Appl Biochem Biotechnol
DOI 10.1007/s12010-016-2339-6

Covalent Immobilization of Human Placental 17β-Hydroxysteroid Dehydrogenase Type 1 onto Glutaraldehyde Activated Silica Coupled with LC-TOF/MS for Anti-Cancer Drug Screening Applications

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Received: 25 July 2016 / Accepted: 22 November 2016

Abstract Human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), a potential target in breast cancer prevention and therapy, was extracted from human placenta and immobilized on nonporous silica (~5 μm) with a covalent method for the first time. The optimum initial enzyme concentration and immobilization time during the immobilization process were 0.42 mg mL^{-1} and 12 h, repectively. The binding was confirmed by scanning electron microscope (SEM) and infrared spectroscopy (FT-IR). It could improve the pH, thermal and storage stability compared to free enzyme. Moreover, the immobilized enzyme could be reused at least four times. A screening method based on it coupled with liquid chromatography–time-of-flight mass spectrometer (LC-TOF/MS) was established, and the half-maximal inhibitory concentration (IC 50) of apigenin for the immobilized enzyme was 291 nM. Subsequently, 10 natural products were evaluated leading to inhibition of the activity of 17β-HSD1 at the concentration of 25 μM, and six of them inhibit the activity over 50%.

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Published online: 08 December 2016

Keywords Human 17β-hydroxysteroid dehydrogenase type 1 · Covalent immobilization · Enzyme stability · Inhibitor screening · LC-TOF/MS

Introduction

Breast cancer is the most prevalent type of cancer in women and the main disease in cancer related to deaths [1]. It represents about one third of all cases according to both the American Cancer Society and the National Cancer Institute of Canada [2]. 17β-Hydroxysteroid dehydrogenase (17β-HSDs) play a key role in the activation of estrogen in breast cancer cells and in which 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is the most active of all known types. Based on the experiments of MCF-7 and T47D in breast cancer cells, it is confirmed that activated estrogens obviously decreased and then cell growth slowed down as a consequence of inhibition of expression of 17β-HSD1 [3]. 17β-HSD1 is also expressed in ovaries, placenta and ectopic endometrium [4]. Many further researches on the enzyme extracted from placenta have been done, such as the description of its biological characteristics [5, 6] and the research of its function [7, 8]. Some reports have described the structure of the complex of 17β-HSD1–estradiol–NADP+ [9] or 17β-HSD1–androstanedione–NADP+ [10] through X-ray diffraction, thus providing an important basis for inhibitor screening.

The common catalytic substrates of 17β-HSD1 include estrone, estradiol, androstenedione, dehydroepiandrosterone and dihydrotestosterone [11, 12]. Aiming at its active site, many active compounds have been screened from nature products [13]. And phytoestrogens, including flavonoids, coumestans and lignans, are known inhibitors of the human 17β-HSD types 1 [14, 15]; flavonoids are plant-derived secondary metabolites, and most of them usually have a similar structure with natural human steroid hormones. Some flavonoids and their structural analogues could interfere with the process of estrogen biosynthesis by binding to ERs and key enzymes [16, 17].

Current inhibitor-screening methods mostly employ ^{3}H-labelled estrone as substrate using free enzymes [18], with the following disadvantages: firstly, radioactivity exists in the detection method; secondly, free enzymes have some obvious flaws such as poor stability and difficulties in recycling from the reaction system, resulting in high cost and waste of enzyme. In view of the problems above, establishment of a novel screening method becomes a pressing demand.

In the present study, we extracted 17β-HSD1 from human placenta and then it was covalently immobilized onto silica particles with the help of glutaraldehyde [19]. First of all, immobilized conditions including initial concentration of enzyme and reaction time were optimized. Secondly, the properties including pH, thermal and storage stability of immobilized enzymes were compared with those of free enzymes. The reusability of immobilized enzymes was also evaluated. Finally, we employed androstenedione as the substrate to analyse the changes of testosterone concentration before and after adding an inhibitor, thereby screening inhibitors from potential active compounds based on LC-TOF/MS. Apigenin was used for screening method validation since it was a known inhibitor. Subsequently, we evaluated the inhibitory activities of seven flavonoids (baicalin, hyperoside, isorhamnetin, kaempferol, puerarin, quercetin, wogonin), one dihydroflavonoid (naringenin) and two structural analogues of flavonoids (bergenin, resveratrol).

Materials and Methods

Materials

Human placenta was supplied by the Second Affiliated Hospital of Nanjing Medical University (with the approval of the ethics committee in the Second Affiliated Hospital of Nanjing Medical University). 3-Aminopropyltriethoxysilane (APTES), androstenedione, estrone, testosterone, baicalin, hyperoside, naringenin and wogonin were purchased from Aladdin Chemistry Co. (Shanghai, China). Bergenin, isorhamnetin, kaempferol, puerarin, quercetin and resveratrol were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Triphosphopyridine nucleotide (NADPH) was obtained from BioSharp Corporation (Hefei, China). Glutaraldehyde (GA, 25%, *w*/*v*, aqueous solution) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionized water was purified through a PL5242 Purelab Classic UV (Pall Co., Ltd., USA). Uniform nonporous silica (5 μm in diameter) was purchased from Kromasil Co. (Sweden). Other chemicals were obtained from common commercial sources without further purification.

Preparation of Human Placental 17β-HSD1

Placental microsomes were prepared using the method according to a previously described procedure [7, 20]. The placenta was obtained directly after normal vaginal delivery at local hospitals and immediately placed on ice. The cotyledon tissue was severed from the chorionic plate, cut into pieces and rinsed thoroughly with sodium phosphate buffer (50 mM, pH 7.4, 1% potassium chloride), then homogenized in a Waring blender with three volumes of sodium phosphate buffer (50 mM, pH 7.4, 0.25 M sucrose) and centrifuged at 2500×*g* (30 min, 4 °C), and the supernatant was subsequently centrifuged at 60,000×*g* (1 h, 4 °C). The washed microsomes (human placental 17β-HSD1) were collected by the addition of ammonium sulphate to the resulting supernatant, suspended in sodium phosphate buffer (50 mM, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM cysteine hydrochloride) and then stored at −80 °C. The concentration of enzyme was determined by the Bradford method [21] with bovine serum albumin (BSA) as standard protein.

Surface Modification of Nonporous Silica

Nonporous silica particles as carriers were silanized with APTES and activated using GA before enzyme immobilization. Briefly, 1 mL APTES was added dropwise to an emulsion of silica particles (2 g) in toluene (200 mL) under gentle stirring and the mixture was refluxed at 90 °C for 24 h. The obtained precipitate was filtered, washed thoroughly with toluene and acetone and dried at 105 °C overnight. Thereafter, 500 mg of amine-functionalized silica particles was dispersed in 45 mL of sodium phosphate buffer (50 mM, pH 7.4), then 5 mL GA (25%, *v*/*v*) was added to the mixture and the reaction was allowed to continue for 12 h at room temperature. Afterwards, the obtained GA-activated silica particles were thoroughly washed with deionized water and finally dried at 70 °C overnight.

17β-HSD1 Immobilization

Immobilization was achieved by the following procedures: Briefly, different volumes (20–200 μL) of placenta microsomes (4.67 mg mL^{-1}) were added to an emulsion of GA-activated silica (50 mg) in 1 mL sodium phosphate buffer (50 mM, pH 7.4). Following stirring for 12 h at 25 °C, the precipitate was washed with the immobilization buffer for three times to remove nonbonded enzymes. Finally, the dry immobilized enzyme was recovered by rotation vacuum concentrators at room tempurature. The loading amount (mg/g) was determined by subtracting the residual protein content in the solution from the initial protein content before immobilization using the Bradford assay.

$$\text{The loading amount (mg/g)} = [(C_0V_0–C_tV_t)/M] \times 100\%$$

Herein, C_0 is the initial concentration of enzyme (mg mL^{-1}), V_0 is the initial volume of enzyme solution (mL), C_t is the concentration of enzyme in the total supernatant (mg mL^{-1}) and V_t is the total supernatant volume (mL). M is the weight of support (g).

Enzyme Activity and Inhibitory Assay

Catalytic activity of free and immobilized 17β-HSD1 was determined by the reduction of androstenedione to testosterone with the assistance of the co-factor NADPH [11]. The reaction was carried out at 37 °C in a final volume of 180 μL containing 10 μL placenta enzyme solution, 150 μL androstenedione (35 μM) and 20 μL of NAPDH (25 mg mL^{-1}) for 10 h, then quenched with 50 μL acetonitrile and centrifuged at 8000×*g* for 5 min. Twenty microlitres of the supernatant was injected into the Agilent 1100 series HPLC system. The activity of immobilized 17β-HSD1 was measured at the same conditions, except that 10 μL free enzyme solution was replaced by 10 μL buffer and 5 mg of immobilized enzyme. The immobilized enzyme was collected by centrifugation at 8000×*g* for 5 min, washed three times and then stored at 4 °C for further use. Androstenedione and testosterone were separated by a Zorbax Eclipse XDB-C18 column (4.6 mm, 150 mm, 5 μm) and eluted isocratically at 30 °C with a mobile phase of acetonitrile/water (60/40, *v*/*v*), containing 0.1% formic acid. The flow rate was 1 mL min^{-1}. The wavelength for detection was 240 nm, and the recording time was 8 min. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 pmol substrate per min at pH 8 and 37 °C.

For inhibitory assay, the procedure of experiment is the same as the immobilized enzyme activity assay except for 10 μL of potential inhibitor at certain concentrations instead of 10 μL buffer. Finally, after centrifugation, 20 μL of the supernatant was injected into HPLC-TOF/MS. The peak area of testosterone could be obtained from extracted ion chromatography (EIC, *m*/*z* 289.2162). The LC-MS analysis of degenerated samples was performed on an Agilent 1260 series HPLC system connected to 6224 TOF via ESI source. A Phenomenex Gemini-C18 column (2 mm, 50 mm, 5 μm) was used for separation. The mobile phase was methanol/water (60/40, *v*/*v*) containing 0.1% formic acid, and the analysis time was 2 min. The flow rate was 1 mL min^{-1}, and the column temperature was set at 30 °C. The MS signals were acquired in positive ionization mode. The instrument settings were as follows: capillary temperature, 350 °C; drying gas, 10 L min^{-1}; nebulizer, 35 psig; Vcap, 3500 V; fragmentor, 135 V; skimmer, 65 V; and

mass range recorded, *m*/*z* 110–1000. The enzyme inhibitory activity was calculated by integrating the area values as follows:

$$\text{Inhibition}\ (\%) = 100-[100 \times (A_{i+}-A_{i-})/(A_{0+}-A_{0-})]$$

where A_{0+}, A_{0-} ,A_{i+} and A_{i-} are defined as the peak areas of 100% enzyme activity (only the solvent with the enzyme), 0% enzyme activity (only the solvent without the enzyme), a potential inhibitor (with the enzyme) and a blank (a potential inhibitor without the enzyme).

Characterization

Scan electron microscopy (Hitachi SU8010, Japan) was used for determination of the morphology of silica particles before and after immobilization, and the IR spectra was recorded by Fourier transform infrared spectrometer (JASCO FT/IR-4100, Japan).

Results and Discussion

Characterization of Immobilized 17β-HSD1

17β-HSD1 was immobilized onto silica with a covalent method. The morphology of silica particles before and after immobilization by SEM is shown in Fig. 1. The SEM micrographs suggested a spherical shape and smooth surface of the GA-function silica (a3), which became quite rough after immobilization because of the enzyme aggregates (b3), so the binding of enzyme and carriers was examined [22]. Moreover, the placenta enzyme immobilized onto silica particles was further confirmed by FT-IR spectroscopy (Fig. 2). The sharp adsorption bonds at 1107 cm^{-1} could clearly be seen in all spectra, which were contributed to the Si–O–Si stretching vibration [23]. Compared with GA-activated silica particles, the adsorption bands (in the spectrum of immobilized 17β-HSD1) at 3285 cm^{-1} could be assigned to N–H and O–H stretching vibration [24], while bands occurring at 1654 and 1534 cm^{-1} in the spectrum were attributed to –CONH– (amide I) and amide II, which arose from the peptide bonds that link the amino acid together [25], indicating the existence of enzymes immobilized on the surface of silica particles.

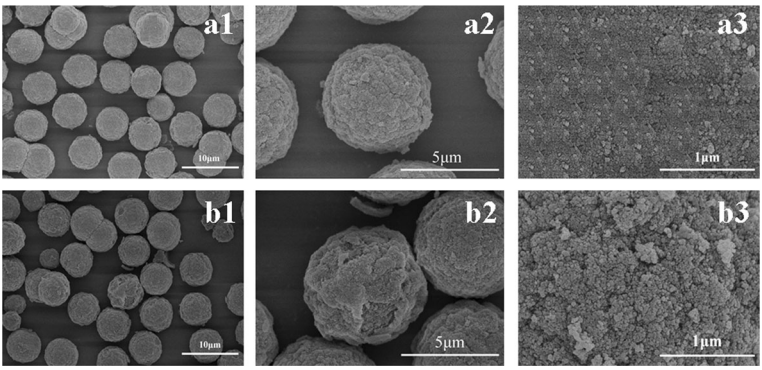


Fig. 1 SEM image of GA-activated silica and immobilized 17β-HSD1 in different scales: *a1* 10 μm, *a2* 5 μm and *a3* 1 μm of GA-activated silica; *b1* 10 μm, *b2* 5 μm and *b3* 1 μm of immobilized 17β-HSD1

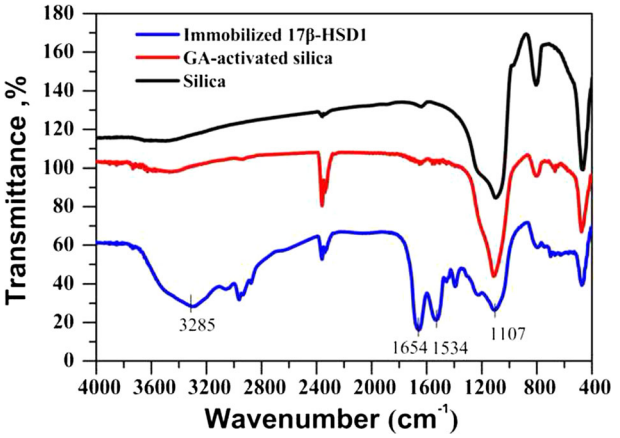


Fig. 2 FT-IR spectra of silica, GA-activated silica and immobilized 17β-HSD1

Selection of Substrate and Incubation Time

As a key enzyme for regulating intracellular level of androgens and estrogens, the most common catalytic substrates are estrone and androstenedione. Compared to androstenedione as substrate, more additional products were produced according to the results of HPLC and LC-TOF/MS when estrone was used as the substrate. It might be because estrone is also the substrate of other enzymes in microsomes. In order to obtain accurate results, we chose androstenedione as the substrate by adjusting the pH of enzymatic reaction to 8 to avoid the competition of substrates among enzymes. It was proved that the environment was specific to 17β-HSD1. In addition, a very good response could be obtained when we used androstenedione as substrate compared to the pool mass response of estrogen, even EIC, as product peak area signal. So, in order to develop a novel method to screen for inhibitors, androstenedione was selected as an ideal substrate. The experimental data showed that the amount of testosterone tended to reach a plateau at 10 h as incubation time increased (Fig. 3). Therefore, 10 h was selected as an optimal incubation time.

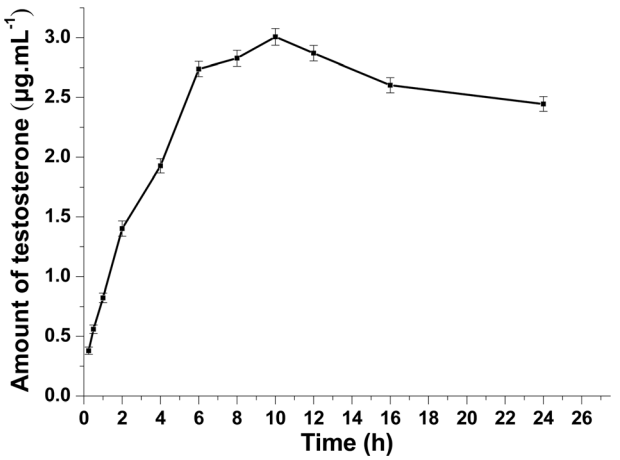


Fig. 3 The effect of incubation time on 17β-HSD1 activity. All assays were performed in triplicate

Effects of Silica Particle Size and Reaction Temperature

Different sizes of nonporous silica particles with diameters of 5, 15 and 30 μm were used as supports. Immobilization on 5-μm silica particles resulted in the highest loading amount and maximum enzyme activity compared to others (data not shown), mostly due to the increased surface area per unit weight of support [26]. The immobilization temperature was carried out at 4, 10, 25 and 37 °C. The activity of the immobilized enzyme increased with the rise of temperature and reached an activity maximum at 25 °C. As the temperature further increased, the activity decreased because of inactivation of the enzyme at high temperature. According to this observation (data not shown), 25 °C was chosen as an optimal temperature.

Effects of Initial Enzyme Concentration and Immobilization Time on Enzyme Loading

The effects of initial enzyme concentration and immobilization time on the immobilization process were investigated. The loading amount increased greatly with the enzyme concentration from 0.00 to 0.42 mg mL^{-1} then levelled off in the range of 0.42–0.78 mg mL^{-1}. And then the immobilization time was also investigated over 24 h. The loading amount initially increased rapidly from 0 to 12 h and then reached a plateau up to 24 h, indicating that the amount of enzyme on the support reaches a maximum value when the enzyme concentration was 0.42 mg mL^{-1} (Fig. 4), and the immobilization time was more than 12 h (Fig. 5). After immobilization, the amount of 17β-HSD1 immobilized onto the support was determined to be 96.25% of initial enzyme, which was giving a loading amount of 8.99 mg in 1 g carrier under optimal conditions. At the same time, the immobilized enzyme could maintain about 96.77% of the activity of initial enzyme.

Effects of pH and Temperature on Enzyme Activity

The optimal pH of free enzyme was 7.4; immobilization on GA-activated silica particles did not alter the optimum pH (Fig. 6). There was no significant difference between the

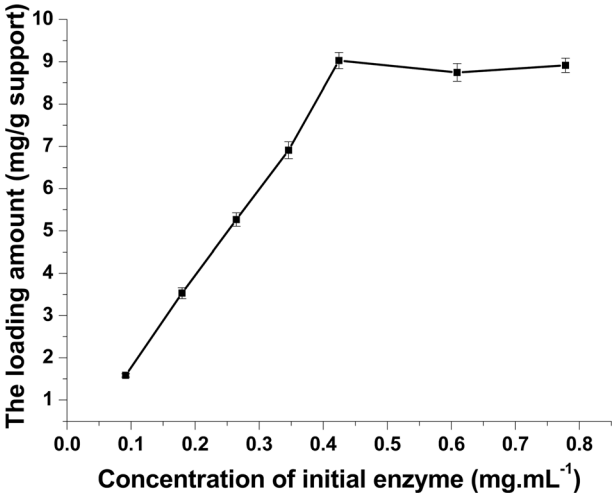


Fig. 4 Effect of initial enzyme concentration on enzyme loading. Immobilization conditions: 20–200 μL 17β-HSD1, 5 mg GA-activated silica in 1 mL phosphate buffer (50 mM, pH 7.4, $t = 25$ °C). All assays were performed in triplicate

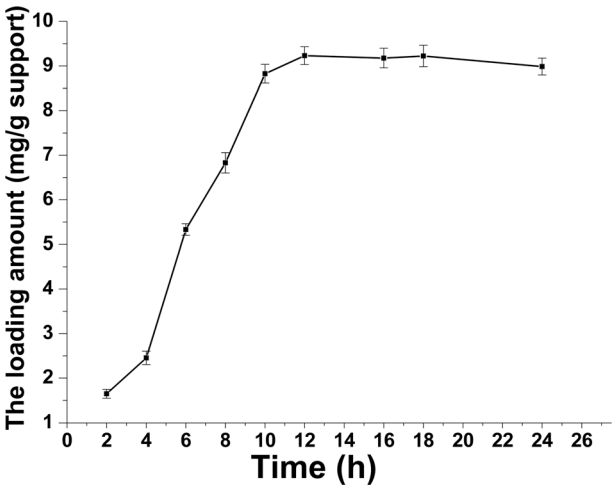


Fig. 5 Effect of immobilization time on enzyme loading. Immobilization conditions: 100 μL 17β-HSD1, 5 mg GA-activated silica in 1 mL phosphate buffer (50 mM, pH 7.4, t = 25 °C). All assays were performed in triplicate

immobilized and free enzyme in terms of pH range. Both of them were active in the pH range of 5–9, but immobilized enzyme had better activity than free one at pH 5 and 9, indicating that the immobilized enzymes increased resistance against denaturation after immobilization. However, another product was produced during incubation besides testosterone at pH 7.4. This is because placenta microsomes are a mixture of different proteins, and the neutral environment is closely mimicking that of the human body; some enzymes in microsomes remain active and catalyse different reactions. Given all this, further study was carried out at pH 8 because of the specific environment to 17β-HSD1 and good activity.

The maximal activity of free and immobilized enzyme was observed to be the same at 37 °C (Fig. 7). However, the immobilized enzyme exhibited greater activity at higher temperatures compared to free enzyme, and the activity of free enzyme declined significantly over 45 °C and lost approximately 70% at 65 °C. In contrast, the activity of immobilized enzyme remained stable from 45 to 55 °C and lost 58% at 70 °C, indicating that immobilized enzyme could be used in a wider temperature range for practical application.

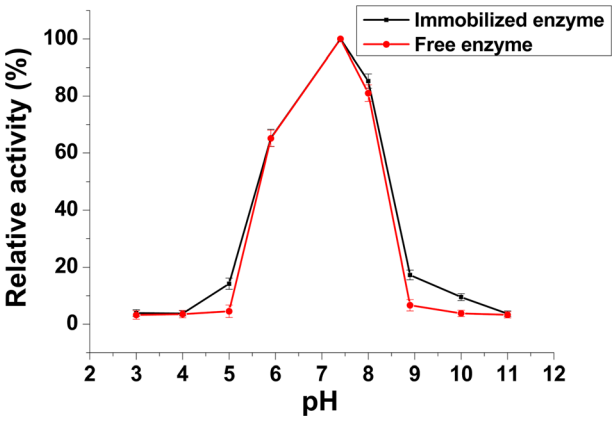


Fig. 6 Effect of pH on enzyme activity. The pH stability of free and immobilized enzymes was compared by immersing them in buffers of varying pH for 1 h at 37 °C. The pH was adjusted using 50 mM citrate buffer (pH 3–5), 50 mM sodium phosphate buffer (pH 6–8), 50 mM Tris–HCl buffer (pH 9) and $NaHCO_3$/NaOH buffer (pH 10–11). All assays were performed in triplicate

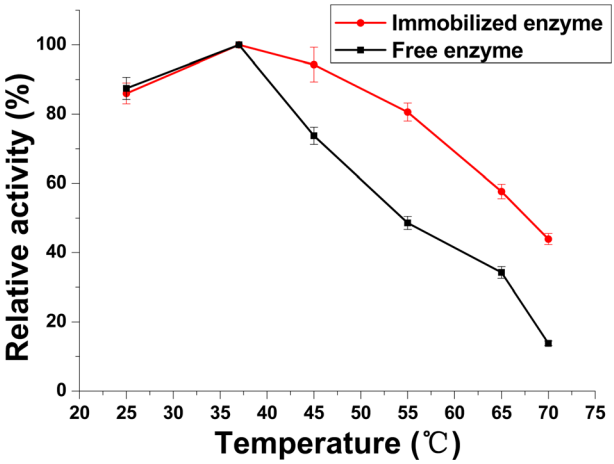


Fig. 7 Effect of temperature on enzyme activity. The activity of free and immobilized enzymes was investigated at various temperatures from 20 to 70 °C. All assays were performed in triplicate

Reusability and Storage Stability of Immobilized Enzyme

In order to investigate the reusability of immobilized enzyme, the activities were evaluated in several cycles. After each cycle, the immobilized enzyme was removed by centrifugation, washed with sodium phosphate buffer (50 mM, pH 8.0) for three times and then used for the next assay. It could remain around 36% of the initial activity after four consecutive operations. The repeated washing and conformational changes of enzyme after each catalytic reaction probably lead to the loss of its activity. The immobilized enzyme also exhibited improved storage stability (37% activity retained) at room temperature after 4 days while the free enzyme almost completely lost its activity under the same conditions. When stored at 4 °C, the immobilized enzyme nearly retained its initial activity within 30 days while the free enzyme lost about 48% of its activity (Fig. 8).

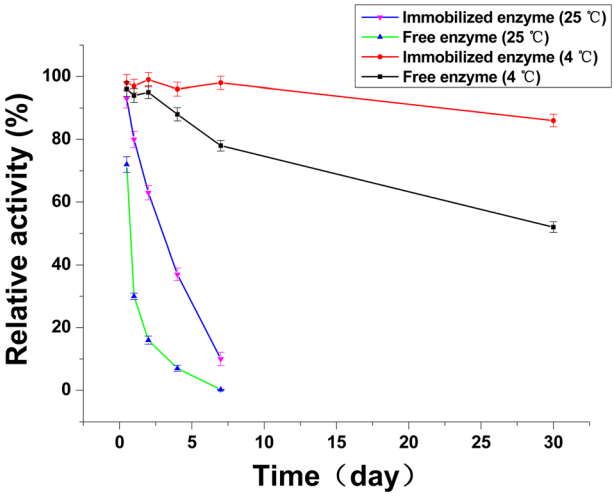


Fig. 8 Storage stability of free enzyme and immobilized enzyme at room temperature and 4 °C. All assays were performed in triplicate

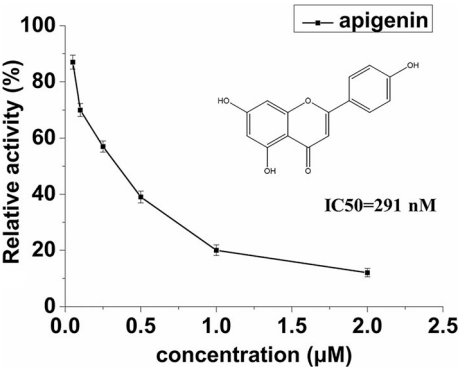
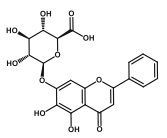


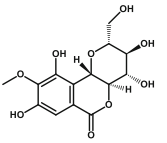
Fig. 9 Graphical representation of the IC50 values of apigenin. The initial enzyme activity (immobilized 17β-HSD1, 685.8 U/g; free enzyme, 78.83 U/mg) is taken as 100%. All assays were performed in triplicate

Screening of 17β-HSD1 Inhibitors

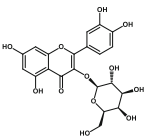
Apigenin, as an active inhibitor towards 17β-HSD1, was reported by literatures [15, 27] and employed to evaluate the inhibitor-screening method. As is seen in Fig. 9, various concentrations of apigenin solution from 50 nM to 2 μM were used to study the efficiency of inhibition, and the IC 50 value was 291 nM, which was close to the reported datum of 300 nM in literature [15].



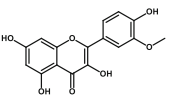
(1)**baicalin**[Root of Scutellaria baicalensis]



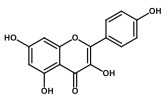
(2)**bergenin**[Bergenia purpurascens;B.crassifoloa;Astilbe macroflora;etc]



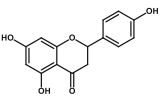
(3)**hyperoside**[Hypericum perforatum L.]



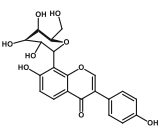
(4)**isorhamnetin**[Fruit of Hippophae fhamnoides L.]



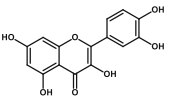
(5)**kaempferol**[Rhizome of Kaempferia galangal L.]



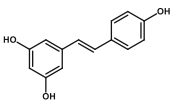
(6)**naringenin**[The core of the fruit of Amacardi-um occidentale L.]



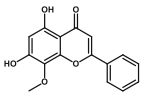
(7)**puerarin**[Root of Pueraria lobata（Willd.）Ohwi]



(8) **quercetin**[Bark and leaf of Quercus iberica]



(9)**resveratrol**[Root and Rhizome of Polygonum cuspidatum Sieb. et Zucc.]



(10)**wogonin**[Root of Scutellaria baicalensis Georgi]

Fig. 10 The structure of 10 compounds for inhibitor screening. Relative plants are included in *square brackets*

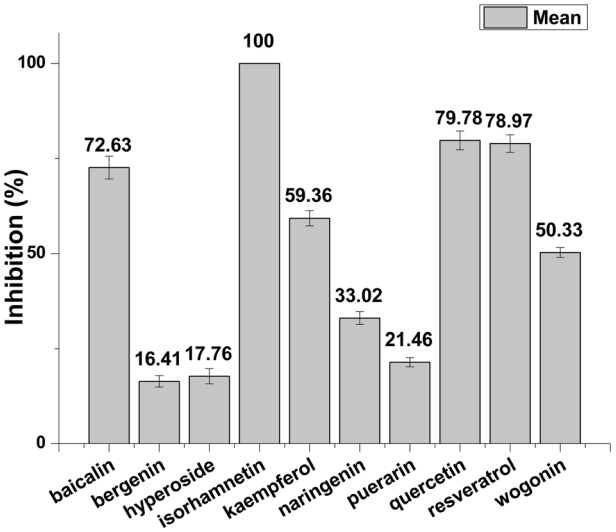


Fig. 11 Inhibition efficiency of different compounds at a concentration of 25 μM. The initial activity of immobilized 17β-HSD1, 685.8 U/g, is taken as 100%. All assays were performed in triplicate

In this study, we have evaluated the inhibitory activities of seven flavonoids (baicalin, hyperoside, isorhamnetin, kaempferol, puerarin, quercetin, wogonin), one dihydroflavonoid (naringenin) and two structural analogues of flavonoids (bergenin, resveratrol) using the inhibitor-screening method. The structures of these compounds and relative plants are shown in Fig. 10. After incubation, Fig. 11 shows that 10 compounds could inhibit the activity of 17β-HSD1 at a concentration of 25 μM and 6 of them inhibit the activity over 50%. The peak area of testosterone from EIC with wogonin (25 μM) and without any inhibitors is shown in Fig. 12.

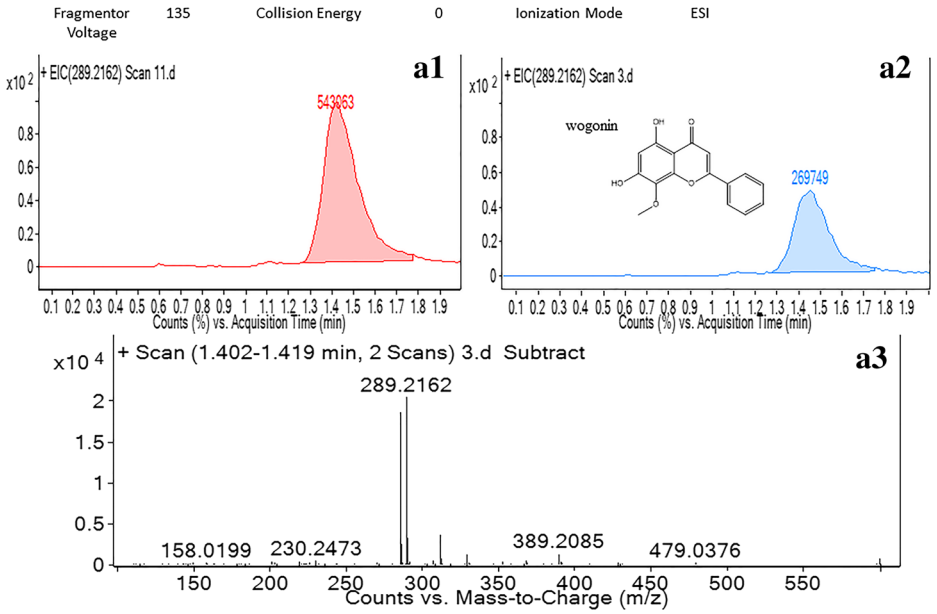


Fig. 12 The EIC (289.2162) of product testosterone. *a1* no inhibitor; *a2* with inhibitor (wogonin at 25 μM); *a3* the spectrum of background subtraction

The results herein reported demonstrate that the screening method based on LC-TOF/MS could be a useful tool for the screening of 17β-HSD1 inhibitors.

Conclusions

In this study, 17β-HSD1 was successfully immobilized onto GA-activated silica and the immobilized conditions were optimized. Moreover, the increased pH, thermal, storage and operational stability indicated the great promise of nonporous silica particles for 17β-HSD1 immobilization. In conclusion, the immobilized enzyme based on LC-TOF/MS, coupled with future experiments, is expected to facilitate the setting up of a low-cost high-throughput system.

Acknowledgements The authors gratefully acknowledge the financial support for this work from the Natural Science Foundation of Jiangsu Province (No. BK20130654) and the National Natural Science Foundation of PR China (No. 81402900).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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