifuged for 10 min at 2000 rpm. The aqueous phase was assayed by HPLC at time zero (C_0) and after shaking to ensure partition (C_w). The partition coefficient was $K_{\text{octanol/water}}$. $K_{\text{octanol/water}} = (C_0 - C_w)C_w$.

2.8. Solid complexes

The solid complexes were obtained from saturated CA solutions in the presence of CDs. The supernatant was removed from the aqueous CA: β-CD, then complex solution by freeze-drying.

2.9. Steroid HPLC analysis

The samples were withdrawn and extracted by chloroform, and dried in vacuum, then the solid extracts were redissolved in eluent (dichloromethane:ether:methanol, 86:12:2 (v/v/v)) and filtered through a 0.45 μm filter. It was assayed by HPLC (Agilent 1100, USA), measuring absorbance at 240 nm. Analysis was performed on a Kromasil 100-5SIL, 250 mm × 4.6 mm (5 μm) column with a flow rate of 0.8 mL min^{-1} at 30 °C. The concentration of CA and PA was determined from calibration curves, making from eluent solutions of standard CA and PA.

2.10. DSC measurements

DSC measurements were performed using a Seiko 2400 (Seiko Instruments, Japan). Samples were hermetically sealed in steel pans

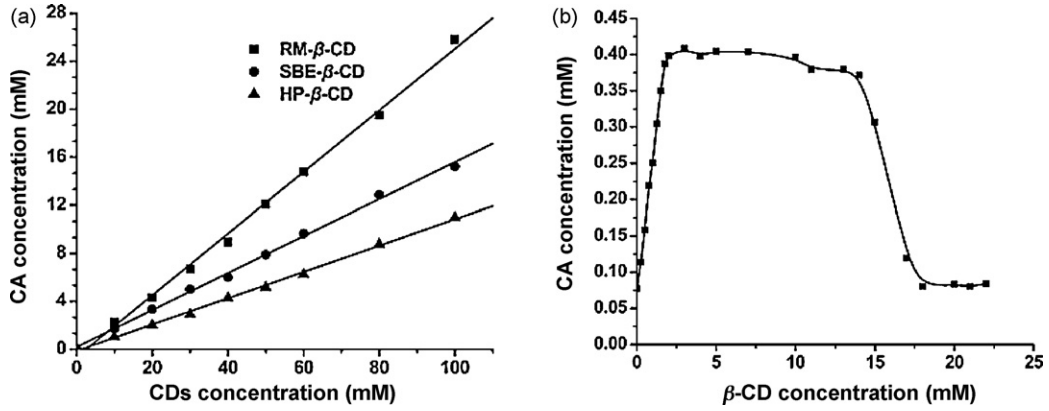

Fig. 1. (a) Solubility curves of the CA in the presence of different concentrations of modified β-cyclodextrins at 34 °C; (b) solubility curves of the CA in the presence of different concentrations of β-cyclodextrin at 34 °C.

and scanned over the temperature range of 20–300 °C at a heating rate of 10 °C min^{-1}.

2.11. NMR

^1H nuclear magnetic resonance (NMR) was recorded in D_2O at 25 °C on a Varian Mercury VX300 spectrometer.

3. Results and discussion

3.1. Phase-solubility study

Phase-solubility diagrams of CA with β-CD, HP-β-CD, SBE-β-CD, RM-β-CD at 34 °C were obtained, the result was shown in Fig. 1.

As shown in Fig. 1a, the differences in the solubility curves of CA with modified CDs were clearly noted. The solubility of CA increased with increasing CDs concentrations. Table 1 shows the increased ability to CA in the order of RM-β-CD > SBE-β-CD > β-CD (low concentration) > HP-β-CD. The solubility of CA in the presence of CDs increased 16.5-, 12.5-, 8.1- and 5.2-fold when RM-β-CD, SBE-β-CD, HP-β-CD and β-CD were 5 mM, respectively. The solubility studies indicated that the CA probably formed inclusion complexes with CDs. However, as shown in Fig. 1b, β-CD solubility curves exhibited a linear increase up to 2 mM (β-CD) for CA after which a plateau was reached, and then followed by decrease in CA concentration. The precipitation of a microcrystalline complex was formed at high β-CD concentration (about 15 mM), indicating a limited solubility of the formed inclusion complex. As can be seen from Fig. 1, according to Higuchi [23], all CDs exhibited A_L-type diagrams in the chosen concentration range, with the exception of β-CD, which exhibited a B_S-type diagram. Straight lines could be fitted to the A_L-type diagrams with regression coefficients (R^2 equal or better than 0.996). All A_L-type diagrams and the initial linear slope on the B_S diagrams had a slope less than unity indicating the probable formation of 1:1 complexes in aqueous solution, while the B_S diagram indicated that β-CD formed 1:1 and 2:1 inclusion complexes with CA. Assuming an 1:1 complex stoichiometry (low β-CD concentration), the inclusion complex stability constants (K_S) for CA with various CDs concentrations were calculated from the slope of the fitted straight line [23]. As shown in Table 1, the values of K_S increased in the order of RM-β-CD > SBE-β-CD > β-CD > HP-β-CD. On the other hand, B_S-type diagram indicated that β-CD first formed 1:1 water-soluble complex with CA, and then another β-CD further encapsulated the other end of CA to form 2:1 water-insoluble complex. So we calculated $K_{2:1}$ which is the association constant of 1:1 complex with the second β-CD [24]. Value of $K_{2:1}$ is 687.0, which would further restrict the dissociation of β-CD-CA.

Phase-solubility studies displayed the different complex and soluble performance of CDs for CA. In contrast to the alkylated CDs, RM-β-CD and SBE-β-CD showed the fairly good complexants and solubilizers, while β-CD exhibited good complexant but very poor solubilizer for CA.

Table 1
Solubility parameters of CA in aqueous solutions of CDs at 34 °C.

CD	Solubility curve type	Slope (1:1)	K_S (1:1)
CA/β-CD	B_S	0.1790	2795.2
CA/HP-β-CD	A_L	0.1098	1581.3
CA/SBE-β-CD	A_L	0.1812	2837.2
CA/RM-β-CD	A_L	0.2565	4422.9

3.2. Solubilization process measurements of steroids by CDs

The solubilization profiles for the systems under study were presented in Fig. 2. It showed that CA formed complexes with CDs very rapidly. CDs complexation of the steroid accelerated its dissolution rate, and in less than 1 min the equilibrium had been achieved, except β-CD of which the last equilibrium took much longer time. Solubilization process curve of β-CD and CA confirmed that β-CD first formed 1:1 water-soluble complex with CA and then another β-CD further encapsulated the other end of CA to form 2:1 water-insoluble complex, which resulted in the decreasing of CA apparent solubility until 80 min the equilibrium achieved. This result was in

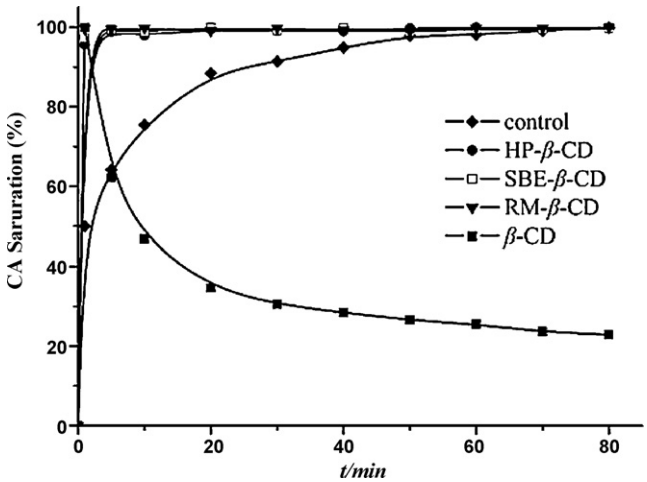

Fig. 2. Dissolution in water of free CA and CA with different CDs in shake flasks rotated at 180 rpm at 34 °C.

Table 2
Partition coefficient $K_{octanol/water}$ of the inclusion complexes in octanol/water system at 34 °C.

	Partition coefficient	Log *P*
CA	42.56	1.62
CA/β-CD	68.21	1.83
CA/HP-β-CD	30.33	1.48
CA/SBE-β-CD	21.04	1.32
CA/RM-β-CD	19.85	1.30

accordance with the phase-solubility studies. As a result, a dynamic equilibrium was established among free, complexed CA, and CA dissolved from complex in aqueous solution.

3.3. Partition coefficient

Several solvent systems have been used to relate partition coefficients to membrane absorption. Because of its polar and non-polar nature, octanol was used to mimic the complex nature of the membrane. The partition coefficients ($K_{octanol/water}$) of the complexes of CA with HP-β-CD, SBE-β-CD, RM-β-CD or β-CD were listed in Table 2, which was in accordance with that reported previously [25]. This result showed that the lipophilic characteristics of CA decreased when it was complexed with CDs. On the other hand, the increased aqueous solubility in the presence of CDs was not directly related to the change in lipophilicity.

3.4. DSC diagrams

Thermal analysis has been reported as a method to characterize CD complexes. DSC diagrams illustrate the DSC profiles of CA, β-CD, physical mixtures and complexes. As shown in Fig. 3, an endothermic transition was observed at about 248.8 °C, corresponding to CA fusion peak. β-CD exhibited transitions at about 103.5 °C. The transitions attributed to β-CD could be extended due to the release of water from the molecules. Sharp and broad endothermic transitions approximating to the CA and β-CD transitions, respectively, were seen in the physical mixture. New characteristic peaks (different than CA and β-CD transitions) at about 240.2, 94.8 and 112.6 °C for the CA: β-CD complexes, respectively. These thermal behavior changes might result from the formation of a new compound through weak interactions between CA and β-CD. This observation implied that the molecular arrangement of steroid in the solid complexes was different from that in their own crystal habit. So the steroid was dispersed at a molecular level in the solid complexes.

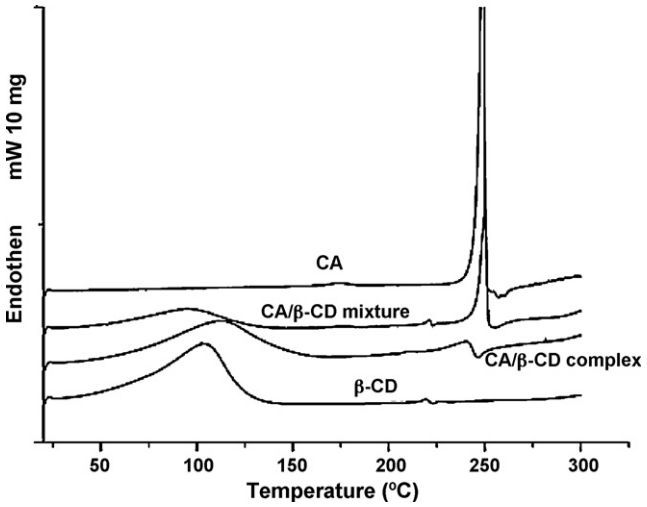

Fig. 3. DSC thermograms of CA, β-CD, CA: β-CD complexes and their physical mixture.

3.5. ROESY NMR

2D NMR spectroscopy has recently become an important method for the investigation of the structure of the CDs host–guest inclusion complexes, while the guest molecule group is included into the CD cavity, the NOE correlations between the protons of the guest and the inner protons of the CD cavity (H3 and H5) will be measured. According to the relative intensity of these cross-peaks, it is possible to estimate the orientation of the guest molecule within the CD cavity. As shown in Fig. 4a, the ROESY spectrum displayed clear NOE cross-peaks between the H5 protons of β-CD and H18/H21 protons of CA, as well as between the H3 of β-CD and H15 proton, and the cross-peaks between H3/H5 of β-CD and H16 protons of CA, which indicated distinctly that the D ring in CA was included into the hydrophobic cavity of β-CD. Moreover, the clear NOE correlations between H5 protons of β-CD and H1/H2 protons of CA, and between the H3/H5 of β-CD and H4 proton of CA, showing that the A ring in CA was included into the hydrophobic cavity of β-CD. From these ROESY data we can deduce a possible binding mode of CA-β-CD complex as illustrated in Fig. 4b. These studies supported further phase-solubility study that CA could form 2:1 inclusion complex with β-CD.

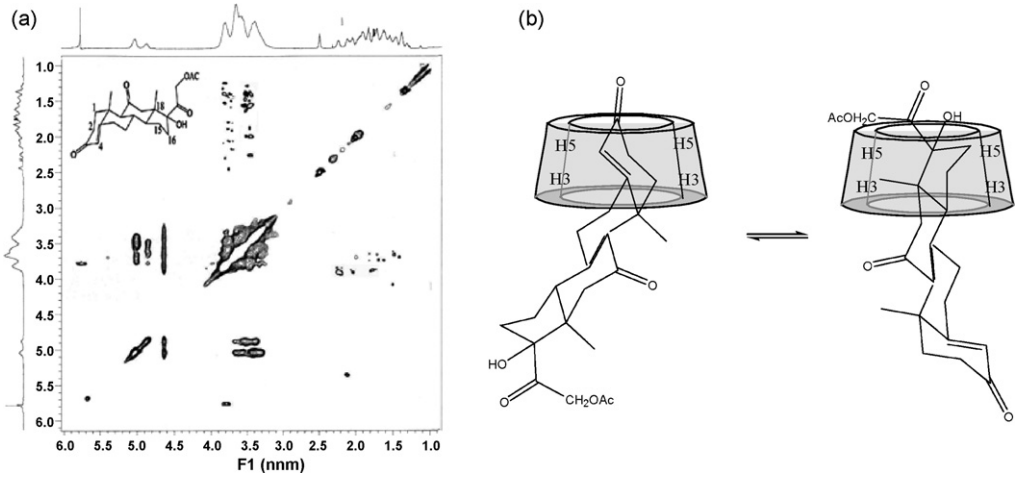

Fig. 4. (a) ROESY spectrum of the inclusion of β-CD-CA in D_2O at 25 °C with a mixing time of 400 nm. (b) Possible structure of the inclusion complex of β-CD-CA based on the ^1H ROESY NMR experiment.

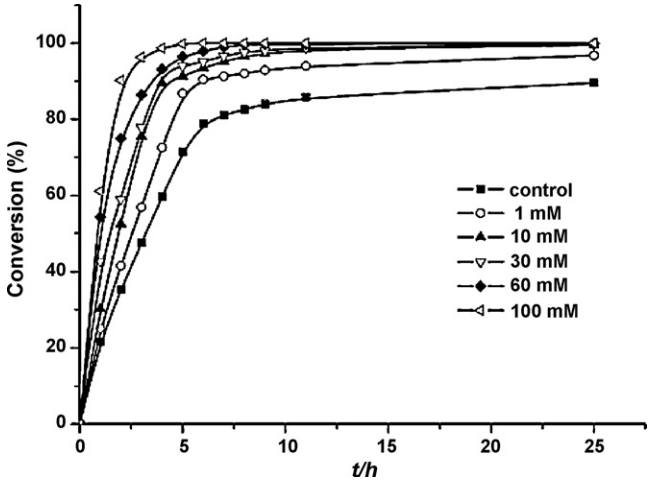

Fig. 5. Time courses of CA (10 g L^{-1}) conversion to PA by *Arthrobacter simplex* cells in homogenous of RM-β-CD at various concentrations.

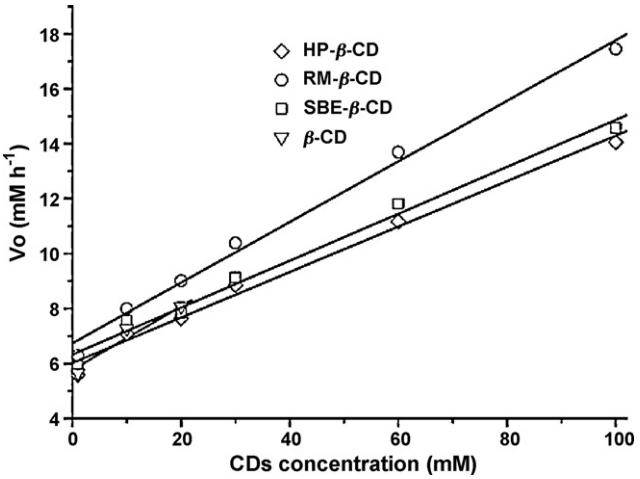

Fig. 7. Initial velocity of CA (10 g L^{-1}) at different concentrations of the various CDs.

3.6. Effect of cyclodextrins on CA biotransformation process

The performance of β-CD and its derivatives was tested in an identical biotransformation system consisting of resting *Arthrobacter simplex* cells in an aqueous dispersion of CA (10 g L^{-1}) at various concentrations of CDs ranging from 1 to 100 mM.

The profiles of the bioconversion of CA of 10 g L^{-1} in 0, 1, 10, 30, 60 and 100 mM RM-β-CD solutions were shown in Fig. 5. The increasing concentration of RM-β-CD from 1 to 100 mM significantly enhanced the bioconversion rates and degree of conversion. RM-β-CD at 100 mM led to a complete biotransformation within 3 h, and 56.9%, 74.5%, 81.6% and 86.4% at concentration of 1, 10, 30 and 60 mM, respectively. While in the absence of RM-β-CD 47.5% within 3 h.

Close examination of the effect of SBE-β-CD, HP-β-CD, β-CD on CA biotransformation process was investigated. As shown in Fig. 6, all CDs exerted an enhancement of the biotransformation. In contrast to 47.5% of control, the addition of CDs lead to an 81.6% conversion within 3 h (30 mM) at a CA concentration of 10 g L^{-1} in the presence of RM-β-CD, while 79.6%, 75.4%, 65.6% degree of conversion of CA in the presence of SBE-β-CD, HP-β-CD at 30 mM and β-CD at 20 mM, respectively. However, β-CD showed different performance on the conversion process. It played positive role at the beginning of conversion but 3 h later β-CD played negative role and resulted in a low degree of conversion. At last, degree of conversion of CA was 99.6%, 99.5%, 99.3%, 79.3% in the presence of RM-β-CD, SBE-β-CD, HP-β-CD at 30 mM and β-CD at 20 mM, respectively, and 89.5% in the absence of CDs. This may be explained by the formation of 2:1 β-CD-CA water-insoluble complex. This insoluble β-CD-CA complex is too strong to become available to the biocatalyst, thus leading to a decline in the bioconversion.

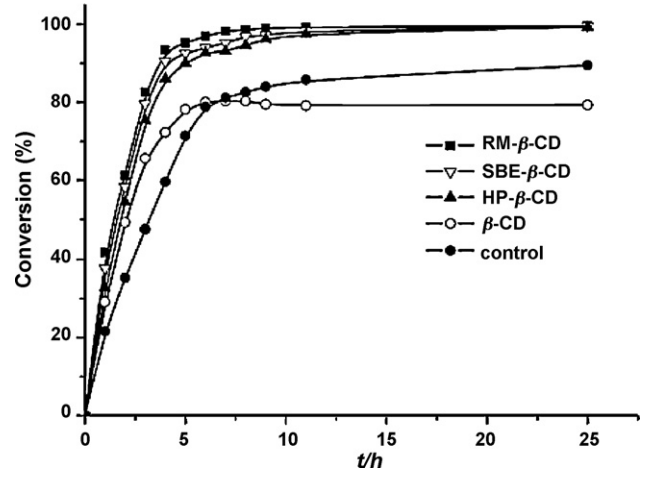

Fig. 6. Time course of CA (10 g L^{-1}) conversion to PA by *Arthrobacter simplex* cells at the medium of different CDs.

In order to illustrate the effect of K_S on the conversion, we tested the V_0 of CA (10 g L^{-1}) bioconversion at different CDs and different concentrations during the initial velocity scale (within 0.5 h). Results were shown in Fig. 7, V_0 is 4.89 mM h^{-1} in the absence of CD, while V_0 increased simply linearly by time with increasing concentration of CDs in the presence of CDs. The highest V_0 was observed in the RM-β-CD, while HP-β-CD was only slightly inferior than either RM-β-CD or SBE-β-CD. Although β-CD played a bad performance in the last conversion phase, β-CD was considerably advantageous with respect to the control at the beginning. During the initial velocity scale β-CD even show better effect than HP-β-CD, but inferior than RM-β-CD in less than 30 mM concentration. These studies indicated that the similar K_S may not induce the prominent influence on the bioconversion, however, the relatively higher K_S of 2:1 β-CD-CA complex would restrict the bioconversion of CA, which resulted in the decline of bioconversion at high concentration. Therefore, this study together with phase-solubility studies implied that CA solubility enhancement resulting from the formation of inclusion complexes played the main role of biotransformation in aqueous solution.

It is well known that hydrophilic CDs cannot penetrate biological membranes [26], so the biotransformation of water-insoluble CA was determined by the CA concentration in aqueous solution, which was attributed to the solution and dissociation rate of CA. It could be expressed by the following equation:

$$CA_S \overset{A}{\rightleftharpoons} CA_{aq} \overset{B}{\longrightarrow} CA_{mem} \rightarrow \text{Product}$$

According to the equation, the hydrophilic CDs acted as true carriers by keeping hydrophobic CA molecules in solution and delivering them to the lipophilic membrane surface where CA partition from the CD cavity into the lipophilic membrane [27,28]. As discussed above, RM-β-CD, SBE-β-CD and HP-β-CD showing good solubilizers and complexants, resulted in relatively higher conversion, while β-CD exhibited better complexant but very poor solubilizer, led to the relatively lower conversion. Therefore, the enhancement of the bioavailability of CA was primarily due to their strong solubilization power.

Furthermore, the complexation and solubilization of CDs for CA in aqueous medium may be interpreted by the host–guest interactions. Many investigations have demonstrated that a good match of the size and shape of the host to those of the guest should greatly favor host–guest inclusion complexation. RM-β-CD, SBE-β-CD and HP-β-CD, leading to a more hydrophobic interior and increasing the flexibility of the modified β-CD by breaking some of the intramolecular hydrogen bonds, could greatly change the original conformation to suit the inclusion of CA, resulting in a significant enhancement of the solubility of CA. While β-CD, having fairly rigid cavity, are not preorganized or flexible enough to allow allosteric conformational changes. At the same time, β-CD possessing numerous hydroxyl groups, favor the formation of dimer by the hydrogen bonding through direct association of the hydroxyl groups of β-CD or through intervening water molecules, thus providing some support to the formation of β-CD-CA 2:1 inclusion complex.

4. Conclusions

In summary, we have studied the influence of CDs on the Δ^1-dehydrogenation of CA to PA by *Arthrobacter simplex* cells. Significantly, the introduction of CDs into the reaction can lead to correspondingly higher bioconversion rates. The results obtained indicated that RM-β-CD, SBE-β-CD and HP-β-CD, showing the better good solubilizers, could result in relatively higher conversion, while β-CD exhibiting the poor solubilizers, could lead to the relatively lower conversion. Therefore, the solubilization of CDs for CA in aqueous medium plays the key role in the biotransformation process. These results provide a convenient and powerful method for solving many problems associated with the process of drug delivery and biotransformation of different novel steroids in aqueous solution.

Acknowledgements

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