Fitoterapia 81 (2010) 437–442

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Preparation of icariside II from icariin by enzymatic hydrolysis method

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ARTICLE INFO

Article history:
Received 18 September 2009
Accepted in revised form 8 December 2009
Available online 22 December 2009

Keywords:
Herba Epimedii
Icariin
Icariside II
Enzymatic hydrolysis

ABSTRACT

It has been reported that icariin and icariside II, two flavonoid glycosides coming from herba epimedii, which have a closely structural relationship, show some pharmacological effects such as preventing osteoporosis, cancer and depression. The content of natural icariside II is very low in herba epimedii, but it is the main component in vivo after the administration of herba epimedii. More icariside II can be obtained from icariin by enzymatic hydrolysis method than by traditional isolation method. This study focuses on finding a simple and feasible method to prepare icariside II from icariin by enzymatic hydrolysis, so as to meet the request for further pharmacologic actions study. Icariin was obtained successively with 90% ethanol extraction, isolation on macroporous resin and purification on silica gel chromatography. Enzymatic hydrolysis conditions were tested for the bioconversion of icariin into icariside II by orthogonal array design. The structures of isolated icariin and produced icariside II were identified by UV, IR, ESIMS, ^{1}H NMR, ^{13}C NMR, and DEPT spectroscope. Enzymatic hydrolysis experiment showed that icariin could be transformed into icariside II with the action of β-glucosidase and the optimum reaction conditions were determined as follows: 50 °C, 0.2 M disodium hydrogen phosphate and citric acid buffer system (pH6.0), the ratio of icariin/enzyme is 1:1 and reaction time 5 h. By using this enzymatic condition, 95.5 mg icariside II (with the purity of 99.1%) was obtained eventually by transforming 200 mg icariin.

1. Introduction

Herba Epimedii (HE), a herb coming from dried aerial part of Epimedium sagittatum (Sieb. et Zncc.) Maxim., has traditionally been used as a tonic, aphrodisiac and antirheumatic drug in China for many years [1]. The main active constituents of HE are flavonoids from which more than 60 kinds of flavonoids have been identified, including icariin and icariside II (Fig. 1). Icariside II, one kind of metabolites from icariin, belongs to flavonoid glycoside. It has been reported that icariside II can enhance the differentiation and proliferation of osteoblasts, facilitate matrix calcification; meanwhile it inhibits osteoclastic differentiation in both osteoblast–preosteoclast coculture and osteoclast progenitor cell culture, and reduce the motility and bone resorption activity of isolated osteoclasts [2,3]. It shows relatively obvious inhibition on the ischemia/reperfusion-induced protein tyrosine kinase activation to protect human umbilical vein endothelial cells [4,5]. Icariside II has also been found to initiate the inhibition of COX-2/PGE_2 pathway and then induce apoptosis mainly via mitochondrial dependent pathway in PC-3 prostate cancer cells [6] and inhibit melanogenesis [7]. It suggests that the

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doi:10.1016/j.fitote.2009.12.006

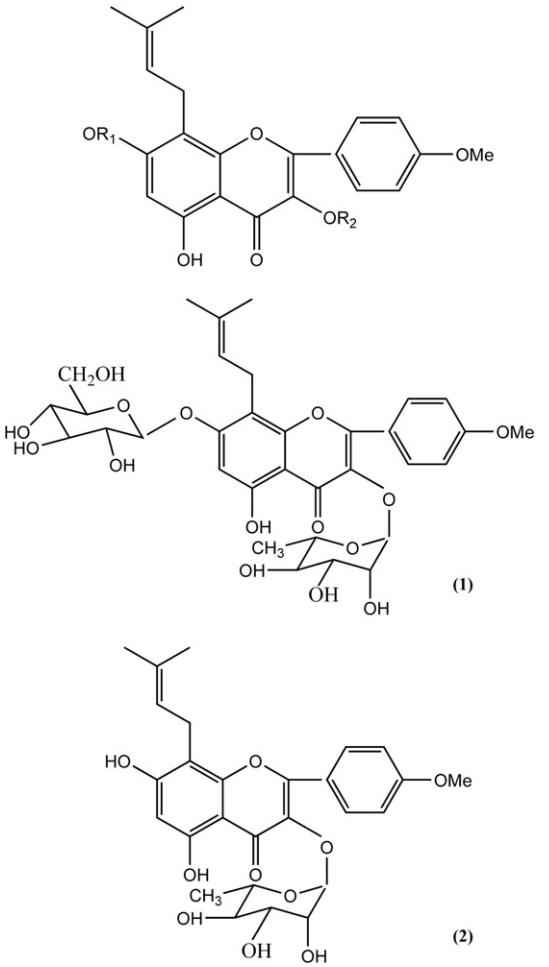

Fig. 1. Chemical structure of icariin and icariside II. R_1 = Glc R_2 = Rha Icariin (1). R_1 = H R_2 = Rha Icariside II (2).

potential use of HE as a therapeutic candidate against various diseases involve the overexpression of HIF-1α [8].

In the above pharmacological studies, most of icariside II was isolated from herba epimedii. It was reported that the content of natural icariside II in herba epimedii was low compared with that of icariin, with the content of 0.63 mg/g–10.86 mg/g for icariin and 0.17 mg/g–1.97 mg/g for icariside II respectively [9]. Due to the low content of natural icariside II in herba epimedii, only small amount of icariside II used for pharmacological study can be obtained by a series of complex extraction and isolation, such as several steps of extraction with different solvent, silica gel column chromatography, Sephadex LH-20 purification and preparative HPLC isolation [6,8]. It is difficult to meet the request for further pharmacologic actions study. Based on the close relationship between the structures of icariin and icariside II, and with the advantage of relatively high content and easy isolation procedure of icariin, enzymatic hydrolysis method can be adapted to transform icariin into icariside II. This method had been reported in some studies. For example, with the action of cellulase, response surface methodology was used to test the optimum hydrolysis conditions and the recommended conditions were an enzyme concentration of 7.5 mg/ml, pH5, 50 °C, and 12 h reaction time [7]. In spite of cellulase can be used to hydrolyze icariin into icariside II, another two hydrolysates were produced simultaneously, such as icariside I and icaritin, which may bring some difficult in further isolation of icariside II. According to the structural type of glucosyl in icariin, β-glucosidase may be the specific hydrolysis enzyme and it has been used to hydrolyse icariin into icariside II [2]. It is more convenient for the isolation and purification of icariside II. But currently no systemic study for the condition of β-glucosidase on transforming icariin into icariside II was reported. The present study describes the isolation of icariin from HE by using column chromatography and screening of the optimum enzymatic hydrolysis condition for transforming icariin into Icariside II with β-glucosidase.

2. Experimental

2.1. Apparatus

The extraction and isolation of icariin from HE were performed by a CTG-10 reflux extraction instrument (Hunan Hengyang Pharmacologic Manufactory) with a total capacity of 100 L, an AB-8 macroporous resin (Tianjing Nankai University Chemical Industry Factory) and silica gel column (Qingdao Hailang silica-gel Drier Factory) successively. The analysis of icariin and icariside II was carried out by a Agilent 1100 HPLC system (American Agilent) which consists of an AutoSample injection (G1313A), QuatPump (G1311A), Column Component (G1316A) and Photodiode Array Detector (G1315A) and Kromasil C_{18} column (4.6×250 mm, Phenomen tech Co, Ltd.). Agilent ChemStation Software (Rev. B. 03.02) was used to deal with the data. The reaction of enzymatic hydrolysis was carried out by a HH-W21-600 thermostat constant temperature implement (Shanghai Medical Thermostatic Equipment Factory). A TGL-16H high speed centrifuge (Zhuhai HEMA Co., Ltd) was used to treat the samples. Qingyang II melt point apparatus (Jinjiang Electric instrument Factory), Finnigan TSQ Quantum Discovery Max Mass Spectrum (Thermo Electron Corporation), Nicolet 8700 FT-IR (Thermo Scientific Instrument) and Bruker Avance 400 NMR spectrometer (Swiss Avance) were used for structure identification.

2.2. Reagents and materials

Ethanol, *n*-butanol, ethyl acetate and chloroform used for extraction and isolation were of an analytical grade. Methanol used for HPLC analysis was an HPLC grade reagent. β-glucosidase was purchased from Fluka (content of 8.92 u/mg). Standard icariin was purchased from Institute for the control of Pharmaceutical and Biological Products (ICPBP).

The Herba Epimedii was purchased from Anhui Guotou Chinese Medicine Company and authenticated by Professor Liu Shoujin, Department of medical plant, Anhui University of Traditional Chinese Medicine, as dried aerial part of Epimedium sagittatum (Sieb. et Zncc.) Maxim.

2.3. Extraction and isolation of icariin

HE (5 kg) was extracted with 90% ethanol (50 L) for 1 h under reflux. The extraction procedure was repeated twice.

The extracts were combined together and concentrated to dryness, then extracted with watered-saturated *n*-butanol for 3 times to obtain crude extracts. The crude extracts were dissolved in boiling distilled water, then subjected to an AB-8 macroporous resin column (100 cm × 5 cm) and eluted with distilled water and 70% ethanol successively. The 70% ethanol eluting was collected and evaporated to dryness to yield Herba Epimedii total flavonoids, and extracted with ethyl acetate for 3 times. These ethyl acetate layers were combined and evaporated to dryness, then was dissolved in appropriate amount of methanol for further separation by silica gel chromatography column (65 cm × 3 cm), using chloroform–methanol (10:1 and 5:1 successively, v/v) system as elution. Each 250 ml elution was collected and monitored with the standard icariin as the control by TLC method as follows: HSGF254 plates (2.5 mm × 7.5 mm), developing with ethyl acetate–butanone–formic acid–water (10:1:1:1, v/v/v/v) and photographed under an ultraviolet lamp with 254 nm wavelength. The fractions containing icariin were combined and evaporated to dryness. The purity analysis of isolated icariin was performed on a Kromasil® C_{18} column (250 mm × 4.6 mm, i.d. 5 μm) at 30 °C, using an isocratic elution of methanol–water (77:23, v/v) as mobile phase, with a flow rate of 1.0 ml/min. The wavelength of detector was set at 270 nm. The structural identification of icariin was carried out by UV, IR, ESIMS, melt point, ^{1}H NMR and ^{13}C NMR.

2.4. Enzymatic hydrolysis of icariin into icariside II

2.4.1. Mono-factor experiment

5 mg icariin was suspended in 500 μl ethyl acetate and 1 mg β-glucosidase was dissolved in 1 ml buffer system comprising 0.2 M disodium hydrogen phosphate and citric acid (DHPCA) respectively. The reaction condition of mono-factor experiment enzymatic hydrolysis for icariin into icariside II was tested included reaction time, temperature, pH of DHPCA and the ratio of substrate/enzyme in reaction mixture. For the effect of each factor on enzymatic hydrolysis, other three factors were fixed and all the reactions were tested in duplicated. The final reaction product was dried by the N_2 blow, and dissolved in 400 μl methanol, and then, 10 μl filtrated solution was injected into HPLC system using a isocratic elution of MeOH–H_2O (77:23, v/v) as mobile phase on a Kromasil® C_{18} column at 1.0 ml/min flow rate and 270 nm wavelength. The content of icariside II was calculated by external standard method. The product of icariside II in each reaction was used to reflect the enzymatic hydrolysis effects.

2.4.2. Optimization of enzymatic hydrolysis of icariin into icariside II with orthogonal array design

To find effective enzymatic hydrolysis condition, an orthogonal array design experiment (OAD) ($L_{18}(3^4)$), including four-factors and three-levels OAD, was used to get the best reaction conditions. The factors and levels were chosen from the former mono-factor test results. In OAD experimental arrangement and analysis of reaction, SPSS software (Version 13.0) was used. Statistical significant was considered at $p < 0.05$. Significance of the different was evaluated by orthogonal design ANOVA method.

2.5. Preparative enzymatic hydrolysis of icariside II by optimum condition

200 mg icariin was transformed into icariside II according to screening optimized condition, and the produced icariside II was extracted from the reaction mixture with ethyl acetate, then dissolved in methanol and subjected to silica gel column, using $CHCl_3$–MeOH (10:1, v/v) as elution. This procedure was similar to the purification of icariin and identification of produced icariside II was carried out by UV, IR, ESIMS, melt point, ^{1}H NMR and ^{13}C NMR.

3. Results and discussion

3.1. Extraction and isolation of icariin

2.2 g icariin was obtained from 5 kg herba epimedii, with the purity of 98.5%, based on the ration of peak area. The identification of isolated icariin was carried out by melt point, ESIMS, UV, IR, ^{1}H NMR and ^{13}C NMR and compared with the data given in references [10,11]. The test results are as follows: yellow needle crystal, molecular formula, $C_{33}H_{40}O_{15}$; mp 228–230 °C; ESIMS, *m/z* (%): 677 $[M+H]^+$; UV λ_{max}^{MeOH}nm (lgε): 223 (sh, 4.30), 271(4.37), 300(3.85), 350(3.80); IR$\nu_{KBr}^{cm^{-1}}$: 3408, 1652, 1611, 1574, 1429, 1363, 837; ^{1}H NMR (DMSO-d_6, 400 MHz): 0.79(3H, d, *J* = *6.0 Hz H-6´´), 1.60(3H, s, H-14), 1.69(3H, s, H-15), 3.49(2H, m, H-11), 3.86(3H, s, OCH$_3$), 5.06 (1H, d, J* = 5.2 Hz H-1´´´), 5.13(1H, t, *J* = 7.0 Hz H-12), 5.29(1H, d, *J* = 1.6 Hz H-1´´), 6.64(1H, s, H-6), 7.14(2H, d, *J* = 9.2 Hz H-3´ H-5´), 7.91(2H, d, *J* = 9.2 Hz H-2´ H-6´), 12.57(1H, s, 5-OH), 3.10–5.36 (protons in rhamnose). ^{13}C NMR (DMSO-d_6, 100 MHz) data was shown in Table 1. (Table 1)

3.2. Results of mono-factor experiments

By using β-glucosidase, icariin could be transformed into icariside II. Fig. 2 shows the HPLC chromatography of icariin solution and its enzymatic hydrolysis product, which contains icariside II. (Fig. 2) The effect of reaction time, temperature, pH value and of the ratio of substrate/enzyme was shown in Fig. 3. The content of icariside II increased with the reaction time going on and reached its maximum at 5 h–6 h, then fell down slightly. Therefore, the best reaction time is 5 h. The content of icariside II increased remarkably with the reaction increasing temperature from 30 °C and reached its maximum at 40 °C, then decreased slowly with the temperature increasing. The content of icariside II increased with the buffer pH increasing and reached the peak value at 6.0, then decreased remarkably. The optimum pH of the reaction was found to be 6.0. For the ratio of substrate/enzyme, the content of icariside II increased with the rising ratio of icariin/β-glucosidase in the mixture and reached its maximum at 1:3, and then fell down slightly. (Fig. 3)

3.3. The result of enzymatic hydrolysis by OAD

Eighteen experiments were performed in duplicated. The selected four factors were reaction time (A), reaction temperature (B), pH of the buffer (C), and the ratio of substrate/enzyme (D). The factors and the levels tested are shown in Table 2. The content of icariside II was analyzed by HPLC

Table 1
^{13}C NMR data of isolated Icariin and enzymatic hydrolyzed Icariside II.

Icariin			Icariside II		
C	δ c	DEPT	C	δ c	DEPT
2	157.24	C	2	156.64	C
3	134.62	C	3	134.41	C
4	178.25	C	4	177.93	C
5	160.48	C	5	161.62	C
6	98.61	CH	6	98.31	CH
7	161.37	C	7	161.23	C
8	108.30	C	8	105.89	C
9	152.98	C	9	153.74	C
10	105.57	C	10	104.13	C
11	21.37	CH_2	11	21.11	CH_2
12	122.10	CH	12	122.25	CH
13	131.02	C	13	130.92	C
14	25.38	CH_3	14	25.33	CH_3
15	17.79	CH_3	15	17.69	CH_3
1′	122.22	C	1′	122.38	C
2′, 6′	130.49	CH	2′, 6′	130.32	CH
3′, 5′	114.03	CH	3′, 5′	113.98	CH
4′	159.04	C	4′	158.81	C
OCH_3	55.45	CH_3	OCH_3	55.42	CH_3
1″	101.97	CH	1″	101.93	CH
2″	69.66	CH	2″	70.03	CH
3″	70.03	CH	3″	70.31	CH
4″	70.63	CH	4″	71.12	CH
5″	70.31	CH	5″	70.57	CH
6″	17.39	CH_3	6″	17.38	CH_3
1‴	100.55	CH			
2‴	73.33	CH			
3‴	76.59	CH			
4‴	71.10	CH			
5‴	77.15	CH			
6‴	60.63	CH_2			

method according to the mono-factor tests. Table 3 lists the data of the orthogonal design ANOVA table of these experiments. (Tables 2 and 3)

The results shown in Table 3 indicate that the reaction temperature and pH value have significant effects ($p<0.05$) on the content of icariside II in enzymatic product. However, the effects of other two factors were slight and insignificant ($p>0.05$). As seen from Table 3, we can find that the influence to the content of icariside II decreases in the order: B> C> D> A according to the p values. According to the results of the OAD experiments, the optimized conditions for transforming icariin into icariside II was as following: reaction time 5 h, 50 °C, pH 6.0 and the ratio of substrate/enzyme was 1:1.

3.4. Preparative enzymatic hydrolysis of icariside II by optimum condition

200 mg icariin and 200 mg β-glucosidase were mixed in 1000 ml DHPCA (pH6.0), and then incubated in 50 °C water bath for 5 h. The final reaction mixture was extracted with ethyl acetate for three times and then evaporated the combined ethyl acetate layer to dryness under reduced pressure. HPLC analysis showed that most icariin (98%) had been transformed into icariside II. The residue was dissolved in 10 ml methanol and subjected to silica gel column chromatography, using $CHCl_3$-MeOH (10:1) as elution to isolate icariside II. Finally, 95.5 mg icariside II was obtained with the purity of 99.1%. The identification of produced icariside II was carried out by melt point, ESIMS, UV, IR, ^1H NMR and ^{13}C NMR and compared with the data given in references [10,11]. The test results are as follows:

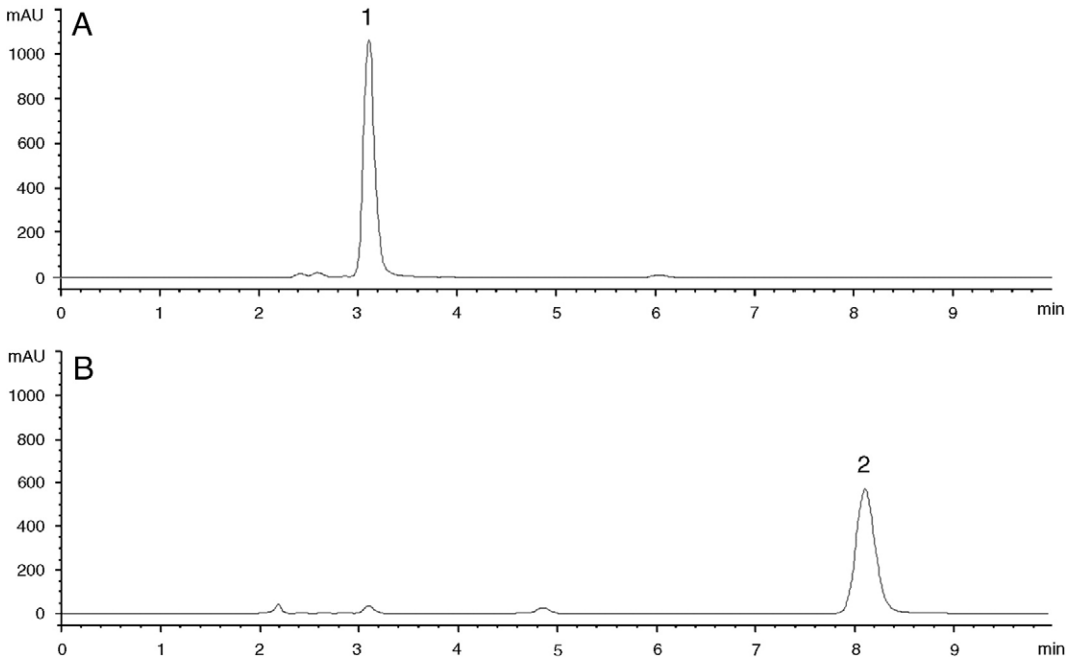

Fig. 2. HPLC chromatography of icariin enzymatic hydrolysis. A and B correspond to the HPLC chromatography before and after enzymatic hydrolysis respectively. Peak 1 and peak 2 correspond to icariin and icariside II respectively. Enzymatic hydrolysis conditions: 2 μl icariin solution (10 mg/ml), 20 μl β-glucosidase solution (1 mg/ml), 50 °C, pH6.0 and 5 h. HPLC analysis conditions: column, Kromasil® C18; mobile phase, MeOH–H_2O (77:23); flow rate, 1.0 ml/min; detection wavelength, 270 nm.

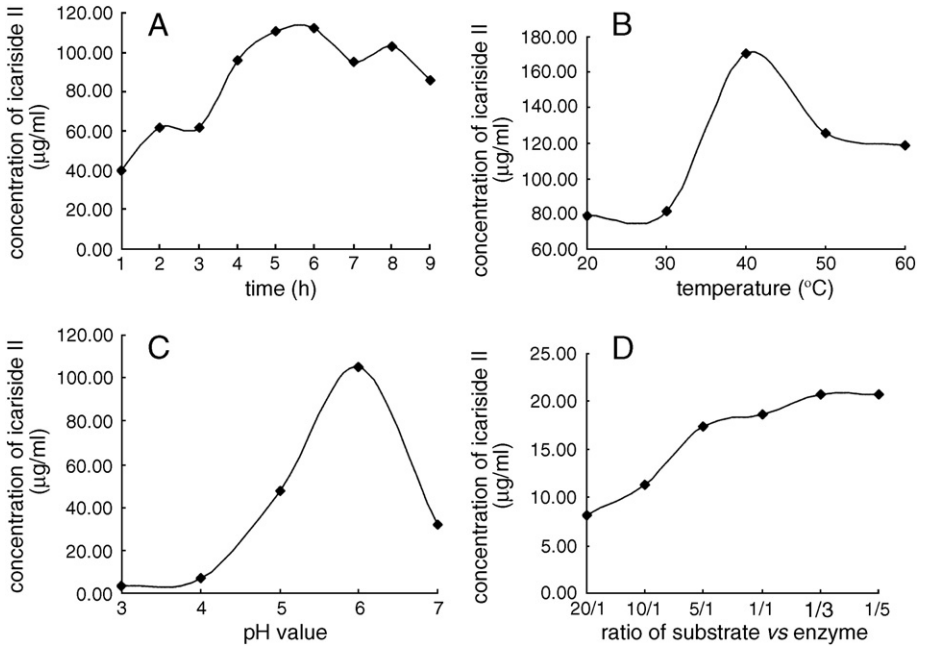

Fig. 3. Results of mono-factor enzymatic hydrolysis. A: Effect of reaction time. 20 μl icariin solution, 20 μl β-glucosidase solution and 960 μl DHPCA (pH6.0) were added in a 1.5 ml eppendorf tube, incubated in a water bath at 40 °C for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h and 9 h respectively. B: Effect of reaction temperature. 20 μl icariin solution, 20 μl β-glucosidase solution and 960 μl DHPCA (pH6.0) were added in a 1.5 ml eppendorf tube, incubated in a water bath at 20 °C, 30 °C, 40 °C, 50 °C and 60 °C respectively for 5 h. C: Effect of pH value of buffer. The reaction was performed by adding 20 μl icariin solution, 20 μl β-glucosidase solution and 960 μl DHPCA (pH3.0, 4.0, 5.0, 6.0 and 7.0 respectively) at 40 °C for 5 h. D: Effect of the ratio of substrate/enzyme. 2 μl icariin solution was mixed with 1, 2, 4, 20, 60 and 100 μl β-glucosidase solution (substrate : enzyme = 20:1, 10:1, 5:1, 1:1, 1:3 and 1:5) respectively. DHPCA (pH6.0) was added to the mixture at a final volume of 1 ml. Then it was incubated in a water bath at 40 °C for 5 h.

green-yellow needle crystal, molecular formula, $C_{27}H_{30}O_{10}$; mp 205–207 °C; ESIMS, *m/z* (%): 515 $[M+H]^+$; UV λ_{max}^{MeOH}nm (lgε): 223 (sh, 4.28), 271(4.31), 300(3.84), 350(3.80); IR$\nu_{KBr}^{cm^{-1}}$: 3420, 1651, 1597, 1511, 1440, 1374, 837; ^{1}H NMR (DMSO-d_6, 400 MHz): 0.78(3H, d, *J* = 6.0 Hz H-6˝), 1.61(3H, s, H-14), 1.70 (3H, s, H-15), 3.50(2H, m, H-11), 3.85(3H, s, OCH_3), 5.16(1H, *t*, *J* = 6.0 Hz H-12), 5.27(1H, d, *J* = 1.6 Hz H-1˝), 6.32(1H, s, H-6), 7.13(2H, d, *J* = 9.2 Hz H-3´ H-5´), 7.87(2H, d, *J* = 9.2 Hz H-2´ H-6´), 10.84(1H, br. s, 7-OH), 12.53(1H, s, 5-OH), 3.04–5.15 (protons in rhamnose). ^{13}C NMR (DMSO-d_6, 100 MHz) data was shown in Table 1.

4. Conclusions

It has been reported that several glycolytic enzymes, including β-glucosidase, cellulase, nariginase and hesperidinase, could be used for the bioconversion of icariin into icariside II, and response surface methodology was used to test the optimum conditions of cellulase hydrolysis, the recommended conditions were an enzyme concentration of 7.5 mg/ml, pH5, 50 °C, and 12 h reaction time [7]. In above studies, three kinds of flavonoids were produced in the enzymatic hydrolysis reaction simultaneously, further isolation of icariside II must be carried out by using a medium-pressure liquid chromatography system, an elution system composed of distilled water and acetonitrile, as well as an ultra pack SI-40D column. In our study, high purity of icariside II can be obtained easily from the final reaction product by solvent extraction and common-pressure column chromatography method. The same factors (reaction time, temperature, pH value of buffer and the ratio of substrate/enzyme) were tested by orthogonal array design and the results showed that: with the action of β-glucosidase, icariin can be transformed into icariside II, and the optimized reaction conditions were 50 °C, pH6.0, reaction time at 5 h and icariin/β-glucosidase (1:1). 95.5 mg icariside II was obtained successfully by transforming 200 mg icariin using the conditions.

Table 2
The factors and levels of the OAD (L_{18} (3^4)).

Level	Factor			
	A (time, h)	B (temperature, °C)	C (pH value)	D (ratio of substrate/enzyme)
1	5	30	5	1:1
2	6	40	6	1:3
3	7	50	7	1:5

Acknowledgements

This study was supported by the National Natural Science Foundation of China for Dr. Shangquan Liu (No. 30840106). The authors also wish to acknowledge the assistance of Professor Jinqi Liu at Anhui Traditional Chinese Medicine College for his NMR spectral analysis.

Table 3
Orthogonal design ANOVA of icariin enzymatic hydrolysis.

Source	Sum of square (ss)	Degree of freedom (*df*)	*F*-ratio	$F_{0.05}$	*p* value	Type of effect
(A) Time	160.224	2	0.634	19	0.553	
(B) Temperature	4572.495	2	18.081	19	0.01	Significant
(C) pH	1073.549	2	4.245	19	0.05	Significant
(D)ratio	420.914	2	1.664	19	0.243	

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