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Biotransformation of Glucosinolates Epiprogoitrin and Progoitrin to (*R*)- and (*S*)-Goitrin in *Radix isatidis*

Zhiyong Xie,[†,§] Yanhong Shi,[†,§] Zhengtao Wang,[‡] Rui Wang,[*,†,‡] and Yiming Li[*,†]

[†]School of Pharmacy and [‡]Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ABSTRACT: *Radix isatidis* is an important traditional Chinese medicine with antiviral efficacy. (*R*)- and (*S*)-Goitrin are its main bioactive constituents in the 2010 edition of the Chinese Pharmacopoeia. (*R*)- and (*S*)-Goitrin are the breakdown products of epiprogoitrin and progoitrin from *R. isatidis*. The biotransformation of glucosinolates epiprogoitrin and progoitrin to (*R*)- and (*S*)-goitrin, however, is still unclear. In the current paper, the biotransformation of glucosinolates was studied. First, the high-performance liquid chromatography methods to analyze glucosinolates and their breakdown products were developed. Then, the biotransformation of individual glucosinolates such as epiprogoitrin and progoitrin was investigated under different pH conditions. Lastly, their biotransformation under five extraction environments was studied. The results showed that (*R*)- and (*S*)-goitrin were the most noteworthy breakdown products. Their relative transformation rates were about 70−80%, and the influence of different extraction environments on the transformation rate was not significant. These results would serve as a theoretical basis for industrial production and quality control and would be helpful for further studies on the biotransformation of glucosinolates.

KEYWORDS: Biotransformation, gucosinolates, *Radix isatidis*

INTRODUCTION

Radix isatidis is the dry root of *Isatis indigotica* Fort. that belongs to the cruciferous isatis plant genus. It is one of the traditional Chinese herbs for antivirus and is always recorded in the 1985−2010 editions of the Chinese Pharmacopoeia. So far, the known chemical constituents from *R. isatidis* are alkaloids, nucleosides, organic acids, glucosinolates and their breakdown products, polysaccharides, and so on. Its pharmacological effects have antiviral, anticancer, and antiendotoxin activities, etc.[1−3]

The antiviral effect of the water extraction fraction of *R. isatidis* has attracted the attention of researchers in many of the chemical constituents and pharmacological effects above, especially that of (*R*)- and (*S*)-goitrin that are the breakdown products of glucosinolates.[4] Thus, (*R*)- and (*S*)-goitrin have been used as the markers of antiviral efficacy in *R. isatidis* in the 2010 edition of the Chinese Pharmacopoeia. We have separated the main glucosinolates, epiprogoitrin and progoitrin, from *R. isatidis*.

(*R*)- and (*S*)-Goitrin are transferred by their glucosinolate prototypes, epiprogoitrin and progoitrin, on the effect of endogenous myrosinase (Figure 1).[5−9] In general, the breakdown pathway of glucosinolates is related to their structures, the pH of the external environment, and other cofactors such as ferrous ion and epithiospecifier protein (ESP). The breakdown products are usually isothiocyanates, thiocyanates, 5-alkyl-oxazolidine-2-thiones, indole-3-carbinols, nitriles, and epithioalkylnitriles.[10−15] Hence, these factors could further complicate the biotransformation of glucosinolates.

The acidic condition of pH 5.0 with or without ferrous sulfate and cysteine and the neutral condition of pH 6.5 are used generally for the breakdown of glucosinolates;[6,15−18] however, the effects of different pH conditions on their breakdown are unclear. Although some biotransformation under specific pH conditions is known, it is still unclear how the biotransformation is on the extraction environment, whether the different extraction environments lead to the different breakdown pathways, and whether the biotransformation on the extraction environment is identical with that on the specific pH condition.

The current study aims to determine the biotransformation of the individual glucosinolates, epiprogoitrin and progoitrin, under different pH conditions and then elucidate that on the extraction environments to instruct the extraction of *R. isatidis* better.

MATERIALS AND METHODS

Materials and Reagents. The roots of *I. indigotica* Fort. were collected from Bozhou, Anhui Province, China, and were identified by Dr. Lihong Wu of the Shanghai R&D Center for Standardization of Chinese Medicines. A voucher specimen was deposited in the Herbarium of Department of Traditional Chinese Medicine Chemistry, School of Pharmacy, Shanghai University of Traditional Chinese Medicine. Potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), sodium acetate (NaAc), glacial acetic acid (HAc), ammonium chloride (NH_4Cl), concentrated ammonia water ($NH_3 \cdot H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), methylene chloride, methanol, and 95% ethanol were all of analytical grade, and L-cysteine (Cys) was of biochemical grade. The above reagents were purchased from the Sinopharm Chemical Reagent Co. (Shanghai, CN). Myrosinase (EC3.2.1.147, 280 u g^{-1}) was obtained from white mustard seed and purchased from Sigma-Aldrich Co. (St. Louis, MO). Epiprogoitrin, progoitrin, (*R*)- and (*S*)-goitrin, and (*R*)-goitrin and (*S*)-goitrin were prepared in the Lab of Department of Traditional Chinese Medicine Chemistry, School of Pharmacy, Shanghai University of Traditional Chinese Medicine, with

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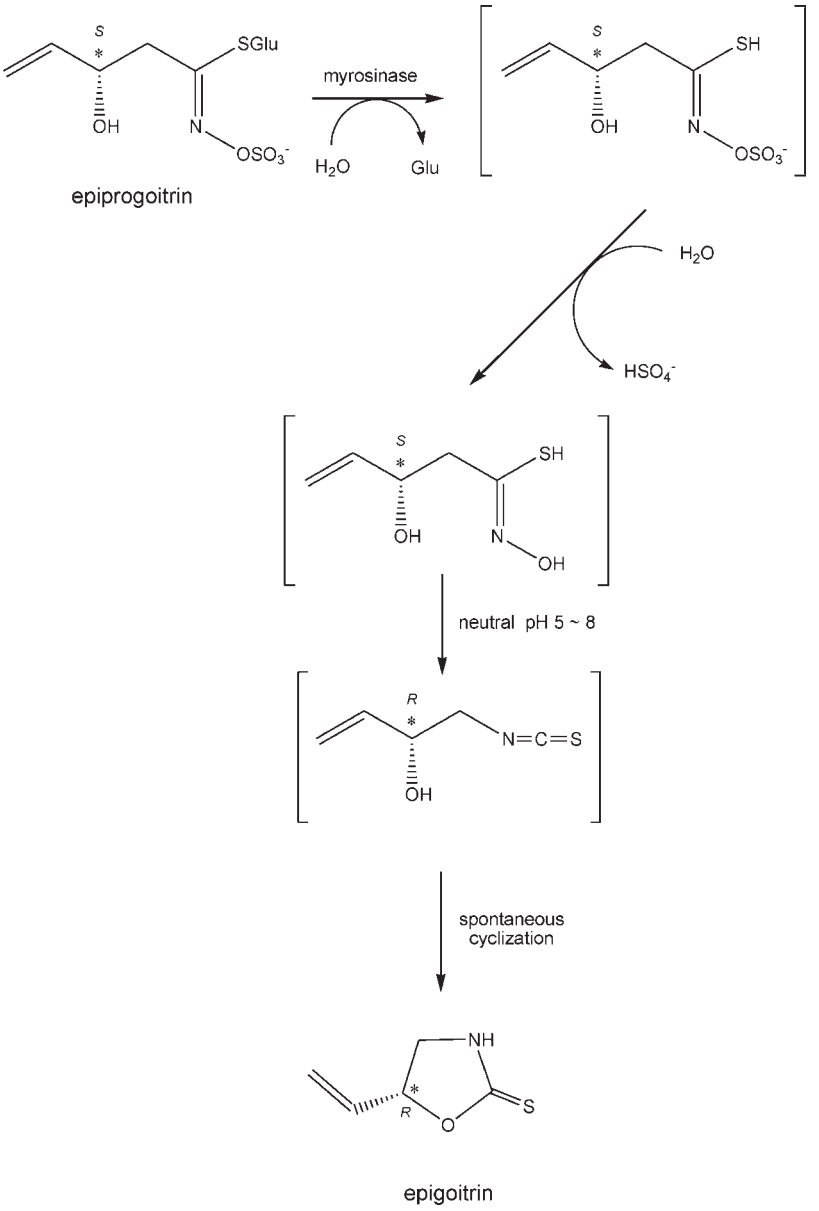

Figure 1. Breakdown pathway of epiprogoitrin. That of progoitrin is analogous to it except that the chiral carbon configuration in side chain is reversed.

98% purity as determined by high-performance liquid chromatography (HPLC). The aqueous solutions of myrosinase, the glucosinolates, and their breakdown products were stored at 4 °C.

HPLC Analytical Methods. The Agilent 1100 series liquid chromatograph was equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment, and a diode array detector. All solutions were filtered through a 0.45 μm filter before use.

HPLC methods for glucosinolate analysis were as follows: A reversed-phase Ultimate XB-C_{18} column (250 mm × 4.6 mm, 5 μm) was used to analyze the glucosinolates at 229 nm and 30 °C. The flow rate was 1.0 mL min^{-1}. The analysis time was 25 min. The mobile phase was prepared from a 0.02% aqueous phosphoric acid water solution (phase A) and acetonitrile (phase B). The gradient program was as follows: 100% A−0% B for 5 min, increased to 85% A−15% B from 5 to 20 min, returned to the initial condition 100% A−0% B at 23 min, and equilibrated for 2 min.

HPLC methods for breakdown product analysis were as follows: A reversed-phase Ultimate XB-C_{18} column (250 mm × 4.6 mm, 5 μm) was used to analyze the breakdown products at 245 nm and 30 °C. The flow rate was 1.0 mL min^{-1}. The analysis time was 30 min. The mobile phase was prepared from water (phase A) and acetonitrile (phase B). The gradient program was as follows: increased from 70% A−30% B to 50% A−50% B from 0 to 25 min, returned to the initial condition 70% A−30% B at 28 min, and equilibrated for 2 min.

Chiral HPLC methods for breakdown product analysis were as follows: A chiral SHISEIDO CD-Ph column (250 mm × 4.6 mm, 5 μm) was used to analyze the breakdown products at 245 nm and 40 °C. The flow rate was 0.5 mL min^{-1}. The analysis time was 20 min. The mobile phase was prepared from water (phase A) and methanol (phase B). The isocratic elution was used with 70% A−30% B.

Breakdown of Two Main Individual Glucosinolates. A suitable amount of epiprogoitrin and progoitrin was dissolved in distilled water to constitute their water solutions, and the solutions were stored for 1 month at room temperature (about 35 °C in August). Then, the solutions were analyzed by the above common and chiral HPLC methods.

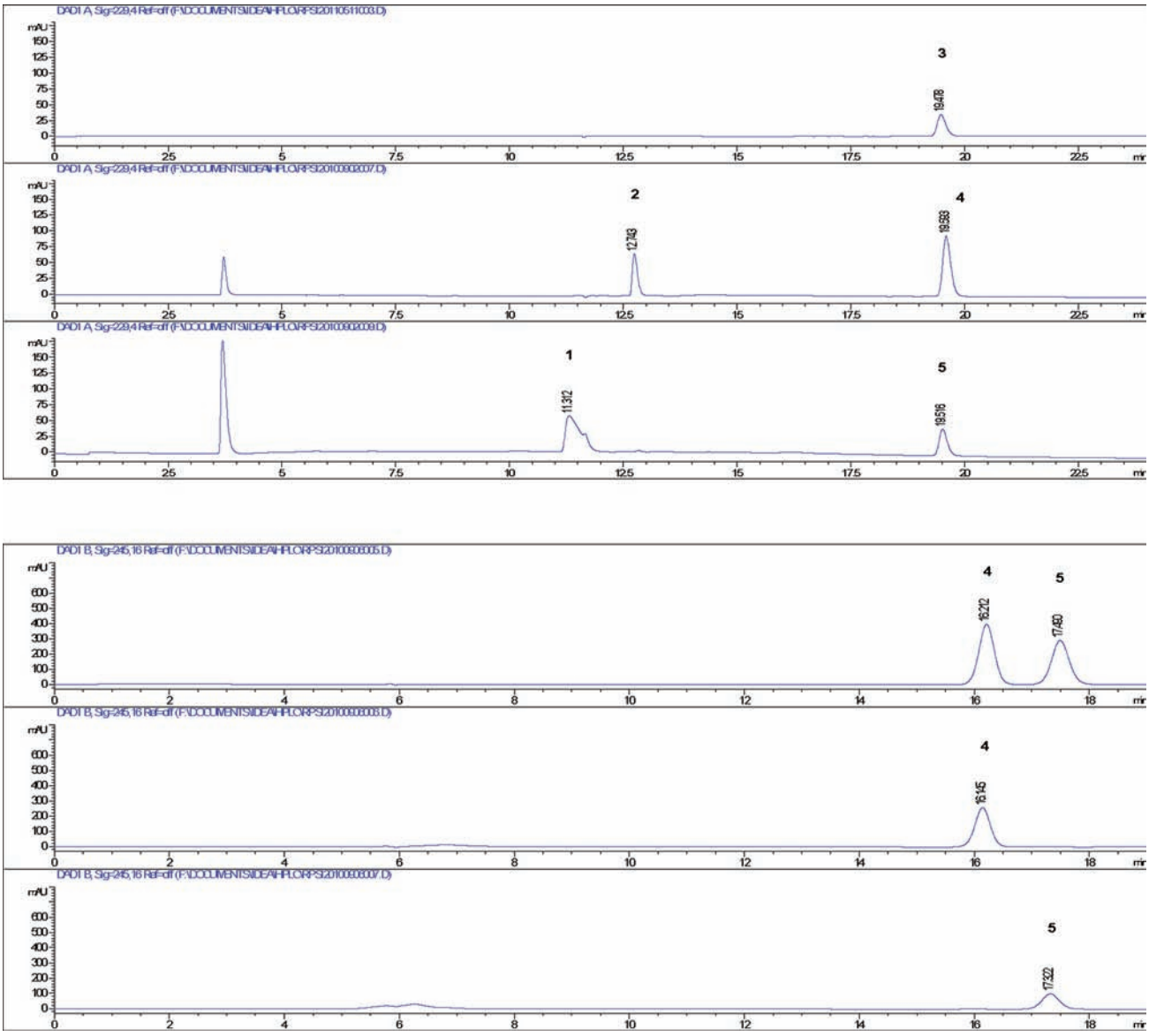

Figure 2. Top: Common HPLC chromatograms of (*R*)- and (*S*)-goitrin in methanol (above), epiprogoitrin with partial hydrolysis in water (middle), and progoitrin with partial hydrolysis in water (below). Peaks: 1, progoitrin; 2, epiprogoitrin; 3, (*R*)- and (*S*)-goitrin; 4, (*R*)-goitrin; and 5, (*S*)-goitrin. Bottom: Chiral HPLC chromatograms of (*R*)- and (*S*)-goitrin in methanol (above), epiprogoitrin with partial hydrolysis in water (middle), and progoitrin with partial hydrolysis in water (below). Peaks: 4, (*R*)-goitrin; and 5, (*S*)-goitrin.

The six pH conditions were designed based on commonly used pH conditions. The practical process was as follows. One milliliter of buffer solution with corresponding pH [buffer solution 1 (BS 1), pH 3.0 NaAc−HAc; BS 2, pH 3.0 NaAc−HAc, $FeSO_4$; BS 3, pH 5.0 NaAc−HAc; BS 4, pH 5.0 NaAc−HAc, $FeSO_4$; BS 5, pH 6.5 KH_2PO_4−NaOH; BS 6, pH 8.0 KH_2PO_4−NaOH; BS 7, pH 10.0 NH_4Cl−$NH_3 \cdot H_2O$; and BS 8, pH 12.0 KH_2PO_4−NaOH] was aspirated and 5 μL of 0.5 mol L^{-1} ferrous sulfate solution, and 10 μL of 0.5 mol L^{-1} L-cysteine solution was added into BS 2 and 4 to reaction concentrations of 2.5 and 5.0 mmol L^{-1}, respectively. Ten microliters of 8.93 mg mL^{-1} myrosinase solution (2.5 u mL^{-1}) and 10 μL of 2.00 mg mL^{-1} glucosinolate solutions (epiprogoitrin and progoitrin) were added into BS 1−8, respectively. The reaction solution was bathed for 1 h at 37 °C. The solution after reaction was extracted twice with 1 mL of methylene chloride per time, and the organic phase was incorporated. The methylene chloride was blown with nitrogen gas at 40 °C. The residue was reconstituted with 0.5 mL of methanol.

Breakdown of Individual Glucosinolates on Five Extraction Environments. Five extraction environments were designed in terms of the extraction conditions of glucosinolates and their breakdown products as follows: environment 1 (E 1), water extraction for 2 h at 37 °C; E 2, 35% ethanol extraction for 4 h at 37 °C; E 3, 70% ethanol extraction for 30 min at 80 °C; E 4, 35% ethanol extraction for 30 min at 85 °C; and E 5, water extraction for 30 min at 90 °C. Four grams of plant material of *R. isatidis* was extracted with 40 mL of the above solvent. After extraction, the aqueous solutions in E 1 and 5 were filtered; however, the ethanol solutions in E 2, 3, and 4 were evaporated under vacuum conditions after filtration, and the residue was reconstituted with an identical volume of water. The five solutions were all diluted 100-fold.

One milliliter of aqueous solution diluted 100-fold from E 1 and 2 was treated with methylene chloride and methanol based on the same method described above to obtain the methanol solutions of breakdown products. The double of 1 ml of aqueous solutions diluted 100-fold from E 3, 4, and 5 was added 10 μL of myrosinase solution, and to one of them

were added 5 μL of ferrous sulfate solution and 10 μL of L-cysteine solution. The reaction solution was bathed for 1 h at 37 °C. Next, the process was identical with that above.

Qualitative Determination of Dynamic Process of Myrosinase-Glucosinolate Reaction. Ten microliters of myrosinase solution was added into 1 mL of buffer solution at pH 6.5 without epiprogoitrin, and 10 μL of epiprogoitrin solution was added into 1 mL of buffer solution at pH 6.5 without myrosinase. The reaction solution was bathed for 2 h at 37 °C. Next, the process was identical with that above.

Table 1. Transformation Rates of Two Main Individual Glucosinolates

	epiprogoitrin	progoitrin
pH 3.0	11% (*R*)-goitrin	7% (*S*)-goitrin
pH 3.0 $FeSO_4$, Cys	ND[a]	ND
pH 5.0	73% (*R*)-goitrin	66% (*S*)-goitrin
pH 5.0 $FeSO_4$, Cys	ND	ND
pH 6.5	81% (*R*)-goitrin	80% (*S*)-goitrin
pH 8.0	81% (*R*)-goitrin	80% (*S*)-goitrin
pH 10.0	86% (*R*)-goitrin	83% (*S*)-goitrin
pH 12.0	81% (*R*)-goitrin	75% (*S*)-goitrin

[a] Not detected. The transformation rates of epiprogoitrin or progoitrin were less than 2%; however, they should be viewed as estimated or not detected approximately (not actually below detection limit) because of variability of bioanalysis.

Ten microliters of epiprogoitrin solution and 10 μL of myrosinase solution were added into 1 mL of buffer solution at pH 6.5; 10 μL of epiprogoitrin solution, 10 μL of myrosinase solution, 5 μL of ferrous sulfate solution, and 10 μL of L-cysteine solution were added into 1 mL of buffer solution at pH 5.0; to a 35% ethanol extract solution at 85 °C diluted 100-fold was added 10 μL of myrosinase solution. The reaction solution was bathed for 1 h at 37 °C. The samples were taken at 0, 10, 20, 30, 40, 50, and 60 min, respectively, and were frozen immediately at −20 °C to check the reaction degree. Another three reactions without myrosinase were also done for 2 h at 37 °C to check the stability of glucosinolates.

RESULTS AND DISCUSSION

Breakdown of Individual Glucosinolates under Different pH Conditions. The partial hydrolysis of epiprogoitrin and progoitrin is shown in Figure 2, top and bottom. As compared with each other, we found that epiprogoitrin was hydrolyzed to (*R*)-goitrin and progoitrin to (*S*)-goitrin.

The breakdown of individual glucosinolates is shown in Table 1. The data indicated that all of the hydrolyses reached 100% completion. For breakdown of epiprogoitrin, if there was the existence of ferrous sulfate, no breakdown products were detected, and only varied amounts of (*R*)-goitrin were detected without the existence of ferrous sulfate. For the breakdown of progoitrin, if there was the existence of ferrous sulfate, no breakdown products were detected, and only varied amounts of (*S*)-goitrin were detected without the existence of ferrous sulfate.

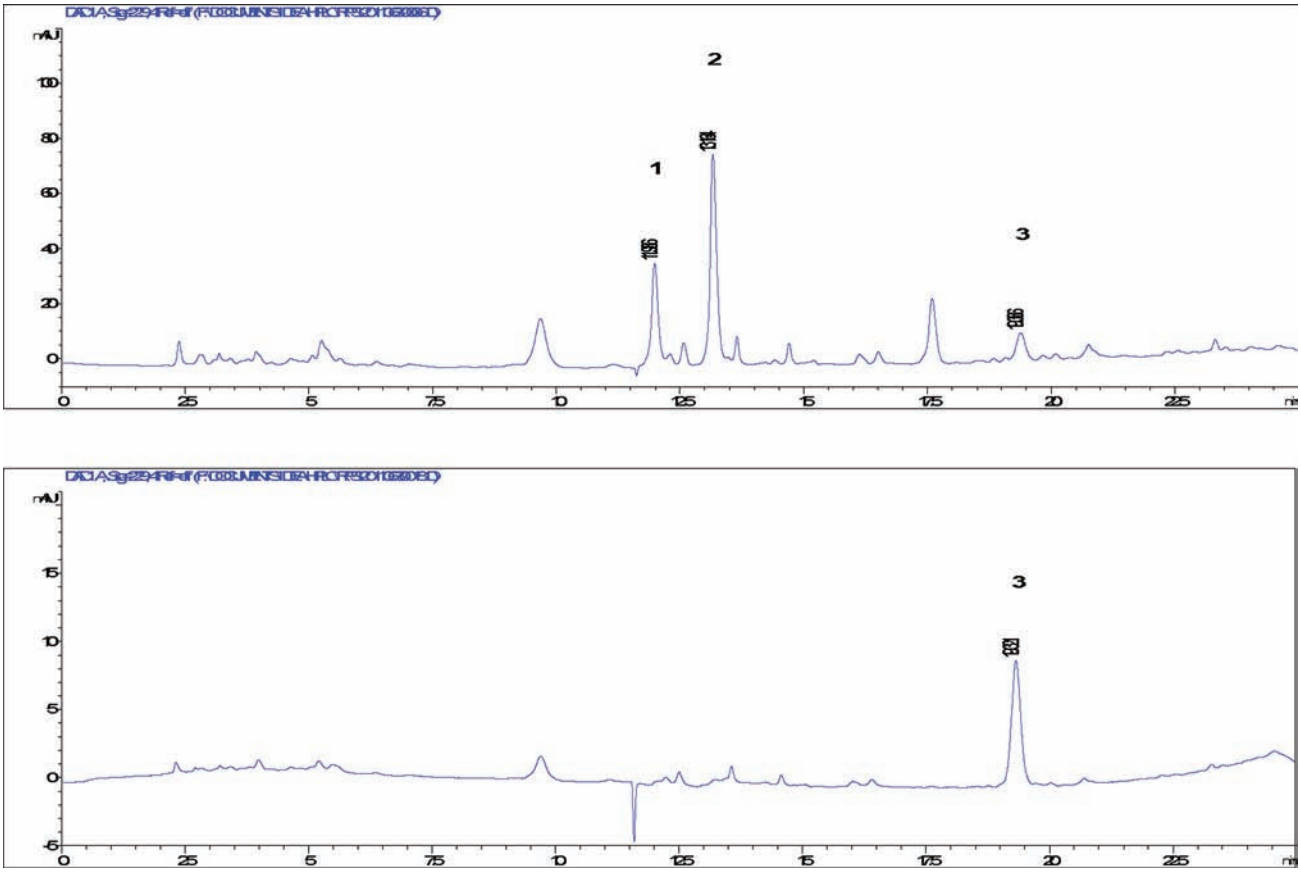

Figure 3. Top: HPLC chromatogram of 70% ethanol extract at 80 °C. Peaks: 1, progoitrin; 2, epiprogoitrin; and 3, (*R*)- and (*S*)-goitrin. Bottom: HPLC chromatogram of 70% ethanol extract at 80 °C after breakdown. Peak: 3, (*R*)- and (*S*)-goitrin.

Table 2. Transformation Rates of Individual Glucosinolates on Extraction Environments

	aqueous solution diluted 100-fold
water, 37 °C, 2 h	71% (*R*)- and (*S*)-goitrin[a]
35% ethanol, 37 °C, 4 h	71% (*R*)- and (*S*)-goitrin[a]
70% ethanol, 80 °C, 30 min	73% (*R*)- and (*S*)-goitrin
70% ethanol, 80 °C, 30 min $FeSO_4$, Cys	ND[b]
35% ethanol, 85 °C, 30 min	66% (*R*)- and (*S*)-goitrin
35% ethanol, 85 °C, 30 min $FeSO_4$, Cys	ND
water, 90 °C, 30 min	65% (*R*)- and (*S*)-goitrin
water, 90 °C, 30 min $FeSO_4$, Cys	ND

[a] The calculation of transformation rates was based on the content of the glucosinolates under 70% ethanol extraction environment at 80 °C. [b] Not detected. The transformation rates of epiprogoitrin or progoitrin were less than 2%; however, they should be viewed as estimated or not detected approximately (not actually below detection limit) because of variability of bioanalysis.

Thus, on the basis of the experiments, (*R*)- and (*S*)-goitrin were the most noteworthy breakdown products, with their transformation rates within about 70−80% between pH 5.0 and 10.0, and the chiral carbon in the R side chain of glucosinolates was not involved in the breakdown reaction, and its configuration remained unchanged.

Usually, many researchers focused on the conditions of pH 6.5 (neutral) and 5.0 with ferrous sulfate and cysteine (acidic).[6,16] Considering the misfit of myrosinase under extreme pH conditions and the possible pH ones in the practical processes, the pH conditions above were designed to investigate the biotransformation of glucosinolates.

Breakdown of Individual Glucosinolates on Five Extraction Environments. The HPLC chromatograms of 70% ethanol extract at 80 °C before and after complete breakdown are shown in Figure 3. Thus, the contents of epiprogoitrin, progoitrin, and (*R*)- and (*S*)-goitrin were 2.14, 5.72, and 0.21 mg g^{-1} or 5.01, 13.41, and 1.61 μmol g^{-1} in dry weight plant material, respectively. Epiprogoitrin and progoitrin were completely broken down to (*R*)- and (*S*)-goitrin after 1 h (Figure 3, bottom).

The breakdown of individual glucosinolates on five extraction environments is shown in Table 2. The data indicated that glucosinolates were broken down completely on water and 35% ethanol extraction environments at 37 °C, and glucosinolates were kept on high-temperature extraction environments because myrosinase was completely inactivated. All of the hydrolyses reached 100% completion. If there was the existence of ferrous sulfate, no breakdown products were detected, and only varied amounts of (*R*)- and (*S*)-goitrin were detected without the existence of ferrous sulfate.

The transformation rates on the five extraction environments were stable relatively and were not disturbed in terms of our experiments reaching about 70%. So, the influence of the different extraction environments on the transformation rate was not significant. These extraction environments were analogous to the conditions of pH 6.5 and 8.0. If glucosinolates needed to be transferred, water extraction at 37 °C was proposed; if they did not need to be transferred, 70% ethanol extraction at 80 °C was proposed.

The hot water extraction was the actual production process to obtain the water-soluble constituents from *R. isatidis* for antivirus purposes.[19] If glucosinolates were needed, usually 70% ethanol/methanol was used to extract the plant material at high temperatures because 70% alcohols and high temperature could inactivate myrosinase.[20] So, the five extraction environments above were designed to check the biotransformation of glucosinolates. The transformation rates of water and 35% ethanol extraction environments at 37 °C were just calculated based on the content of the glucosinolates under 70% ethanol extraction environment at 80 °C.

Qualitative Determination of Dynamic Process. Only the glucosinolate or myrosinase could not initiate the enzymic catalytic reaction, and they were stable in 2 h at 37 °C. The reaction time of 1 h was sufficient to complete the reaction. The reaction was completed after 30 min. The decrease in epiprogoitrin or/and progoitrin corresponded to the increase in (*R*)- or/and (*S*)-goitrin. So, (*R*)- and (*S*)-goitrin were the breakdown products of epiprogoitrin and progoitrin based on qualitative determination of dynamic process of the myrosinase−glucosinolate reaction.

In conclusion, (*R*)- and (*S*)-goitrin were the most noteworthy breakdown products. Their relative transformation rates were about 70−80%, and the influence of different extraction environments on the transformation rate was not significant. These results would serve as a theoretical basis for industrial production and quality control and would be helpful for further studies on the biotransformation of glucosinolates.

AUTHOR INFORMATION

Corresponding Author

*Tel: 86 21 51322191. Fax: 86 21 51322193. E-mail: ymlius@163.com (Y.L.). Tel: 86 21 51322181. Fax: 86 21 51322193. E-mail: ellewangtcm@gmail.com (R.W.).

Author Contributions

§These authors contributed equally to this paper.

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