Appl Microbiol Biotechnol (1991) 35:731-737
017575989100212A

Applied and Microbiology Biotechnology

Microbial transformations in a cyclodextrin medium. Part 2. Reduction of androstenedione to testosterone by *Saccharomyces cerevisiae*

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Received 26 February 1991/Accepted 24 May 1991

Summary. An intensive and systematic investigation of the reductive transformation of androstenedione (AD) to testosterone by *Saccharomyces cerevisiae* was undertaken in the presence of natural and chemically modified cyclodextrins (CD). The bioconversion was significantly larger in the presence of β- and γ-CD and hydroxypropyl-β-CD but only slight in the presence of α-CD and dimethyl- and trimethyl-β-CD. The performance of the various cyclodextrin media was interpreted in the light of the measured phase solubility diagrams of AD. Further investigation focused on biotransformation of the β-CD-androstenedione complex, the formation of which was studied by differential scanning calorimetry and X-ray powder diffractometry and stoichiometry determined by ^{1}H-nuclear magnetic resonance. A mechanism whereby CDs reduce the effective inhibitory concentrations of substrate and product as well as facilitate transport of the complexed substrate through the yeast cell wall has been suggested for the CD-promoted biotransformation.

Introduction

Cyclodextrins (CDs) combine an ability to form inclusion compounds with a biocompatibility with microbes (Bar 1989a). This useful combination prompted their use in microbial transformations of water-insoluble organics (Uvardy et al. 1983; Hesselink et al. 1989) and toxic or inhibitory substrates (Bar 1989a).

In the first part (Bar 1989b), the biotransformation of benzaldehyde and vanillin by *Saccharomyces cerevisiae* was investigated in a medium of α- and β-CDs, which were shown to significantly alleviate the inhibitory effects of the water-soluble substrates. We now extended our studies to include a water-insoluble substrate, a steroid. Thus, the present study was aimed at comprehensively investigating the yeast-mediated reduction of androst-4-ene-3,17-dione (AD) to testosterone in aqueous media of natural and chemically modified CDs. The reductive biotransformations of organic compounds by yeast cells has been recently reviewed by Ward and Young (1990) and the specific 17β-reduction of steroids by yeast has also been a subject of several investigations (Koshcheyenko and Sukhodolskaya 1985; Skryabin and Koshcheyenko 1987). In this study, we focused primarily upon the effect of CDs on the microbial conversion while equally investigating the physico-chemical interaction between CDs and the substrate and product in the bioconversion medium.

Materials and methods

Materials. All steroids were purchased from Sigma (St. Louis, Mo., USA), β-CD (Kleptose) was a generous gift from Roquette Frères (Lille, France). The purity, the water content and the source of all CDs used in this study are summarized in Table 1.

Microorganism. A strain of *S. cerevisiae* was employed for fermentations and bioconversion studies and was stored as a lyophilized biomass.

Table 1. Specifications of natural and chemically modified cyclodextrins (CDs) used in this study

Cyclodextrin	Abbreviated name	Water content (%)	Purity of dry matter (%)	Source
α-CD	Alpha	8.6	98.7	Chinoin
β-CD	Beta	9.6	100.0	Roquette Frères
γ-CD	Gamma	8.9	100.0	Chinoin
Heptakis(2,6-di-*O*-methyl)-β-CD	Dimeb	1.5	100.0	Chinoin
Heptakis(2,3,6-tri-*O*-methyl)-β-CD	Trimeb	0.9	[a]	Chinoin
Hydroxypropyl-β-CD[b]	Hyprob	2.2	98.75	Chinoin

[a] Trimeb had a melting point range of 157-159° C
[b] The assay for hydroxypropyl groups in Hyprob is 19.7%, and its average molecular weight is estimated to be 1417

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Fermentative activity. A sterile 250-ml round-bottomed flask, equipped with a magnetic bar, was loaded with 50 ml fermentation medium and a known amount of CD and was placed in thermostated water bath at 30° C. The medium (pH 6.6) consisted of 9.05 g/l of KH_2PO_4, 6.33 g/l of Na_2HPO_4, 1.5 g/l of Bacto yeast-nitrogen base (Difco, Detroit, Mich., USA) and a varying concentration of glucose. The flask was then connected to a gas burette and the volume of gas evolved during the fermentation was measured as previously described (Bar 1989b).

Microbial transformation of AD. A volume of 19.6 ml sterile buffered glucose medium with an accurately weighed amount of CD was loaded into 100-ml erlenmeyer flasks. The buffered glucose medium (pH 6.6) consisted of 9.23 g/l of KH_2PO_4, 9.52 g/l of Na_2HPO_4 and a varying amount of glucose. The flask content was warmed to ca. 60° C and a solution of the steroid in 0.2 ml dimethylformamide (DMF) was added dropwise under vigorous manual swirling. In cases of high steroid loading, the DMF solution was pre-heated prior to addition. In either case, heavier and wettable white precipitates formed upon cooling to room temperature. The slurry of CD-steroid complexes was supplemented with 0.6 ml of concentrated yeast-nitrogen-base solution (75 g/l) and the bioconversion-fermentation was initiated by inoculating with 0.1 g dry weight (DW) of yeast. The shake flasks were incubated at 30° C in a New Brunswick (Edison, NJ, USA) Controlled Environment shaker at 150 rpm. Since agitation of a microbial slurry tends to build up a mass of solids adhering to the flask walls, care was taken to redisperse it twice daily. Furthermore, samples withdrawn from a suspension of solids agitated in a rotary shaker may be poorly representative of the whole flask content and consequently unreliable. Therefore in kinetics experiments, a series of flasks was incubated under identical conditions and the whole content of each flask was subjected at a time to product analysis.

Product analysis. The steroid product and substrate in the contents of each flask were extracted with 30 ml ethyl acetate following addition of excess NaCl. The extraction was facilitated by warming the mixture to ca. 50° C in a water bath. After centrifugation at 4100 *g* for 5 min, the organic phase was separated and evaporated at 40° C in a rotary evaporator. A volume of 10 ml chloroform was added to the remaining solids in order to dissolve the steroids and subsequently dried with anhydrous $MgSO_4$. Aliquots of this chloroform solution were mixed with a methanol solution of hydrocortisone as an internal standard and subsequently analysed by HPLC in a Varian (Palo Alto, USA) 8500 liquid chromatograph connected to a UV spectrophotometer Uvidec 100 III (Japan Spectroscopic, Japan) operating at 254 nm. Analysis was performed on a Lichrospher Si 60 column (Merck, Darmstadt, FRG) with an eluent consisting of 94.5% chloroform, 5.0% methanol and 0.5% glacial acetic acid, flowing at 50 ml/h.

Residual glucose in the fermentation medium was assayed by the dinitrosalicylic acid method. Ethanol was determined in a Tracor (Austin, Texas, USA) 540 gas chromatograph with a flame ionisation detector, over a Porapak Q (Supelco, Penn., USA) column, using *n*-propanol as an external standard.

Solubility measurements of AD in cyclodextrin solutions. The isotherms were measured and constructed according to the method of Higuchi and Connors (1965). An excess amount of finely ground AD was added to 20 ml of an aqueous CD at a varying concentration in 50 ml erlenmeyer flasks. The flasks were tightly sealed (to avoid evaporation), and incubated at 30° C in a New Brunswick Scientific Controlled Environment shaker at 150 rpm for 10 days. Twice a day, any solids found adhering to the flask wall were manually re-dispersed. After 10 days, the agitation was interrupted and the flasks were left overnight in the incubator for settling. Aliquots of the supernatant liquid were removed with a pre-warmed pipette and filtered through a 0.2-μm filter paper (Schleicher & Schuell BA-83, Dassel, FRG). The filtrates were diluted with aqueous ethanol (50% v/v) and analysed spectrophotometrically at 235 nm.

Analysis of the β-CD-AD complex. The precipitates obtained from a selected number of the solubility experiments were analysed by ^{1}H-nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and X-ray powder diffractometry.

For ^{1}H-NMR, air-dried precipitates were dissolved in deuterated dimethylsulphoxide (d_6-DMSO) and the spectrum was recorded with a Varian VXR-300S FT-NM spectrometer after 50 scans with a pulse delay of 5 s.

For DSC the thermograms of the above precipitates, water-crystallized β-CD, AD and their mixture were recorded on a Mettler (Zurich, Switzerland) TA400 system at a heating rate of 5° C/min from 30 to 400° C.

For X-ray powder diffractometry, air-dried precipitates were crushed and pulverized and their spectrum was recorded with a Philips (Eindhoven, Netherlands) diffractometer with a monochromated (CuK) radiation. The range of diffraction angles scanned was $2\theta = 5$–$45°$.

Measurement of dissolution rate of free and complexed AD. Cyclodextrin-AD complexes were first prepared by two methods: the DMF method at 60° C as for the microbial transformation and the 10-day agitation method at 30° C as for the solubility measurements. The precipitates were filtered and washed with water and subsequently suspended in 20 ml water or bioconversion medium at a total equivalent concentration of 2 g/l of AD. The 100-ml erlenmeyer flasks were incubated under conditions identical to those employed for the bioconversion (30° C and 150 rpm). Other flasks containing only 2 g/l of AD in either water or bioconversion medium served as controls. Intermittently, a 0.5-ml aliquot of the slurry was withdrawn and filtered through a 0.2-μm filter. The filtrate was diluted with water and its optical absorbance was measured at 235 nm. The absorbance values were expressed as a percentage of the highest ultimate absorbance measured.

Results

The reduction of AD was performed by yeast cells growing in a fermentation broth. Under these conditions, some degree of experimental variability in bioconversion efficiency was observed in the fermentation systems. Consequently, great care was taken throughout this study to employ identical conditions with the same yeast inoculum in each set of comparative experiments.

Effect of chemically modified CDs on yeast fermentative activity

The methylated heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (Dimeb) and heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (Trimeb) as well as the hydroxypropylated hydroxypropyl-β-cyclodextrin (Hyprob), were found to be non-toxic to the fermenting yeast cells, as evidenced by the virtually identical rate and amount of CO_2 evolution during anaerobic fermentation of glucose (20 g/l). The non-toxicity of the chemically modified CDs is worth noticing since these CDs, unlike the natural ones, possess greater surface activity which could be deleterious to the cell wall and membrane (Szejtli 1988).

Table 2. Effect of cyclodextrin type on the conversion (%) of androstenedione (AD) (2 g/l: molar CD:AD ratio 3:2) by *Saccharomyces cerevisiae* [5 g dry weight (DW)/l] after 120 h at 30° C

Cyclodextrin	Control	α-CD	β-CD	γ-CD	Dimeb	Trimeb	Hybrob
Conversion	27	39	85	90	36	37	78

Table 3. Effect of β-CD-AD molar ratio on the bioconversion (%) of an initially constant concentration of AD (2 g/l) by *S. cerevisiae* (5 g DW/l) at 30° C after 96 h

β-CD:AD	0	0.5	1.0	1.5	2.0	3.0
Conversion	26	38	45	58	59	38

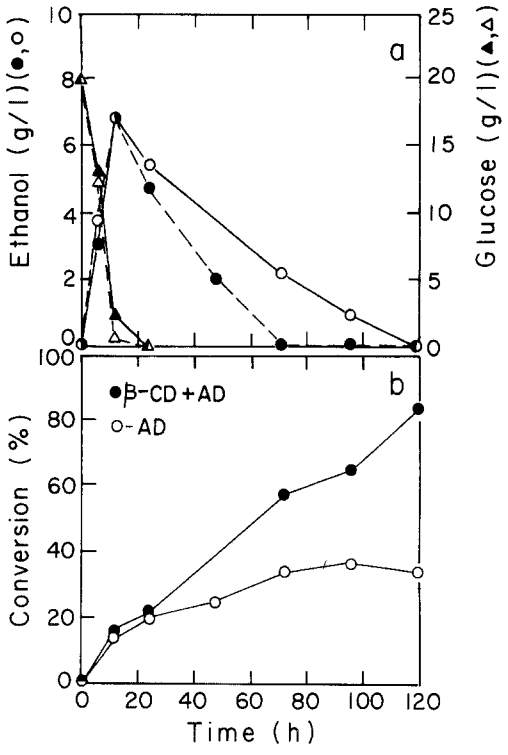

Fig. 1. Time course of simultaneous fermentation (**a**) and bioconversion (**b**) of free (*open symbols*) and complexed androstenedione (AD) (2 g/l) with β-cyclodextrin (β-CD) (*filled symbols*, molar CD:AD ratio 3:2) by *Saccharomyces cerevisiae* [5 g dry weight (DW)/l] at 30° C

Effect of CD type on the bioconversion

Table 2 compares the bioconversion degrees of free and CD complexed AD after a fermentation time of 120 h. In contrast to the lowest conversion of 27% of the free steroid, considerably higher conversion degrees of 85, 90 and 78% were obtained with AD complexed with β-CD, γ-CD and Hyprob respectively, α-CD and the methylated CDs introduced little advantage over the control. Since γ-CD and Hyprob are more expensive than β-CD, the latter was chosen for further investigation.

The formation of complexes between CDs and water-insoluble steroids may take up to a few days for completion. To speed up the process, a concentrated steroid solution in a water-miscible solvent, DMF, was added dropwise to a warm (ca. 60° C) aqueous solution of CD and biotransformation was initiated by adding the biocatalyst. Obviously, the amount of the CD-complexed steroids formed is dependent upon the molar ratio between the two components. For the experiments described in Table 2, the molar ratio CD:AD was 3:2, a ratio reasonable assumed to be identical with the reported ratio (Uekama et al. 1982) of 3:2 for β-CD:testosterone (the latter is the bioconversion product). However, various other ratios were subsequently investigated.

Effect of the β-CD:AD ratio on bioconversion

The conversion of a constant concentration of AD (2 g/l) previously complexed with an increasing proportion of β-CD is shown in Table 3. Increasingly higher conversions were obtained up to a molar ratio of 1.5. At much higher CD concentrations, the bioconversion was reduced.

Time course of the fermentation-bioconversion

Since the bioconversion was performed by fermenting cells, the progress of steroid transformation (Fig. 1b) was monitored together with glucose consumption and ethanol production (Fig. 1a) in the presence and absence of β-CD. All sugar (20 g/l) was practically consumed in the first 12 h but only a small fraction (20%) of the steroid substrate was biotransformed. Further substrate was converted into product as ethanol was oxidized in the shake flasks open to the air. After 120 h, 83% of the CD-complexed AD was converted into testosterone in contrast to 30% of the converted free steroid.

Effect of sugar content on the bioconversion

Increasing glucose concentration not only led to richer yeast growth but also to higher bioconversion of AD. Initial glucose concentrations of 2, 10, 20 and 50 g/l, fermented by an identical inoculum (5 g DW/l), led to conversion degrees of 27, 45, 57 and 87% respectively, after 96 h. The yeast growth could not be monitored by measuring the optical density because of the presence of the CD-steroid crystals. Interestingly, some bioconversion (10%) was also observed by the initial yeast inoculum of 5 g DW/l in the absence of added sugar.

Solubility diagrams of β-CD-AD

Solubility diagrams of a compound in CD solutions are generally generated by measuring the total dissolved

compound concentrations in a series of CD solutions equilibrated with an equal excess amount of the guest compound (G). The total concentration of the dissolved compound is then plotted as a function of the initial cyclodextrin concentration and the phase-solubility diagrams thus obtained are generally classified (Higuchi and Connors 1965) as either type A, whereby a soluble inclusion compound is formed, or type B, whereby an inclusion compound with a definite solubility is formed. With type A, the concentration of the dissolved compound increases linearly with the initial CD concentration and the interaction between the free and the included compound in the solution is given (Szejtli 1988) by:

$$m\mathrm{CD}_{aq} + n\mathrm{G}_{aq} \overset{K_s}{\rightleftharpoons} [\mathrm{CD\text{-}G}]_{aq} \quad (1)$$

where K_s is the stability constant of the complex and m and n are stoichiometry coefficients. When $m=n=1$, the K_s of the 1:1 inclusion compound can be determined from the phase solubility diagram (Szejtli 1988) by:

$$K_s = \frac{\text{slope}}{S_0(1\text{-slope})} \quad (2)$$

where S_0 is the saturation G concentration with no CD and the slope represents the change of dissolved G per change of CD concentration. With type B, an additional equilibrium between the dissolved and the precipitated [CD-G]s complex is superimposed on Eq. 1.

The steroid product, testosterone, has previously been the subject of solubility studies and its phase diagram (Lach and Pauli 1966) as well as its stability constants (Uekama et al. 1982) with α-, β- and γ-CDs have been determined. Consequently, the present study has concentrated mainly on the substrate, AD. The solubility diagrams of AD with the natural and chemically modified CDs are shown in Fig. 2. It can be seen that the AD solubility (S_0) in water (0.18 mM at 30° C) can be substantially increased in the presence of CDs, especially the chemically modified CDs (Fig. 2b). The solubility enhancement is represented by the slope factor (Table 4) and was calculated from the initial part of the straight line in the diagrams. The β- and γ-CDs, which yielded B type curves, have maximum complex solubilities (S_{max}) of 0.42 and 2.88 mM, respectively. The stability constants as well as other solubility parameters of the various complexes are listed in Table 4. It can be seen that the slope and K_s values of Dimeb are larger than those of Trimeb and this testifies to the greater inclusion ability of Dimeb, similar to the observation made by Imai et al. (1984).

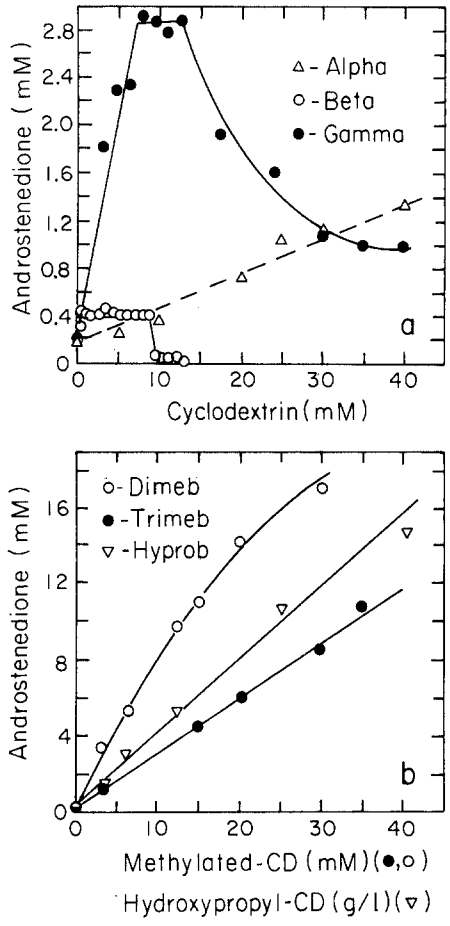

Fig. 2. Phase-solubility diagrams of androstenedione in solutions of natural cyclodextrins (CD) (**a**), methylated β-CDs and hydroxypropyl-β-CD (**b**) in water at 30° C. See Table 1 for full names of the CDs

Table 4. Solubility parameters of androstenedione in aqueous solutions of natural and chemically modified cyclodextrins at 30° C

CD	Slope (mol/mol)	K_s (M^{-1})	Type	S_{max} (mM)
α-CD	0.029	160	A	—
β-CD	0.69	12360	B	0.42
γ-CD	0.72	14280	B	2.88
Dimeb	0.74	15810	A	—
Trimeb	0.28	2160	A	—
Hyprob	0.39	3500	A	—

K_s, stability constant of the complex; S_{max}, maximum solubility of the complex

Characterisation of the β-CD-AD complex

Since the CD of choice for the biotransformation was β-CD, the β-CD-AD complex was investigated in further detail. The composition of a CD complex, obeying the Type B phase solubility diagram, can in principle be determined from the plateau region in the diagram (Szejtli 1988). However, this method, which involves low values of the substrate and complex concentrations, is prone to inaccuracies. For instance, Lach and Pauli (1966) used this method to determine a molar ratio of β-CD:testosterone of 2.03:1 in contrast to another reported ratio of 3:2 (Hirayama and Uekama 1987) calculated by the same method. Consequently, we chose to determine directly the stoichiometry of the CD-AD complexes from the recorded ^{1}H-NMR spectrum. The molar ratio of CD-AD (Fig. 3) was calculated

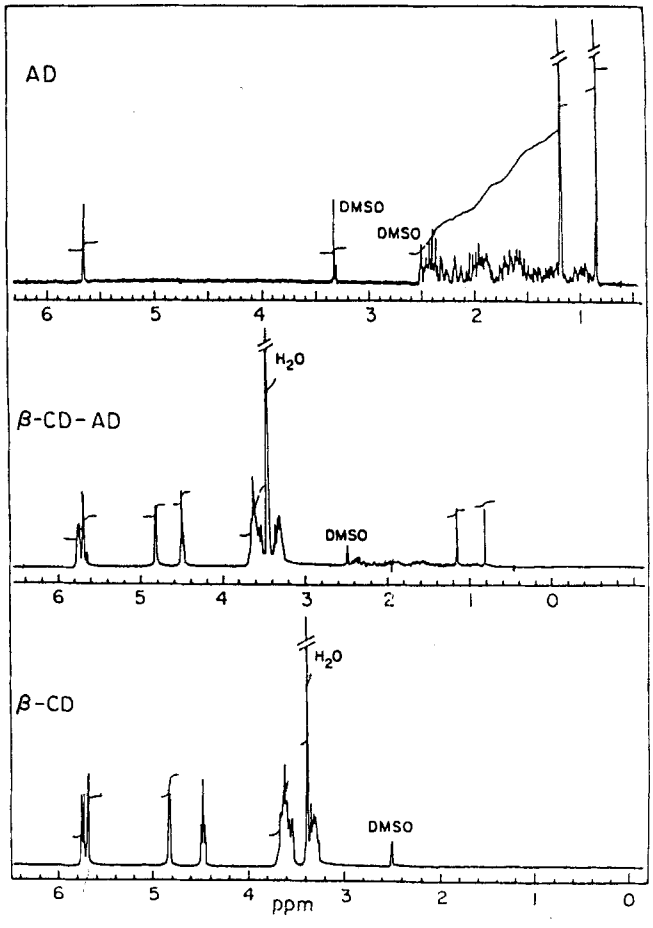

Fig. 3. ^{1}H-Nuclear magnetic resonance spectra of AD (*upper*), β-CD (*lower*) and of their complex (β-CD-AD) (middle), all dissolved in deuterated dimethylsulphoxide (DMSO)

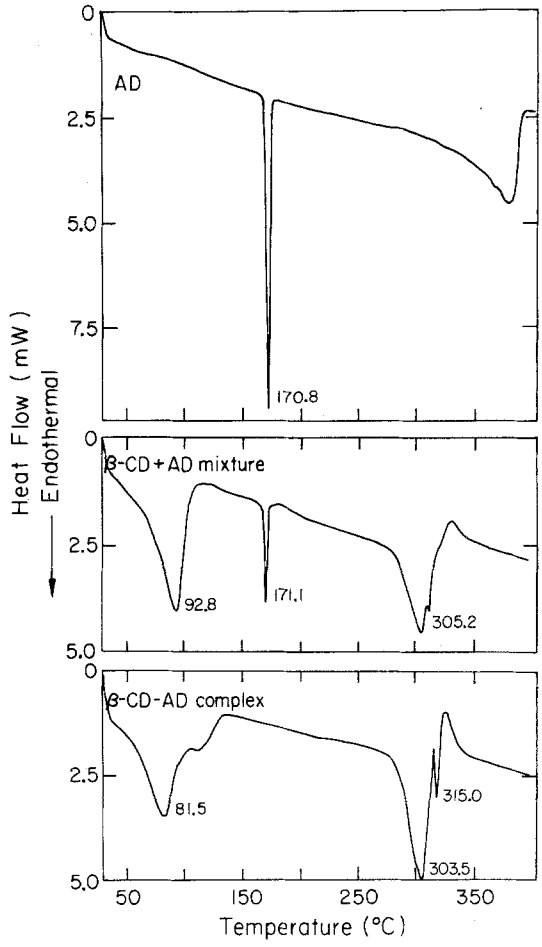

Fig. 4. Thermograms, by differential scanning calorimetry, of AD, a mechanical mixture of β-CD and AD and of the β-CD-AD complex with a heating rate of 5° C/min

from integration signals to be 1.60, which practically implies a ratio of 3:2. To ascertain the unique physicochemical structure of the complex, DSC and powder X-ray diffraction comparative analysis of β-CD and AD recrystallized from water and of their complex was performed.

Figure 4 shows that the endothermal melting peak (170.8° C) is present in the DSC thermogram of the mechanical mixture β-CD and AD crystals but absent in that of β-CD-AD complex. Furthermore, the diffractogram of the complex (Fig. 5) contains distinctly new diffractions, absent in diffractograms of the separate AD and β-CD. Thus, both DSC and X-ray diffractometry testify unequivocally to the formation of a new chemical species, the β-CD-AD complex.

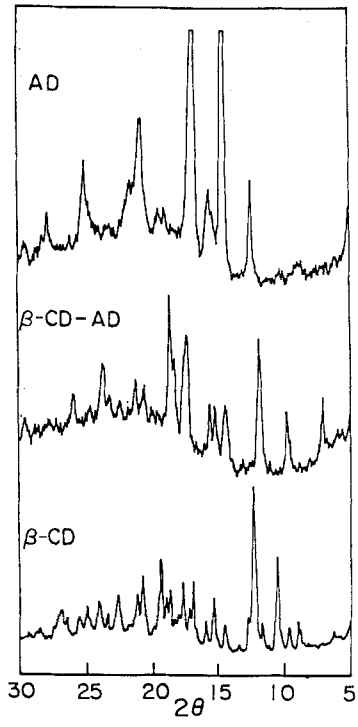

Fig. 5. X-Ray powder diffractograms of AD, β-CD and the β-CD-AD complex (molar CD:AD ratio 3:2)

Dissolution rates of free and complexed AD

Bioconversion of a steroid with a very poor aqueous solubility of 0.18 mM inevitably raises the question of its availability as a soluble substrate to the biocatalyst. Figure 6 shows the dissolution rates in water of AD. The β-CD-AD complex prepared rapidly at 60° C, as in the procedure of the microbial transformation, and of the β-CD-AD complex formed slowly during 10 days of

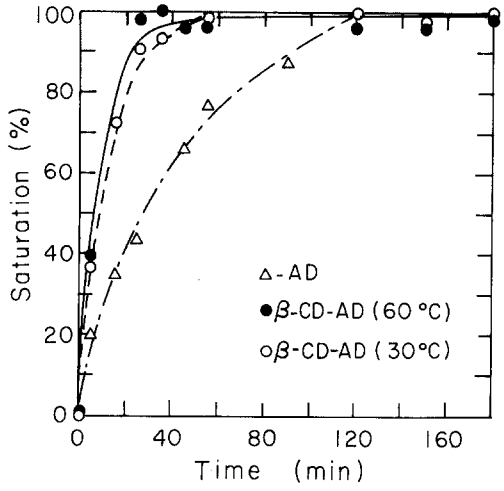

Fig. 6. Dissolution in water of free AD, and β-CD-AD complexes prepared rapidly at 60° C or slowly at 30° C in shake flasks rotated at 150 rpm at 30° C

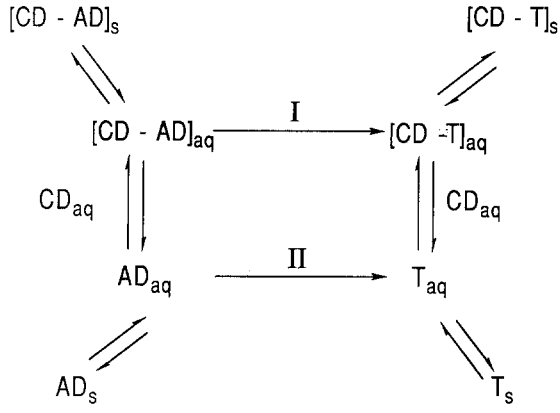

Fig. 7. Scheme showing various interactions between the free and complexed, dissolved and solid species of the reactant and steroid product: *T*, testosterone

agitation of the two components (see Materials and methods). Since the kinetics of dissolution are dependent on the stirring conditions, these experiments were performed under conditions identical to those of the bioconversion. Free AD saturated water after ca. 120 min while complexed AD from the two preparations reached saturation after only ca. 40 min. Similar dissolution profiles were observed in a bioconversion medium with no yeast cells. It is thus seen that CD complexation of the steroid not only enhanced its aqueous solubility but also accelerated its dissolution rate.

Discussion

Addition of CDs to fermentation-bioconversion medium introduces a physico-chemical interaction with steroids. The substrate initially present in the medium as solid complexes of [CD-AD]s, soon dissolves in the aqueous medium as $[CD\text{-}AD]_{aq}$. The latter partly dissociates to yield free dissolved AD_{aq}, which in turn could precipitate to solid ADs. Analogous equilibria certainly hold for the testosterone product, *T*. The array of the various interactions between the free and complexed, dissolved and solid species of the reactant and steroid product is shown in Fig. 7.

Obviously, for a substrate to be biotransformed, it must first be taken up by the microbe. Usually, a substrate can be taken up as a dissolved species, or as a substrate solubilized by a compound secreted by the microbe or through microbial adhesion to an insoluble substrate (Goswami et al. 1983). A microscopic examination of the bioconversion system revealed no adhesion of yeast cells on solid particle. Furthermore, *S. cerevisiae* is not known to excrete hydrophobic compounds capable of solubilizing AD. Thus, uptake of dissolved molecules of AD would seem quite reasonable. Figure 7 suggests that both dissolved free AD_{aq} (pathway II) and complexed $[CD\text{-}AD]_{aq}$ (pathway I) could be substrates for the biotransformation. The free AD species is certainly the sole substrate in bioconversion systems with no CDs. Since AD is sparingly soluble in water, it was judged important to determine whether its biotransformation was controlled by its dissolution rate.

Figure 1 clearly reveals that the bioconversion progressed slowly during the first 80 h. However, Fig. 6 shows that AD saturated the aqueous medium after only 2 h. A similar enhancement of bioavailability by CDs has been reported for other compounds (Szejtli 1988). The initial substrate uptake rate, determined from the bioconversion curve of the control experiment (Fig. 1), was 30 mg/l per hour. This rate is much lower than the initial dissolution rate measured from Fig. 6 of 4.8 g/l per hour, thus implying no control by mass transfer from the solid to the aqueous phase. This same situation prevailed in the bioconversion of β-CD-AD complex: a high dissolution rate of the complexed AD (9.1 g/l per hour) in contrast to its low uptake rate (36 mg/l per hour).

Thus, it appears that the overall process was controlled by intrinsic bioconversion kinetics. These in turn could reasonably be influenced inter alia by a substrate transfer into the microbial cell or perhaps by a possible inhibitory effect of the free substrate or product on the enzymatic conversion or microbial metabolism. A higher transfer substrate rate to the cells, as a result of the better distribution of the steroid in the CD solution cannot however explain the discrepancies between the low conversion degrees obtained in the presence of α-CD, Dimeb or Trimeb (Table 2) and their solubilizing power (Table 4). However, complexation of either steroid would certainly reduce the concentration of the free dissolved species, thus alleviating the inhibitory effect. Some supporting evidence for such an inhibitory effect can be seen in Fig. 1 where ethanol consumption in the absence of β-CD was slower and the bioconversion practically ceased after 70 h. Furthermore, among the natural CDs, the significantly high bioconversion degrees obtained with β- and γ-CDs (Table 2), are compatible with the very high corresponding stability constants of 1.24×10^4 and 1.43×10^4 of complexed androstenedione.

The very mild enhancement effect of α-CD (Table 2) may be attributed to the much lower K_s of 160 (Table 4). A similar compatibility may be suggested between the bioconversion degrees and the stability constants of testosterone complexed with α- ($K_s = 134$), β- ($K_s = 7540$) and γ- ($K_s = 1.65 \times 10^4$) CD measured by Uekama et al. (1982). It is important to recall that all these K_s values, which are calculated on the assumption of a molar CD:steroid ratio of 1:1, reflect only roughly the complexing ability of CD since the actual stoichiometry of the complexes could well be different as the experimentally determined ratio is 3:2 for CD:AD. Interestingly, this stoichiometry was found to coincide with the optimal biconversion observed upon investigating the effect of β-CD:AD ratio (Table 3). An exaggerated loading of CD as in the experiment with a β-CD:AD ratio of 3:1 (Table 3), apparently led to a substantial decrease in the free substrate concentration (the equilibrium in Eq. 1 was shifted to the right) and consequently to a decline in the bioconversion.

While the bioconversion stimulation by natural CDs can be simply explained by a reduced effective concentration of an inhibitory free substrate according to pathway II (Figure 7), it is more difficult to interpret the effect of the chemically modified CDs. Among the latter CDs, only Hyprob significantly enhanced the bioconversion, while the methylated CDs, Trimeb and Dimeb, exerted only slight stimulation (Table 2) despite their considerable complexing abilities (Table 4). In general, Hyprob is known to be the best biocompatible CD among the three modified CDs and for this reason it has found applications as a harmless injectable drug carrier (Szejtli 1988). Yet, both Dimeb and Trimeb were also biocompatible with yeast since they exerted no deleterious effect on its fermentative activity. One may therefore suggest that $[CD\text{-}AD]_{aq}$ could also interact with the yeast cells and provide a substrate for bioconversion (pathway I in Figure 7).

The species $[CD\text{-}AD]_{aq}$ would probably have first to penetrate the cell wall before AD further proceeds to the catalytic site. Yeast cell wall is composed primarily of polysaccharides (Reed 1982) and these would be capable of interacting with the cyclic oligosaccharides, CDs, via the numerous hydroxyl groups. Thus, hydrogen bonding between the yeast cell wall and the steroid-including CDs could enhance the affinity of these to the cells and facilitate their penetration through the cell wall. Unlike the hydroxyl-bearing natural CDs and hydroxypropyl-CD, the methylated Trimeb and Dimeb, which have a considerably reduced capacity for hydrogen bonding, could exhibit less affinity to the cells and consequently a retarded uptake by the cells. Thus, Trimeb and Dimeb, despite their substantially high complexing ability (Table 4), showed only a slight enhancement upon the biotransformation (Table 2). Furthermore, this mechanism whereby $[CD\text{-}AD]_{aq}$ interacts directly with the microbial cell, could well provide an additional advantage of a diminished cell inhibition by the encapsulated substrate.

The enzymatic conversion per se most probably occurs on the free AD inside the cell (Ward and Young 1990). Since the reductive biotransformation requires regeneration of a reduced nicotinamide cofactor, increasingly higher loadings of glucose yielded correspondingly higher conversions as a result of both the larger supply of reducing power and the larger yeast growth. The low conversion observed in the absence of added sugar could be reasonably attributed to initially present intracellular reserve material. Similarly, the cells in Fig. 1 kept converting AD despite the virtually complete consumption of glucose and ethanol. It is equally unlikely that the large molecule of cyclodextrin-steroid is taken up into the cell. Even though microorganisms can degrade CDs (Szejtli 1988). *S. cerevisiae* does not excrete cyclodextrinase (Bar 1989b) and therefore the final bioconversion medium contained a product complexed with CD.

Acknowledgement: The authors are grateful to Casali Foundation for supporting this research.

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