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FULL PAPER

Continuous Production of Ursodeoxycholic Acid using Two Cascade Reactors with Coimmobilized Enzymes

Ming-Min Zheng,[a] Fei-Fei Chen,[a] Hao Li,[a] Chun-Xiu Li*[a] and Jian-He Xu*[a,b]

Abstract: Ursodeoxycholic acid (UDCA) is an effective drug for the treatment of hepatitis. In this study, 7α-hydroxysteroid dehydrogenase (7α-HSDH) and lactate dehydrogenase (LDH), as well as 7β-HSDH and glucose dehydrogenase (GDH), were co-immobilized onto an epoxy-functionalized resin (ES-103) for catalyzing the synthesis of UDCA from chenodeoxycholic acid (CDCA). Through optimizing the immobilization pH, time and the loading ratio of enzymes to resin, the specific activities of immobilized LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 were 43.2 U g^{-1} and 25.8 U g^{-1}, respectively, which are 12- and 516-fold higher than that under the initial immobilization conditions. Continuous production of UDCA from CDCA was subsequently achieved by using immobilized LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 in two serial packed bed reactors. The yield of UDCA reached nearly 100% and lasted for at least 12 h in the packed bed reactors, which is superior to the batch-wise reaction. This efficient continuous approach developed in this study may provide a feasible route for large-scale biotransformation of CDCA into UDCA.

Introduction

In China, bear bile has a long history of use to prevent and cure diseases, and the major pharmacologically active ingredient in this bile is ursodeoxycholic acid (UDCA).[1] UDCA promotes the transformation and excretion of cholesterol. Thus, UDCA is used widely in the treatment of gallstones, biliary cirrhosis and even liver cancers.[2] Production of UDCA is generally achieved using chemical methods with the main disadvantages including poor selectivity, a large number of by-products and low yield.[3] Additionally, chemical synthesis may cause various environmental problems.[4] In our previous study, we adopted a two-step reaction strategy to implement successfully the biotechnological synthesis of UDCA from its epimer chenodeoxycholic acid (CDCA), which is remarkably more efficient and environmentally friendly.[5]

The initial cascade strategy includes four enzymes in a one-pot system: 7α–hydroxysteroid dehydrogenase (7α–$HSDH_{Ec}$ from *E. coli*) and lactate dehydrogenase (LDH) for the first reaction step, and 7β-$HSDH_{Rt\text{-}M1}$ with glucose dehydrogenase (GDH) for the second reaction step.[5,6] In the second reaction step, residual 7α-$HSDH_{Ec}$ from the first step could work together with GDH to convert the intermediate 7-oxo-lithocholic acid (7-oxo-LCA) back to CDCA. Thus, to circumvent the undesired reverse reaction in the second step, heat-inactivation is used between the first and second reaction steps. However, heat treatment takes time and effort, which is not conducive to industrial applications and by contrast, a sequential synthesis approach would be more ideal.[7] Therefore, we have considered the use of enzyme immobilization to spatially separate the catalysts of the two reaction steps in an effort to maintain continuous processing.

Immobilized enzymes are used commonly in industrial applications with many advantages, such as improved operation stability, feasible enzyme recycling, reduced cost and an increase in organic solvent tolerance.[8]

Traditional immobilization methods can be divided into three categories: entrapment, cross-linking, and covalent bonding with carrier.[8b] The entrapment method involves fixing an enzyme or a cell inside the network of a polymer. The commonly used carriers are organic polymers (e.g., calcium alginate, polyacrylamide gel), inorganic silica, hollow fibers, or microcapsules.[9] The entrapment method is simple, but enzyme leakage is likely to occur. In general it is necessary to further improve stability by cross-linking. Cross-linking involves covalent bonding between catalysts using a crosslinking agent with bifunctional or multifunctional groups. The stability of the immobilized enzyme prepared by this method is appreciable, but the reaction process is very intense and complex, which can influence the activity of the enzyme and lead to a low activity recovery.[10] Immobilization is also a widely used method to tether enzymes on a specific carrier by affinity adsorption, ionic force, or covalent binding. A variety of carriers have been developed for immobilization, including organic polymers, biopolymers, hydrogels and inorganic solids.[11] In addition to traditional immobilization methods, new materials or methods have also been developed and successfully applied for immobilizing biocatalysts in recent years. For example, a catalyst immobilized onto magnetic nanoparticles can be quickly and conveniently separated from the reaction system,[12] and an

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immobilized enzyme on a nanoflower structure was formed by chelation between an enzyme and transition metal ions.[13]

Bioreactors are used for biochemical reactions, and this equipment is a key factor for realizing highly efficient industrial bioconversions.[14] A packed bed reactor (PBR) is a commonly used continuous flow reactor with immobilized enzymes.[15] The mechanical damage to the enzyme in the PBR is relatively small, and the enzyme load per unit volume is high. Furthermore, the use of a continuous process avoids the physical loss of the enzyme during a batch reaction, which requires the recycling of the catalysts (e.g., via centrifugation or filtration).[16] However, a continuous process is only suitable for a substrate solution that does not contain any solid particles and also has a low viscosity.

Herein, we have attempted various immobilization methods for co-immobilizing 7α-$HSDH_{Ec}$ and LDH, as well as 7β-$HSDH_{Rt\text{-}M1}$ and GDH. By comparing the protein loading and activity recovery, we chose a resin for immobilizing enzymes and optimized the immobilization conditions. Finally, the coimmobilized enzymes were recruited in two packed bed reactors to achieve a two-step continuous reaction for efficient production of UDCA.

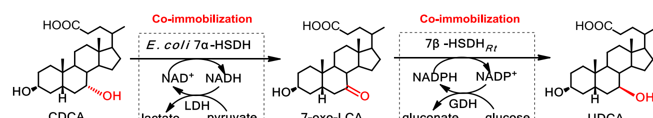

Figure 1. The scheme of cascade enzyme reactions for the synthesis of ursodeoxycholic acid from chenodeoxycholic acid.

Results

Immobilization of enzymes onto epoxy-resins

In order to achieve continuous synthesis in a packed bed reactor for production of UDCA, we first tried to immobilize the four desired enzymes for use in the cascade reactions (Fig. 1). Initially, a number of immobilization carriers or methods were tested, including gel entrapment, cross-linking with glutaraldehyde, metal-organic frameworks (MOFs)[17] and inorganic crystal nanoflowers; however, the activities of the resulting immobilized enzymes were very low (data not shown). Finally, we chose commercially available resins with active epoxy groups as a carrier to immobilize the enzymes. The epoxy groups of polymer resins can covalently bind with the amino groups of the enzymes. The enzyme mixtures, LDH and 7α-$HSDH_{Ec}$, and GDH and 7β-$HSDH_{Rt\text{-}M1}$, were co-immobilized onto six commercially available resins with different functional groups and compositions. The initial test of immobilizing LDH-7αHSDH and 7βHSDH-GDH with three primary amino resins displayed poor activity recovery (data not shown). Compared with the immobilized enzymes on other epoxy-functionalized resins, LDH-7αHSDH@ES-103 exhibited the highest activity (3.6 U g^{-1}), and 7βHSDH-GDH@ES-103 also exhibited comparable activity (0.05 U g^{-1}) in comparison with 7βHSDH-GDH@ES-101. In contrast, 7βHSDH-GDH@ES-1 displayed no detectable activity (Table 1). Nevertheless, the immobilization conditions required further optimization because of very low activity recovery.

Table 1. Activities and activity recoveries of LDH-7αHSDH and 7βHSDH-GDH coimmobilized on epoxy-functionalized resins.[a]

Entry	Resin carrier	Skeleton property	Particle size (μm)	LDH-7αHSDH		7βHSDH-GDH	
				Specific loading (U g^{-1})	Activity recovery (%)	Specific loading (U g^{-1})	Activity recovery (%
1	ES-1	hydrophilic	150~300	2.5	4.0	n.d.	n.d.
2	ES-101	hydrophobic	150~300	2.7	4.4	0.06	0.07
3	ES-103	hydrophobic	100~250	3.6	5.7	0.05	0.06

n.d.: not detected
[a] The immobilization was performed in 2 M potassium phosphate buffer (pH 7.8) at 20°C for 15 h with 10 and 50 $mg_{protein}$ g_{resin}^{-1} for LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103, respectively.

Optimization of immobilization conditions

Under different pH conditions, amino acid residues on the surface of proteins exhibit different charges, which may affect protein immobilization. Thus, enzymes were immobilized onto resins under different pH conditions. The results showed that immobilization under acidic conditions were clearly better than under alkaline and neutral pH conditions (Fig. S1). The immobilization pH showed a significant effect on the activity of the immobilized enzymes. As a result, pH 6.0 and 5.5 were chosen as the immobilization pH values for LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103, respectively.

The immobilization time was further optimized and the results are shown in Fig. S2. Increasing the incubation time caused a gradual increase in the activity of the immobilized enzymes until the incubation time reached 20 h. Finally, we optimized the loading ratio of enzyme to resin, which is also a critical parameter for immobilization (Fig. 2). The amount of protein bound to the resin increased with increasing protein concentration. Concurrently, the percent activity recovery decreased gradually, mainly because the amount of free protein increased gradually as the protein load concentration increased. Therefore, considering the activity and activity recovery, we chose 10 and 50 $mg_{protein}$ g_{resin}^{-1} for preparing LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103, respectively. The ultimate conditions for immobilization were: 5 mL citrate buffer (2 M, pH 6.0) containing 5 mg LDH-7αHSDH was incubated with 0.5 g of resin ES-103 at 20°C for 20 h, and 5 mL citrate buffer (2 M, pH 5.5) containing 25 mg 7βHSDH-GDH was incubated with 0.5 g of ES-103 at 20°C for 20 h. Under such optimal conditions, the activity of LDH-7αHSDH@ES-103 was 43.2 U g^{-1} with an activity recovery of 37%, whereas that of 7βHSDH-GDH@ES-103 was 25.8 U g^{-1} with an activity recovery of 30% (Table 2, entry 4 and Table S1). The specific loading and activity recovery of the immobilized

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enzymes were obviously improved when compared with those values under the initial immobilization conditions.

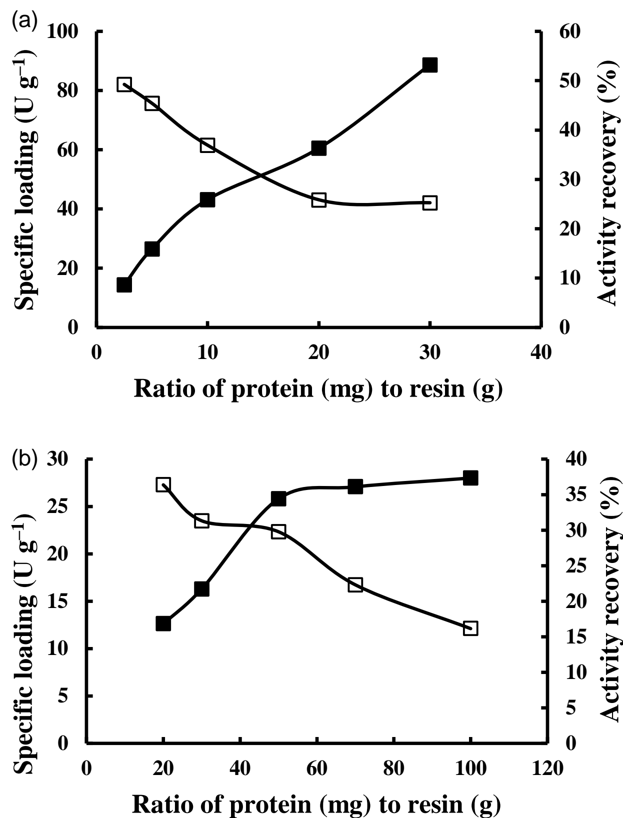

Figure 2. Specific loading (filled symbols) and activity recovery (open symbols) of the LDH-7αHSDH@ES-103 (A) and 7βHSDH-GDH@ES-103 (B) achieved at various ratios of protein to resin. The immobilization pH of LDH-7αHSDH@ES-103 was 6.0 and that of 7βHSDH-GDH@ES-103 was 5.5. The immobilization time was 20 h.

Table 2. Specific loading and activity recoveries of LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 under various immobilization conditions.

Entry	Stage	LDH-7αHSDH@ES-103		7βHSDH-GDH@ES-103	
		Specific loading (U g^{-1})	Activity recovery (%)	Specific loading (U g^{-1})	Activity recovery (%)
1	Before optimization	3.6	5.7	0.05	0.06
2	After optimizing immobilization pH	34	23	7.0	9.9
3	After optimizing immobilization time	41	35	25	30
4	After optimizing the loading ratio of enzyme to resin	43	37	26	30

Characterization of immobilized enzymes

In order to characterize the immobilized enzymes, we examined the effects of temperature and pH on the activity of immobilized enzymes and compared those values with that of the free enzymes. Both the optimum temperatures of LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 were lower than those of the free enzymes (Fig. S3). The comparison of pH optima between the immobilized and free enzymes is shown in Fig. S4. Both the pH optima of the two groups of immobilized enzymes were shifted to more alkaline pH conditions, which is beneficial for the cascade synthesis of UDCA from CDCA considering the effective coupling of the oxidative and reductive reactions.[6] This shift to alkaline conditions is probably because of the chemical bonding between the resin and the enzyme.

Thermal stability is important for industrial applications. The residual activities of the immobilized enzymes 7βHSDH-GDH@ES-103 were both higher than those of the free enzymes after preincubation for 2 h between 20 and 50°C. Although at low temperatures, the residual activities of LDH-7αHSDH@ES-103 were similar to those of the free enzymes, at high temperatures, such as 60°C, they were significantly higher than those of the free enzymes (Fig. S5). The half-lives at 30°C for the immobilized and free LDH-7αHSDH were 23.0 h and 16.4 h, respectively, and those of the immobilized and free 7βHSDH-GDH were 2.9 h and 2.1 h, respectively (Fig. 3). From the results, we observe that the half-lives of the immobilized enzymes are slightly longer than those of the free enzymes.

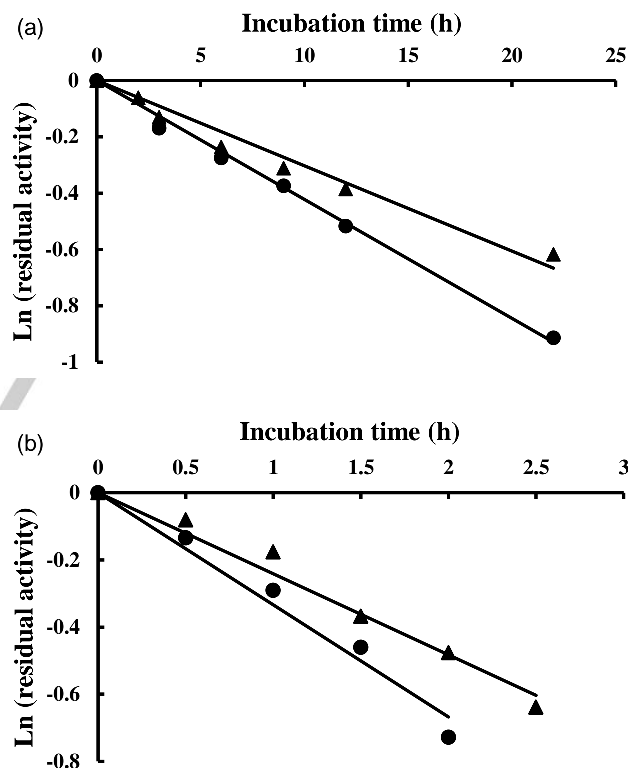

Figure 3. Comparison of the half-lives at 30°C between the immobilized (triangles) and free enzymes (circles). (a) LDH-7αHSDH and (b) 7βHSDH-GDH.

For any industrial application, recycling and reuse of enzymes is an important issue to address. Therefore, we first examined the batch-wise reactions of immobilized enzymes LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103. As illustrated in Fig. S6A, LDH-7αHSDH@ES-103 reached nearly 100% conversion in the first five batches, but the conversion began to

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decline from the sixth batch. At the end of the tenth batch reaction, the residual activity of LDH-7αHSDH@ES-103 detected was 44%. For 7βHSDH-GDH@ES-103, the bioconversion started to decline from the third batch (Fig. S6B). This difference in the batch reactions of the immobilized enzymes might be due to their stability, because the thermal stability of LDH-7αHSDH@ES-103 is better than that of 7βHSDH-GDH@ES-103. In the batch reactors, the activity of immobilized enzymes might be destroyed by mechanical stress. Moreover, physical loss of enzymes is unavoidable during the process of recovering enzymes. Hence, we adopted the packed bed reactor for subsequent preparation of UDCA.

Continuous synthesis of UDCA in cascade bioreactors

A packed bed reactor provides a continuous reaction and reduces maximally the loss of enzyme activity. Accordingly, we tried to construct two PBRs for the cascade synthesis of UDCA from CDCA (Fig. 4).

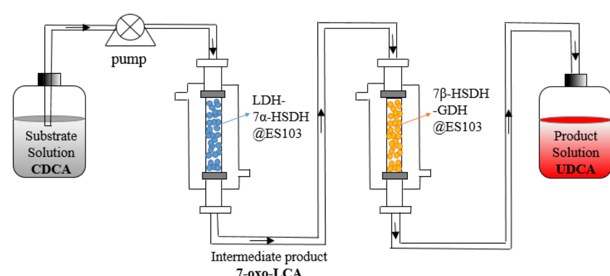

Figure 4. Sketch of the packed bed reactors in the cascade for continuous synthesis of UDCA from CDCA.

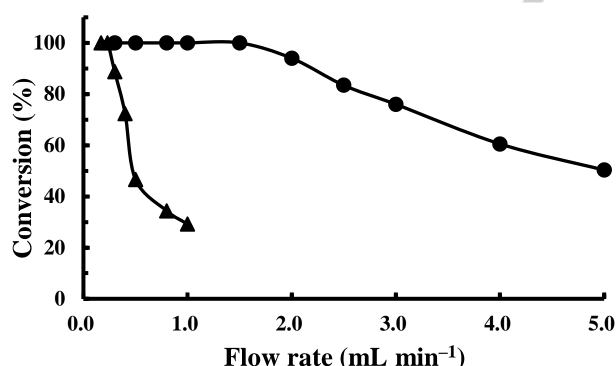

Figure 5. Conversion of substrate by the immobilized enzyme LDH-7αHSDH@ES-103 using a variety of flow rates through the packed enzyme reactor. The substrate solution was fed either from the top to bottom (circles) or from the bottom to top (triangles).

Initially, we examined individually the performance of a PBR with the first step reaction, which converts CDCA to 7-oxo-LCA. Since different flow patterns may affect the mass transfer in a reaction and the pressure drop of the bed,[18] we investigated the effect of upward and downward flow of the substrate solution in the PBR. A portion of immobilized particles, LDH-7αHSDH@ES-103 (3.5 g, *ca.* 5 mL), was added into a packed bed reactor and the conversion degrees of substrate were determined at different flow rates (0.3–5.0 mL min^{-1}) (Fig. 5). The conversion of substrate in a downward flow was always higher than that of the upward flow at any flow rates. This is probably because the packed bed is in a partially fluidized state with the upward flow, in which back-mixing and channeling are unavoidable, resulting in a decrease of substrate conversion. Therefore, in the following studies, the flow direction from top to bottom was selected and CDCA was completely converted when the flow rate was below 1.5 mL min^{-1}.

A similar PBR containing the same amount of the second immobilized enzyme, 7βHSDH-GDH@ES-103 (3.5 g, 5 mL), was connected in series to the aforementioned PBR with the 1st enzyme. The second PBR did not transform the intermediate 7-oxo-LCA into UDCA completely (Fig. 6), because both the activity and thermostability of 7β-HSDH-GDH@ES-103 are lower than those of LDH-7αHSDH@ES-103. Therefore, we increased the loading of the 2nd enzyme 7βHSDH-GDH@ES-103 in the second PBR. With an increase in the loading of 7βHSDH-GDH@ES-103, the content of UDCA in the product solution displayed an upward trend at a variety of flow rates. When the loading volume ratio of the two catalysts (2nd/1st) was increased from 1:1 to 5:1, the CDCA in the feeding solution was completely converted into UDCA when the flow rate was less than 0.8 mL min^{-1}, corresponding to a space velocity (SV) of 9.6 h^{-1} for the 1st PBR or 1.9 h^{-1} for the 2nd PBR (Fig. 6). The space-time yield of UDCA in such a cascade reactor system could reach 88.5 g L^{-1} d^{-1}. Subsequently, we observed that the conversion of substrate could still reach nearly 100% even after a continuous reaction for 12 h in the cascade PBRs, albeit at a slower flow rate (0.5 mL min^{-1}).

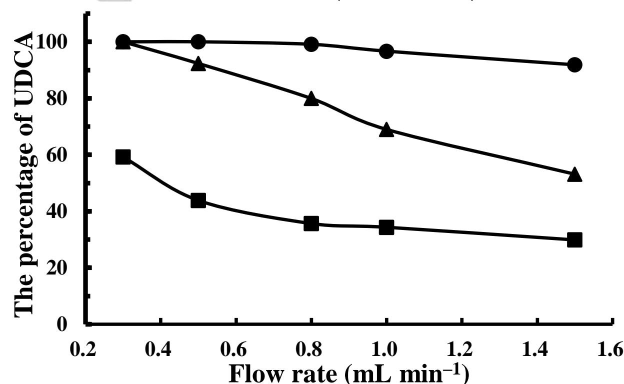

Figure 6. Dependence of the UDCA titer on the flow rate of the substrate solution through the cascade enzyme reactors, with a varied ratio of immobilized enzyme bed volumes (reactor 1 over reactor 2) of 1:1 (squares), 1:3 (triangles) or 1:5 (circles).

Discussion

Enzyme immobilization is a common strategy used in industrial applications, although it remains underdeveloped for cascade bioreactions.[7] Cascade reactions involving multiple enzymes usually suffer from problems of biocatalyst instability and recyclability, and these problems hamper the use of cascade reactions in industrial applications. In this study, we achieved the co-immobilization of multiple enzymes onto commercially available epoxy-functionalized resins (ES-103) and successfully

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catalyzed the production of UDCA. Epoxy-functionalized resins can immobilize various enzymes via covalent binding interactions (the epoxy groups of resins were mainly bound to the amino groups of the enzymes), which are more stable than physical adsorption because covalent bonding to the surface reduces substantially (over other forms of immobilization) the shedding of immobilized enzymes from the surface during reactions and recycling procedures.[8b]

By optimizing the immobilizing pH the specific loads of LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 were increased by 9- and 140-fold, respectively (Table 2, line 2). After optimizing the immobilization time, the activity loading and recovery of the immobilized enzymes had been improved significantly (Table 2, line 3). Finally, the maximal activities of LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103 were 43.2 U g^{-1} and 25.8 U g^{-1}, respectively, in contrast to 3.6 U g^{-1} and 0.05 U g^{-1} under the initial immobilization conditions, representing improvements by 12- and 516-fold, respectively (Table 2, lines 1 and 4).

Although we successfully immobilized enzymes, in the batch reaction, the enzyme could not be recycled more than five batches, which may be a consequence of mechanical shear stress and physical loss of the enzymes. PBRs could be used to circumvent these limitations. After optimizing the volume ratio of these two immobilized enzymes, the cascade PBRs maintained conversion at 100% for at least 12 h, which was significantly better than the batch operation. Furthermore, the continuous operation of cascade PBRs with the four immobilized enzymes for the synthesis of UDCA from CDCA overcomes the drawbacks of employing free enzymes in the batch reactions where the enzymes used in the first reaction step have to be heat inactivated prior to performing the second reaction step.

The co-immobilization of multi-enzymes has been documented in recent years. For instance, Liu et al. reported the co-immobilization of glucose oxidase (GOx) and horseradish peroxidase (HRP) on zeolitic imidazolate frameworks (ZIF-8).[19] The GOx and HRP/ZIF-8 composite displayed high activity, selectivity and stability. Shortly afterwards, Ge and Liu et al. reported the co-localization of multi-enzymes by inorganic nanocrystal–protein complexes.[20] This method is simple and effective. Riva et al. reported the coimmobilization of 12α-HSDH and glutamate dehydrogenase for the preparation of 12-ketochenodeoxycholic acid, where the immobilized enzymes could work for a long time (2 months).[21] The coimmobilization of 7α- and 7β-HSDHs was also reported previously. For UDCA preparation, the 7α-HSDH and 7β-HSDH from *Xanthomonas maltophilia* were immobilized on a kind of resin. However, the yield of UDCA was only 75% and the reaction took a too long period (7 days) using immobilized 7α- and 7β-HSDHs.[22] Recently, Wang et al. coimmobilized 7α- and 7β-HSDHs from *Clostridium absonum* DSM599. Tauroursodeoxycholic acid (TUDCA) was obtained from taurochenodeoxycholic acid (TCDCA) within 5 h using the immobilized enzymes. However, the yield of TUDCA was only 62.5%.[23] Similar works on the production of 12-ketoursodeoxycholic acid have been reported by Weuster-Botz[25], Riva[26] and Werner Hummel[27] et al. In our work, UDCA was continuously produced in two cascade PBRs, with nearly 100% yield and the space-time yield (88.5 g L^{-1} d^{-1}) was higher than the other reports (Table 3). Thus, the cascade reactor with resin-immobilized enzymes reported in this work seems more efficient and better suited for the enzymatic production of UDCA as a substitute of pharmaceutically important bear bile powder.

Table 3. Comparison of biocatalyst performance between this study and literatures for the biosynthesis of UDCA and analogs using immobilized enzymes or cells.

Substrate load (mM)	Yield (%)	Space-time yield (g L^{-1} d^{-1})	Ref.
0.58	75	0.02	[22]
1.0 [a]	40	0.16	[24]
2.0 [b]	62.5	3.0	[23]
10.0	99	88.5	This study

[a] UDCA was prepared using immobilized cells.
[b] The substrate used was taurochenodeoxycholic acid (TCDCA) and the product formed was tauroursodeoxycholic acid (TUDCA).

Conclusions

Four enzymes including LDH-7αHSDH and 7βHSDH-GDH were pairwise co-immobilized on an epoxy-functionalized resin by covalent binding. After optimizing the immobilization conditions, both the specific loading and activity recovery of the immobilized enzymes were significantly improved by 12- and 516-fold, respectively. A continuous reaction process has been constructed successfully for facile enzymatic synthesis of UDCA from cheap and more abundant CDCA using pairwise coimmobilized enzyme particles, LDH-7αHSDH@ES-103 and 7βHSDH-GDH@ES-103, as biocatalysts. Furthermore, the cascade packed bed reactor designed and constructed in this study could efficiently synthesize UDCA with an approximately 100% yield and an excellent space-time yield of 88.5 g L^{-1} d^{-1}, which represents a new and promising technology for practical applications.

Experimental Section

Materials

The standards of ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA) and 7-oxo-lithocholic acid (7-oxo-LCA) were purchased from Shanghai Siyu Chemical Technology Co., Ltd. (Shanghai, China). The resins used for immobilizing enzymes were purchased from Tianjin Nankai Hecheng Science and Technology Co., Ltd. (Tianjin, China). The vector pET-28a(+) was purchased from Novagen (Shanghai, China). All other chemicals or reagents used in this work were of reagent grade and obtained commercially. The genes of 7α-$HSDH_{Ec}$, LDH, 7β-$HSDH_{Rt\text{-}M1}$ and GDH were prepared as described previously.[5,6]

Coexpression of related genes

Initially, we investigated the effects of two genes in different tandem arrangements of plasmids on the activities of enzymes. Then we chose the coexpressed plasmids whose activities of expression were relatively higher.

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7βHSDH-GDH: The purified PCR fragment of 7β-HSDH$_{Rt\text{-}M1}$ (T189V/V207I) was digested with *Bam*HI and *Eco*RI and ligated into expression vector pET-28a(+). The GDH gene was amplified and ligated into the aforementioned vector pET-28a(+) which had already contained the gene of 7β-HSDH$_{Rt\text{-}M1}$ after digested with *Eco*RI and *Xho*I. A ribosomal binding site (rbs) was inserted between the two genes. The recombinant expression was described in our previous report.[5]

LDH-7αHSDH: The method to construct the recombinant plasmid of LDH-7αHSDH is similar to 7βHSDH-GDH. The gene of LDH was digested with *Nde*I and *Bam*HI and the gene of 7α-HSDH$_{Ec}$ was digested with *Bam*HI and *Xho*I. The recombinant expression was described in our previous report.[5]

Enzyme immobilization procedure

The enzyme pairs, 7β-HSDH$_{Rt\text{-}M1}$ and GDH, as well as 7α-HSDH$_{Ec}$ and LDH, were coimmobilized onto a series of epoxy-functionalized resins (ES-1, ES-101, ES-103).

The procedure of coimmobilizing enzymes onto an epoxy-functionalized resin was as follows: 0.5 g resins were washed 3 times with 3 mL potassium phosphate buffer (2 M, pH 7.8). To the resultant resins as collected by filtration, was added 5 mL of crude enzyme solution (2 M potassium phosphate buffer, pH 7.8) containing the coexpressed proteins of 5 mg LDH-7αHSDH or 25 mg 7βHSDH-GDH and the mixture was incubated at 20°C and 170 rpm. After 15 h of incubation, the immobilized enzymes were collected by filtration and washed with water until no protein outflow was detected.

Activity assay of coimmobilized enzymes

The activities of free LDH-7αHSDH and 7βHSDH-GDH were measured using the standard method as described previously.[5] The activities of the immobilized enzymes were measured with the following procedure: the immobilized enzymes of LDH-7αHSDH (4 mg) were added to 1 mL of potassium phosphate buffer (100 mM, pH 8.0) containing 10 mM CDCA, 30 mM sodium pyruvate and 0.5 mM NAD^+; While the immobilized enzymes of 7βHSDH-GDH (10 mg) were added to 1 mL of potassium phosphate buffer (100 mM, pH 8.0) containing 10 mM 7-oxo-LCA, 30 mM glucose and 0.5 mM $NADP^+$. Then the mixtures were shaken (1000 rpm) at 30°C for 10 min using a Heating Thermo Shaker Mixer MHR 23 (Digital HLC, Germany). The samples were then acidified to pH 3.0 by adding 1 M HCl and extracted with ethyl acetate. The organic layer was evaporated and methanol was added for HPLC analysis. The HPLC analysis was performed as described previously (Fig. S7).[5] One unit of activity was defined as the amount of enzyme catalyzing the transformation of 1.0 μmol CDCA or 7-oxo-LCA per minute under the assay conditions. The percent activity recovery is the ratio of the total activity of immobilized enzymes to that of the total activity of free enzymes used.

Optimization of coimmobilized conditions

Optimization of immobilization pH. The coexpressed enzymes, LDH-7αHSDH (5 mg) or 7βHSDH-GDH (25 mg), were dissolved in 5 mL different buffers with varied pH (2 M, citrate pH 5.0–6.0, phosphate pH 6.0–8.0). Then 0.5 g epoxy-functionalized resins were added and incubated at 20°C and 170 rpm for 15 h.

Optimization of immobilization time. Cell free extracts of LDH-7αHSDH (5 mg) or 7βHSDH-GDH (25 mg) were dissolved in 5 mL of 2 M citrate buffer (pH 6.0 or 5.5). Then 0.5 g of an epoxy-functionalized resin was added and incubated at 20°C and 170 rpm for different periods of time.

Optimization of enzyme loading ratio. To 5 mL of 2 M citrate buffer (pH 6.0 or 5.5) containing varied amounts of LDH-7αHSDH or 7βHSDH-GDH, were added 0.5 g of an epoxy-functionalized resin and the resulting mixture was incubated at 20°C and 170 rpm for 20 h.

Characterization of resin-coimmobilized enzymes

Activity of the resin-immobilized enzymes, LDH-7αHSDH@ES-103 or 7βHSDH-GDH@ES-103, as well as that of the free enzymes LDH-7αHSDH or 7βHSDH-GDH, were measured at different reaction temperatures in a range of 20–50°C in potassium phosphate buffer (100 mM, pH 8.0). The optimum pH for immobilizing LDH-7αHSDH@ES-103 or 7βHSDH-GDH@ES-103, as well as that for free LDH-7αHSDH or 7βHSDH-GDH was determined using the method as described above in different buffers with various pH (5.5–10.0).

To study the thermostability of coimmobilized enzymes, LDH-7αHSDH@ES-103 or 7βHSDH-GDH@ES-103 as well as their free counterparts were incubated in potassium phosphate buffer (100 mM, pH 8.0) for 2 h at a temperature in the range of 20–60°C, respectively, then the residual activities were measured, while the initial enzyme activity (without incubation) was defined as 100%. The half-lives of LDH-7αHSDH@ES-103, 7βHSDH-GDH@ES-103 as well as those of the free enzymes were examined by incubating the immobilized or free enzymes in the same potassium phosphate buffer (100 mM, pH 8.0) at 30°C, and measuring the residual activities at different periods of incubation time.

Batch reactions of coimmobilized enzymes

Batch reactions were carried out in a triangle flask to assess the reusability of immobilized enzymes. LDH-7αHSDH@ES-103 (0.1 g) was added to 2 mL potassium phosphate buffer (100 mM, pH 8.0) containing 10 mM CDCA, 30 mM sodium pyruvate and 0.5 mM NAD^+. Similarly, 7βHSDH-GDH@ES-103 (0.2 g) was added to 2 mL potassium phosphate buffer (100 mM, pH 8.0) containing 10 mM 7-oxo-LCA, 30 mM glucose and 0.5 mM $NADP^+$. The reaction mixtures were incubated at 30°C and 180 rpm for 1 h. Then samples of the reaction mixture were analyzed for measuring the substrate conversion and the immobilized enzyme was recovered by filtration for use in the next batch of reaction.

Construction and operation of packed bed reactors

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The resin-coimmobilized enzyme particles of LDH-7αHSDH@ES-103 (3.5 g, *ca.* 5 mL) were packed into a water-jacketed glass column (D:16 mm, H: 60 mm). A substrate solution containing 10 mM CDCA, 30 mM sodium pyruvate, 0.5 mM NAD^+ and 100 mM potassium phosphate buffer (pH 8.0) was continuously fed into the immobilized enzyme column either from top to bottom or from bottom to top using a peristaltic pump (BT100-1F, Baoding Longer Precision Pump Co., Ltd., China) at a variety of flow rates (0.3–5.0 mL min^{-1}).

The coimmobilized enzyme particles of 7βHSDH-GDH@ES-103 (3.5 g, 10 g or 17.5 g) were packed into another water-jacketed glass column (D:30 mm, H: 55 mm). These two enzyme columns were connected in series by a rubber hose. A substrate solution, containing 10 mM CDCA, 0.5 mM NAD^+, 0.5 mM $NADP^+$, 30 mM sodium pyruvate, 30 mM glucose, and 100 mM potassium phosphate buffer (pH 8.0), was sequentially flowed through these two enzyme columns from top to bottom at a variety of flow rates (0.3–1.5 mL min^{-1}) using the aforementioned peristaltic pump. The reaction temperature was maintained at 30°C by circulating a thermostat water in the jackets surrounding the enzyme columns.

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Keywords: Cascade reaction • Coimmobilized enzymes • Continuous operation • Packed bed bioreactor • Ursodeoxycholic acid

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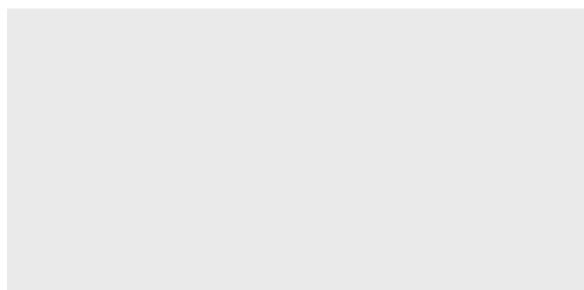

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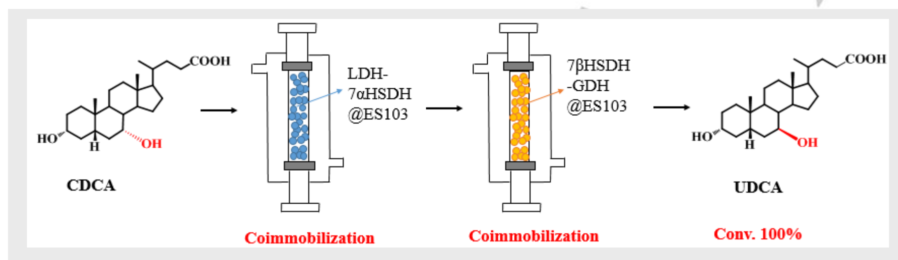

Ming-Min Zheng, Fei-Fei Chen, Hao Li,Chun-Xiu Li and Jian-He Xu**

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Title
Continuous Production of Ursodeoxycholic Acid using Two Cascade Reactors with Coimmobilized Enzymes

Four enzymes including LDH-7αHSDH and 7βHSDH-GDH were pairwise coimmobilized on an epoxy-functionalized resin. Two cascade packed bed reactors were constructed with the coimmobilized enzymes for continuous synthesis of ursodeoxycholic acid from the cheap and easily available chenodeoxycholic acid.

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