Asian Journal of Chemistry; Vol. 24, No. 10 (2012), 4487-4490

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Successive Extraction of Glycyrrhizic Acid and Liquiritin from Licorice with Cellulase and Pectinase

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(*Received*: 3 August 2011; *Accepted*: 7 May 2012) AJC-11418

A simple and environment amicable method for successive extraction of glycyrrhizic acid (GA) and liquiritin from licorice root was developed using cellulase and pectinase for pretreatment (licorice root: cellulose: pectinase = 650:2:1, pH 5.0-6.0, 50 ºC, 160-180 min). 10 % ammonia as a solvent was verified to be more effective than 10 % ethanol for extracting glycyrrhizic acid from licorice based on pretreatment with pectinase and cellulose, resulting in 45.61 % of glycyrrhizic acid purity. Subsequently, 10 and 60 % of aqueous ethanol solution were recruited to successively elute glycyrrhizic acid from the enzyme-digested extracts in D101 macroporous adsorption resin (MAR) and the glycyrrhizic acid purity was increased to 86.33 %. After extraction of glycyrrhizic acid, the residue of licorice root was treated with the above two kinds of enzymes, extracted with 90 % ethanol, 0.32 % of liquiritin purity was achieved. Eluted by macroporous adsorption resin AB-8, the purity of liquiritin was up to 2.78 %. Purified by polyamide, the purity was elevated to 10.89 %. Compared with previous protocols, this method is benign to the environment and the employed enzymes and macroporous resin are suitable for large-scale separation of glycyrrhizic acid and liquiritin from licorice root successively.

Key Words: Licorice, Liquiritin, Glycyrrhizic acid, Cellulase, Pectinase, Extract successively.

INTRODUCTION

Four hundred years ago, human beings have made use of licorice (gancao in Chinese, *Glycyrrhiza uralensis* Fisch, *Glycyrrhiza inflata* Bat., *Glycyrrhiza glabra* L.) as a remedy in China. Licorice is recruited in both western medicine and Chinese herbal medicine[1]. In China, an old saying "nine of ten prescriptions contain licorice (Shi Fang Jiu Cao in Chinese)" indicates the wide employment of licorice in compound prescription. Glycyrrhizic acid (GA) and licorice favonoids (LF) especially liquiritin therein, are main active ingredients in licorice and are well-known for their versatile functions such as antiinflammation, antiulcer, antihepatotoxic, antiAIDS and antiSARS-coronavirus[2-4]. Moreover, glycyrrhizic acid is a popular food and cosmetics additive. The sweet taste of the root is just attributing to glycyrrhizic acid, which is reported to be 50-170 times sweeter than sucrose[1]. For liquiritin in favonoids, it was revealed to exert antidepressant-like effect by anti-oxidative function in animal and to increase 5-HT and norepinephrine (NE) in the mouse hippocampus[5,6]. So far, various methods have been developed for the extraction of glycyrrhizic acid or liquiritin from licorice, including microwave-assisted extraction (MAE)[7], aqueous two-phase system[8], pressurized hot water extraction[9], subcritical water[10], high-speed counter-current chromatography, *etc*.[11]. However, these methods are not very applicable in industry due to high cost or low efficiency. The employment of enzymes for volatiles extraction from celery seeds has been processed with an increase in oil yield[12], so alike was the polysaccharide extraction from pumpkin[13]. Till now, almost no attempt has been done for extracting liquiritin from licorice by using enzyme, let alone the successive extraction of glycyrrhizic acid and liquiritin. To extract glycyrrhizic acid and liquiritin successively, efficiently and environmental friendly, a serial processes were developed in this work by employing enzymes (cellulase and pectinase) to degrade the cell wall, followed by elution with macroporous adsorption resins (MARs) or polyamide in succession.

EXPERIMENTAL

Herbs and reagents: Licorice was purchased from TongRenTang Co. Ltd., Beijing, China. The botanical origin was authenticated by Prof. Xi-zhen Ge in Biochemical Engineering College, Beijing Union University. A voucher specimens were deposited at this college; cellulose (activity of 6×10^5 U/g) and pectinase (activity of 8×10^5 U/g) were

purchased from Sunson Group, Beijing, China. All chemicals were of analytical or chromatography grade.

Extraction methods

Enzyme pretreatment: Licorice was powdered, sieved through a 20-mesh sieve, mixed with the complex enzyme. In a word, 0.8 g cellulase and 1.6 g pectinase were added to 500 g licorice power according to pre-experiment, the pH of water solution was adjusted to 5-6 with citric acid. The material was thoroughly mixed and incubated in an incubator at 50 ± 2 ºC for 160-180 min, the enzyme-pretreated licorice was obtained.

Extracted by 10 % ethanol: The enzyme-pretreated licorice was extracted three times with continuous stirring at 70 ºC for 3 h by 10 % ethanol. The supernatant solutions were harvested, filtered by a Whatman No. 1 filter paper, after centrifugation at 3800 g for 20 min, the pH of the supernatants were adjusted to 2 with sulfuric acid to gain some sediments, which were collected, then the pH were adjusted to 6-7 by adding 1 N NaOH, the supernatants were collected and evaporated in a rotary evaporator. The concentrated extracts without enzyme pretreatment were processed as control sample (in both cases, material were extracted by 10 % ethanol).

Extracted by 10 % ammonia: The enzyme-pretreated licorice was extracted three times with continuous stirring at 70 ºC for 3 h by 10 % ammonia. The supernatant solutions were combined just as operation with 10 % ethanol as section The control sample without enzyme pretreatment was extracted by 10 % ammonia simultaneously.

Purification of glycyrrhizic acid: Macroporous adsorption resins including D101, X-5, AB-8, NKA-9 and XDA-8 were purchased from the Chemical Plant of NanKai University (Tianjing, China). As preliminary experiment, the separation characteristics of four kinds of macroporous adsorption resins were systematically investigated by means of the static adsorption/desorption experiments and D101 was selected as a suitable resin for glycyrrhizic acid purification owing to its higher adsorption. The extracts were subjected to macroporous resin D101, eluted by water to get rid of impurities, then eluted step-by-step with 10, 60 and 90 % ethanol, obtaining three fractions. The glycyrrhizic acid in different elution was determined by HPLC and the glycyrrhizic acid from licorice was obtained.

Determination of glycyrrhizic acid: Glycyrrhizic acid was assayed by HPLC. Preparative HPLC: Shimadzu, analytical HPLC, LC-10AT vp HPLC pump, CTO-10AS vp thermostated column compartment, SPD-10A vp detector and controller. Column: diamonsil C_{18} (250 mm × 4.6 mm, 5 μm, DIKMA, American), mobile phase: MeOH:0.2mol/L ammonium acetate: acetic acid = 67:33:1, isocratically eluted at a flow rate of 1.0 mL/min and 35 ºC, detection: 254 nm, injection volume of all sample and standard solutions was 20 μL. HPLC analysis was based on the peak area.

Preparation of licorice liquiritin: The licorice herbal residues were harvested after glycyrrhizic acid extraction, treated with compound enzyme in pre-experiment conditions: 1.8 g cellulase and 0.9 g pectinase were added to 500 g herbal residues with 1000 mL water, pH (5.0-6.0), 50 ± 2 ºC for 3 h, then extracted three times by 90 % ethanol with continuous stirring at 70 ºC for 2 h. After centrifugation at 3000 g for 20 min, the supernatants were collected and evaporated in a rotary evaporator.

Aqueous ethanol solution selected for eluting favonoids with macroporous adsorption resin AB-8: Macroporous adsorption resins including D101, X-5, AB-8, NKA-9 and XDA-8 were systematically investigated by means of the adsorption/desorption experiments. According to the preliminary experiment, AB-8 was selected as a suitable resin for LF purification, owing to its higher adsorption. The extracts were subjected to macroporous resin AB-8, eluted by water to get rid of impurities, eluted successively by 30, 50 and 95 % ethanol. The LF in different elusions was determined by thin layer chromatography (TLC).

Liquiritin rarefied by polyamide: The eluted products from AB-8 were separated by polyamide (150-200 mesh, purchased from Biochemistry and Plastic Factory of Luqiaosijia, Taizhou, Zhejiang Province, China) column chromatography using distilled water to get rid of impurities, then eluted with 50 and 95 % EtOH step-by-step. The 50 and 95 % EtOH fractions were dried at 60 ºC under vacuum to yield liquiritin.

HPLC analysis of liquiritin in licorice favonoids: To quantify the liquiritin concentration in licorice favonoids, the HPLC analytical parameters including separation column, mobile phase and elution mode were explored. Consequently, the optimized chromatographic condition was below. Preparative HPLC: Shimadzu, analytical HPLC, LC-10AT vp HPLC pump, CTO-10AS vp thermostated column compartment, SPD-10A vp detector and controller. Column:diamonsil C_{18} (250 mm × 4.6 mm, 5 μm) (DIKMA, American), the flow rate used was 1.0 mL/min, column temperature was maintained at 30 ºC, detection: 237 nm; the mobile phase consisted of A (acetonitrile) and B (0.05 % phosphoric acid aqueous solution), gradient elution was indicated in Table-1. Injection volume of all sample and standard solutions was 20 μL. The liquiritin was calculated according to standard curve.

TABLE-1
GRADIENT COMPOSITION OF MOBILE PHASE FOR HPLC ANALYSIS OF LIQUIRITIN

t (min)	0.01	8	35	36	40	50
A (%)	5	19	50	90	19	19
B (%)	95	81	50	10	81	81

RESULTS AND DISCUSSION

Glycyrrhizic acid extraction: The ammonical and ethanolic extraction methods were compared in this study. The glycyrrhizic acid extracted by 10 % ammonia was 21.81 g (Fig. 1, Table-2), which was higher than that of using 10 % ethanol solvent (18.12 g) and the purity of glycyrrhizic acid in extracts was 25.08 and 27.54 %, respectively. Furthermore, pretreated by complex enzyme, extracted by 10 % ammonia, the extracted amounts was 31.53 g and glycyrrhizic acid purity in extract was 45.61 %, which was higher than enzyme + 10 % ethanol (27.35 g, 39.38 %). The glycyrrhizic acid content of licorice depends upon factors such as the species, harvesting season, pretreatment, the place of origin and age, hence, its level may range probably from 0.02-3.37 %[14-16]. The present

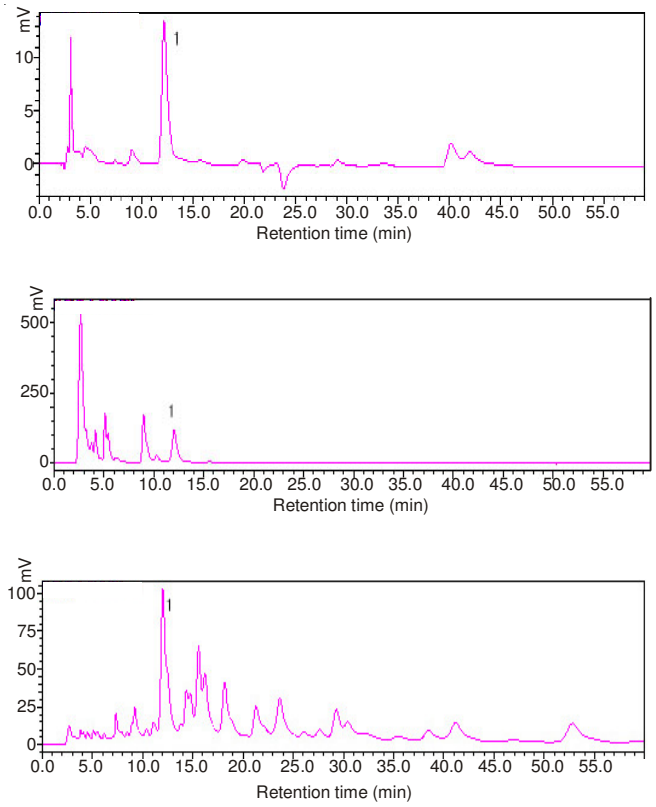

Fig. 1. Typical chromatograms of standard glycyrrhizic acid (a), extracted by 10 % ammonia (b) and enzymolysis extracts by 10 % ammonia (c), (peaks: 1 = glycyrrhizic acid)

TABLE-2
COMPARISON OF FOUR EXTRACTING METHODS FOR GA

Extract methods	Weight of extract (g)	GA of extracts (g)	GA of extracts (%)	GA in material (%)
10 % EtOH	18.12	4.99	27.54	1.00
10 % ammonia	21.81	5.47	25.08	1.09
Enzyme + 10 % EtOH	27.35	10.77	39.38	2.15
Enzyme + 10 % ammonia	31.53	14.38	45.61	2.88

from 1.00-2.88 % (Fig. 1), the herbal material was from the same origin except the pretreatment. According to preliminary experiments, it was found that enzyme pretreatment could efficiently extract active ingredients from the material[17]. It is known that cellulase and pectinase can degrade the cell wall and thus substantially improve the extraction efficiency. Therefore, the glycyrrhizic acid extraction from licorice samples was conducted by the pretreatment with cellulase and pectinase, which lead to a higher extract weight and glycyrrhizic acid purity. Overall, as alkaline water, 10 % ammonia was considered to be the better solvent for extracting glycyrrhizic acid from licorice after treated with pectinase and cellulose. Therefore, 10 % ammonia as extractant enabled more glycyrrhizic acid from compound enzyme-treated licorice than that of using 10 % ethanol (Fig. 1). Hence, considering the simultaneous extraction, 10 % ammonia was used in the subsequent experiments.

Aqueous ethanol solution selected for isolating glycyrrhizic acid: Many kinds of macroporous adsorption resins were employed to purify glycyrrhizic acid[18]. In this study, D101 macroporous adsorption resin was selected to isolate glycyrrhizic acid and eluted by 10, 60 and 90 % aqueous ethanol solution successively. The enzyme-pretreated extracts could be eluted by 10 % aqueous ethanol solution largely but not completely, afterwards, the remaining glycyrrhizic acid was eluted off from the column by washing continuously with 60 % aqueous ethanol solution, little glycyrrhizic acid was kept in 90 % of aqueous ethanol solution (Fig. 2), thus the 10 and 60 % aqueous ethanol solution fractions were combined to gain glycyrrhizic acid, the purity was 86.33 %. Therein, 10 % of aqueous ethanol solution, successively 60 % of aqueous ethanol solution was commended to elute glycyrrhizic acid from D101 macroporous adsorption resin for the enzyme + 10 % ammonia extracts.

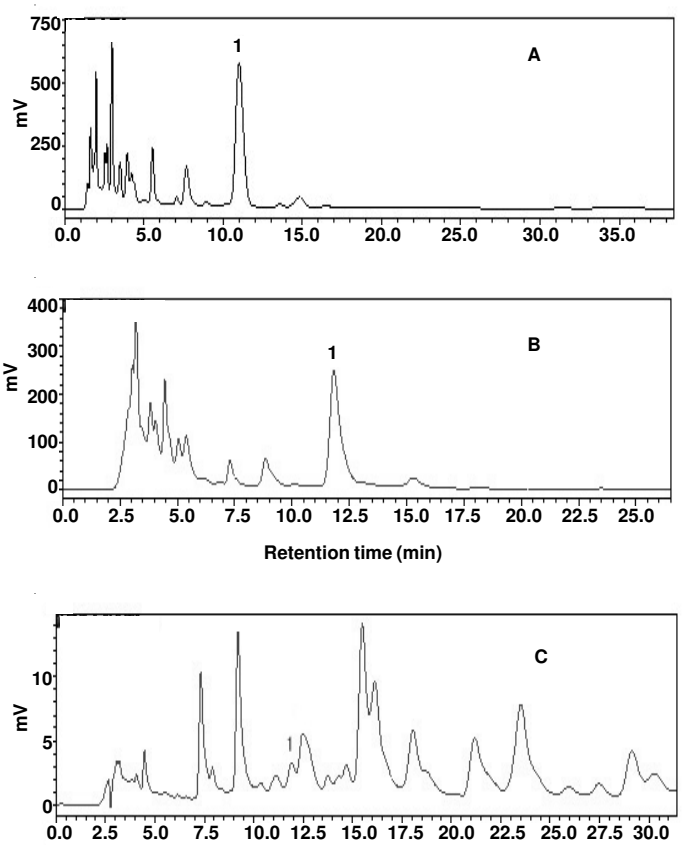

Fig. 2. Typical chromatograms of enzymolysis extracts eluted by 10 % EtOH (a), 60 % EtOH (b) and 90 % EtOH (c), (peaks: 1 = glycyrrhizic acid)

Aqueous ethanol solution for isolating LF by macroporous adsorption resin AB-8: AB-8 was selected as a suitable resin for LF purification. The enzyme pretreated extracts were subjected to macroporous adsorption resin AB-8, the differences of the components were shown in TIC chromatogram, as a TLC result, little LF was kept in water and 30 % ethanol flushed fractions, LF was preserved largely in 50 and 95 % ethanol elusion (Fig. 3), therefore the above two fractions were collected, combined and commended for further rarefied by polyamide.

Purified by polyamide of liquiritin in licorice and HPLC analysis: The concentration of liquiritin in licorice favonoids was determined by HPLC (Fig. 4). While the herb residues pretreated by complex enzyme, extracted by 90 %

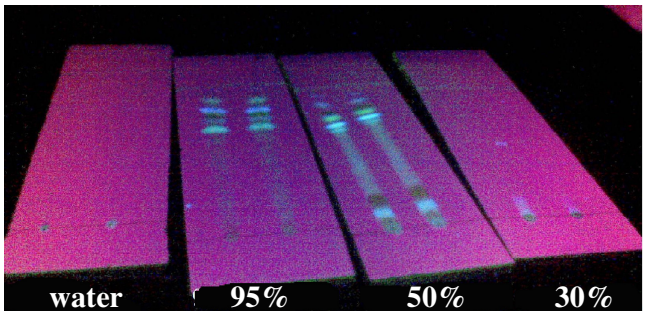

Fig. 3. TLC of LF eluted by ethanol (water; 95 % ethanol; 50 % ethanol; 30 % ethanol)

ethanol, the content of liquiritin became 0.32 % as against 0.21 % in no-complex enzyme sample (Fig. 4). When 50 and 95 % ethanol was selected for eluting the extracts in macroporous adsorption resin AB-8, the liquiritin in extracts was 2.78 %, then purified by polyamide, the liquiritin in enzymolysis extracts was up to 10.89 %.

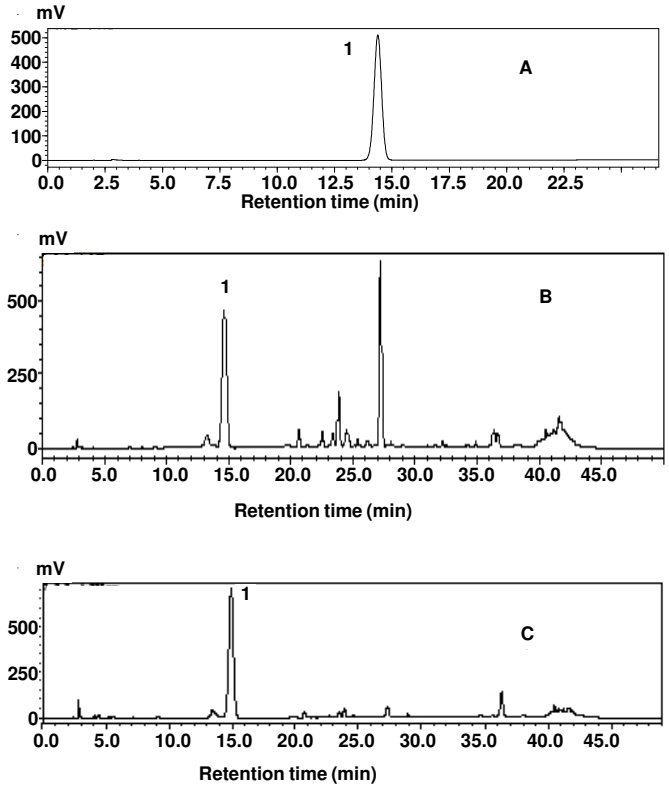

Fig. 4. Typical chromatograms of standard liquiritin (a), extracts (b) and enzymatic extracts (c) rarefied by polyamide, (peaks: 1 = liquiritin)

Conclusion

In this present study, the preparative separation and purification process of glycyrrhizic acid and liquiritin with compound enzyme from licorice root extracts were successfully established. It was showed that the yields of glycyrrhizic acid and liquiritin were greatly enhanced by using cellulose and pectase to treat the licorice roots. Extraction conditions for liquiritin and glycyrrhizic acid from licorice root have been determined. The employment of compound enzyme has been verified to be an efficient strategy for extracting glycyrrhizic acid and liquiritin from licorice root, followed by the extraction with 10 % ammonia, purified by different kinds of macroporous adsorption resins and polyamide. Compared with the conventional techniques, the compound enzyme-based protocol has been testified to be of higher efficiency, simplicity, low cost and less labour.

ACKNOWLEDGEMENTS

This study was supported by Science and Technology Development Project of Beijing Educational Committee, China (KM200911417011, KM200611417013).

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