Korean J. Food & Nutr. Vol. 27. No. 3, 532~537 (2014)
http://dx.doi.org/10.9799/ksfan.2014.27.3.532

THE KOREAN JOURNAL OF
한국식품영양학회지
FOOD AND NUTRITION

Isolation and Purification of Berberine in Cortex Phellodendri by Centrifugal Partition Chromatography

Jung-Bae Kim and †Byung-Ho Bang*

Dept. of Food and Cookery, Sangji Youngseo College, Wonju 660 220-713, Korea
**Dept. of Food and Nutrition, Eulji University, Seongnam 461-713, Korea*

Centrifugal Partition Chromatography에 의한 황백으로부터 Berberine의 분리 및 정제

김 중 배 · †방 병 호*

상지영서대학교 식품영양조리과, *을지대학교 식품영양학과

국문요약

황백(Cortex Phellodendri: CP)은 황벽나무(*Phellodendron amurense*)의 건조된 수피로부터 얻어진다. 이 수피는 한국의 전통 한약제로서 설사, 황달, 무릎과 발의 통증, 요도관 및 피부 감염증에 폭넓게 사용되어 왔다. 이들 기능성 성분의 분리 및 정제는 박층 크로마토크래피, 컬럼 액체 크로마토크래피 및 HPLC와 같은 여러 분석법들이 동양의 약초연구에 이용되어 왔다. 본 연구는 CP로부터 berberine을 분리하기 위해 향류분배 크로마토크래피법(CPC)으로 효과적으로 수행하였다. 두 용매의 CPC 최적조성은 n-butanol: acetic acid: water(4:1:5 v/v/v)이었다. 이동상의 유속은 1,000 rpm 회전력에서 상승법으로 분당 3 mL 속도로 전개시켰다. CPC에서 분리된 분획분은 prep-HPLC로 정제하였다. ^{1}H-NMR 스펙트럼은 4.10과 4.20 ppm에서 3H(－OCH_3), 6.10 ppm에서 2H의 (－OCH_2O－) proton signal의 공명이 관찰되었다. 2개의 방향족 proton은 이중결합 패턴을 보였다. H-11과 H-12 doublet은 각각 7.98과 8.11에서 나타났다. ^{13}C-NMR 스펙트럼에서는 C2와 C3의 methylenedioxy group(－OCH_2O－), C9과 C10에 methoxy group(－OCH_3)이 4개의 치환된 형태로 보였다. 분리 · 정제된 berberine의 화학구조는 ^{1}H, ^{13}C-NMR, ESI-MS 데이터 분석으로 확인하였다.

Key words: CP, HPLC, berberine, cortex phellodendri, *Phellodendron amurense*

Introduction

Since ancient times, many oriental countries have been using traditional herbal medicines (THM) in order to treat all kinds of diseases as well as to maintain health. It has been generally accepted that the efficacy of THM can be attributed to the synergistic activity of various major and minor components of herbs. The identification of the components of THM is of great importance in controlling their quality and gaining a better understanding of their pharmacological effects. THM are a mixture of hundreds of compounds with various polarities; thus, the isolation and purification of biologically active compounds require tedious and time- consuming processes such as extraction and solvent extraction, open column chromatography and preparative HPLC (Yin et al. 2009; Yang et al. 2010)

Cortex Phellodendri (CP) is derived from the dried bark of Phellodendron amurense. It has been widely used as a drug in traditional Korean medicine for treating diarrhea, jaundice, swelling pains in the knees and feet, urinary tract infections, and infections of the body surface (Lee et al. 2005; Min & Cho 2008; Zhu et al. 2011). Modern pharmacological studies elucidate that CP has anti-microbial, anti-inflammatoy and anti-diarrhea

† Corresponding Author: Byung-Ho Bang, Dept. of Food and Nutrition, Eulji University, Seongnam 461-713, Korea. Tel: +82-31-740-7132, Fax: +82-31-740-7370, E-mail: gunnerbh@eulji.ac.kr

properties (Seneviratne et al. 2007). The constituents of CP are numerous and diverse, including alkaloids and flavonoids (Hu et al. 2010; Jeon & Kim 2013). It has been believed for a long time that berberine is the major active ingredient of CP (Chen et al. 2010).

As a counterpart to conventional separation methods, an alternative chromatography technique, centrifugal partition chromatography (CPC), has been utilized to obtain pure compounds from the extracts of natural products (Osamu et al. 2008; Jeon & Kim 2013). Traditional isolation methods such as liquid solid chromatography have various problems when separating secondary metabolites from the plants. These problems which including time consuming process, tedious repeated chromatography and adsorption in stationary phase do not affect the preparative CPC technology of the liquid- liquid chromatographic techniques (Kim et al. 2006; Kim et al. 2010; Lee et al. 2013).

CPC is a liquid-liquid partition chromatography technique that does not use a solid support matrix, thereby resulting in no irreversible adsorption of the sample onto the solid matrix and less peak tailing and contamination (Poucault AP 1994). High selectivity is obtained by careful choosing the biphasic solvent system; thus, allowing the separation of compounds with very similar structures. This method has been widely applied for preparative separation of various natural products such as alkaloids, flavonoids, and hydroxyanthraquinones (Kim & Kim 2007; Yin et al. 2009; Kim JB 2011; Zhang et al. 2011).

We previously reported the structural elucidation of palmatine with antibacterial components from CP (Kim et al. 2013). In this paper, we describe the successful preparative separation and purification of berberine from methanolic extract Cortex Phellodendri (CP) using CPC with a two-phase solvent system composed of n-butanol/acetic acid/water (4:1:5).

Materials and Methods

1. Apparatus

Preparative CPC was performed using a LLB-M (Model LLB-M, Series 1000) high performance CPC (Sanki Instruments Ltd, Tokyo, Japan). The CPC system was equipped with a 321 pump (Gilson, Middleton, WI, USA), a UV-VIS detector (S-3702 Soma, Sōma City, Japan), a fraction collector (FC-203B, Gilson), and a 1 mL sample loop. The analytical high performance liquid chromatography (HPLC) system consisted of a binary Gilson 305 pump, a UV detector (M720 Youngin, Seoul, South Korea), and a 506C interface module (Gilson). Nuclear magnetic resonance (NMR) (300 MHz for ^{1}H-NMR and 225 MHz ^{13}C NMR) spectra were measured in methanol-D4 (MeOD, 99.9%) using a Bruker Biospin DSX-300 spectrophotometer (Billerica, MA, USA) and mass spectra, and the molecular weights of the compound were measured by positive electrospray ionization mass spectroscopy (ESI-MS) (Applied Biosystems, Foster City, CA, USA) at the Korea Basic Science Institute (Daejeon, South Korea).

2. Crude extract sample preparation

Dried CP was obtained from a Wonju Oriental medicine market (Kwangwon Province, Korea). Dried CP (200 g) was extracted with 2 L of 80% methanol for 48 hr. The extract was concentrated in a rotary vacuum evaporator (Eyela, Japan) in order to obtain the crude extract (25 g). The extract was dissolved in 100 mL water and extracted with butanol. A portion of the residue obtained after the evaporation of the butanol was dissolved as the lower layer in the CPC solvent system.

3. CPC separation procedure

The two-phase solvent system was composed of n-butanol: water (1:1). The solvent mixture was mixed vigorously in a separatory funnel and equilibrated at room temperature in order to obtain the upper and lower phases. The upper organic phase was used as the mobile phase, whereas the lower aqueous phase was employed as the stationary phase. The lower phase (stationary phase or aqueous phase) of the two-phase solvent system was pumped into partition cells in the ascending mode at a flow rate of 15 mL/min without rotation. When all cells were completely filled with the stationary phase, the upper phase was pumped at a flow rate of 3 mL/min at a rotor speed of 1,000 rpm. After equilibrium was established, as indicated by the glow of the mobile phase solvent from the CPC system outlet, a water sample solution was injected into the CPC system (Poucault AP 1994). The eluate from the CPC was monitored in the UV at 280 nm; then, the fractions were collected in an 10 mL/tube with a Gilson FC 203B fraction collector.

4. HPLC analysis

The CPC-separated fraction and crude extract were analyzed by HPLC. A J'sphere ODS-H80 column (4 μm particle size, 120Å, 150 × 4.6 mm, YMC Co. Ltd., Tokyo, Japan) was used. The mobile phase was composed of 25% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) in a gradient system. The

flow rate was 1 mL/min with UV absorbance detection at 280 nm. Preparative HPLC was performed using a reversed phase column (Gemini 5 μm, 110 Å, 100 × 21.28 mm, Phenomenex, Torrance, CA, USA) with 25% acetonitrile in 0.1% aqueous TFA at a flow rate of 4 mL/min and monitoring at 280 nm.

Results and Discussion

1. Choice of two-phase solvent system

Purification procedures were carried out by CPC and preparatory HPLC in order to identify the CP. The dried Cortex Pellodendri (200 g) were extracted at room temperature with 80% methanol for 48 hr. The methanolic extract was evaporated *in vacuo* at 50℃, and the concentrated extract was suspended in 10% methanol and extracted successively with butanol. A portion of the residue (28 g) obtained after evaporating the butanol was dissolved in the lower layer of the solvent system and then pumped into the column at 3 mL/min.

The choice of the solvent system is a critical step in the method development of a CPC separation as it is the correct choice of the two solvent mixtures that will ensure the successful separation of the compounds in the mixture. The partitioning coefficient parameter (*K*) is a of paramount importance as the value of this parameter is going to determine the retention time for a compound in the column.

Successful separation of natural products by CPC depends on selecting an optimum two-phase solvent system with an ideal partition coefficient (*K*) range for the target compound of 0.2 and 2 (Kim & Kim. 2007; Kim JB. 2011; Kim et al. 2013). If $K > 10$, the eluted peaks are broad and the excessive mobile phase volume and time are required to complete a CPC run. The compounds do not separate under *K* conditions < 0.2. To identify a suitable two-phase solvent system, n-butanol-isobutanol-acetic acid-water and a system with various ratios we applied. A two-phase solvent system composed of n-butanol: acetic acid: water presented the appropriate *K* values. As a result of the partitioning behavior of peaks IIb, the two-solvent system composed of n-butanol:acetic acid:water (4:1:5 v/v/v) resulted in a favorable partitioning value of $K = 1.02$, between the two layers.

2. CPC separation

The dried CP methanol extract was separated and purified by CPC with a n-butanol: acetic acid: water (4:1:5) solvent system. As shown in Fig. 1, the fractions were collected for approximately

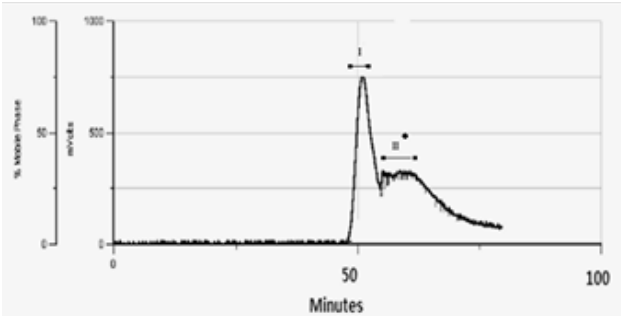

Fig. 1. Preparative centrifugal partition chromatography (CPC) separation of the methanol Cortex Phellodendri extracts. Solvent system: n-butanol/acetic acid/water (4:1:5), flow rate, 3 mL /min; rotation speed, 1,000 rpm; detection, 280 nm.

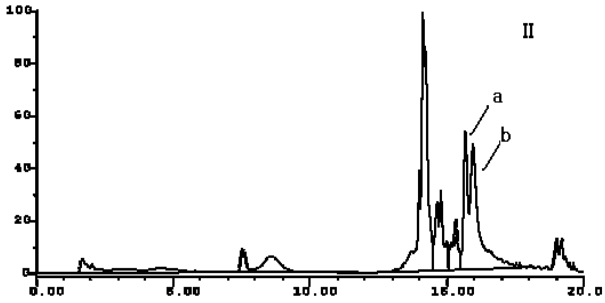

Fig. 2. Centrifugal partition chromatography (CPC) chromatogram of a Cortex Phellodendri methanol extract together with high performance liquid chromatography (HPLC) chromatograms of CPC peak fractions II. Column, J'sphere ODS-H80, 4 μm particle size, 120 Å, 150 × 4.6 mm, YMC Co. Ltd.; elution solvent, 0 − 3 min 15% acetonitrile, 3 − 10 min 15 − 50% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) in a gradient system. Elution time, 20 min; detection, 280 nm.

80 min and were grouped as I and II.

The separated fractions were analyzed by HPLC and the results indicated that the extract contained several compounds including peaks IIa and IIb (retention times, 15.6 and 16.2 min, respectively) and some unknown compounds (Yang et al. 2010). Fig. 2 shows the typical HPLC profiles of the CPC separated fractions. Peak IIa was reported in the previous literatures (Kim et al. 2013) as palmatine.

3. Structural elucidation of peaks IIb

The structural identification of CPC fraction peak IIb was carried out by ESI-MS, ^{1}H and ^{13}C NMR. The 1H and ^{13}C NMR date are shown in Table 1.

Table 1. ^{1}H and ^{13}C NMR data for compound IIb

Position	^{1}H ppm	^{13}C ppm
1	7.64	105.3
2		148.7
3		150.8
4	6.95	108.2
5	3.25	28.3
6	4.92	56.0
8	9.76	145.2
9		144.6
10		151.0
11	8.11	126.8
12	7.98	123.3
13	8.68	120.3
4a		130.3
8a		122.1
12a		134.0
13a		138.5
13b		120.7
$-OCH_2O-$	6.10	102.5
$-OCH_3$	4.10	C9 61.3
	4.20	C10 56.4

The ^{1}H NMR spectrum revealed that the resonances at δ 4.10 and 4.20 ppm corresponded to three ($-OCH_3$) protons, whereas those at δ 6.10 ppm corresponded to two protons ($-OCH_2O-$). Four cyclo protons (H-5 and H-6) only show a simple triplet-triplet pattern. In the spectrum of cyclo protons the signal due to the two identical protons of H-5 in split into a triplet by the H-6 hydrogen. The signal form the H-6 hydrogen is in turn split into a triplet. H-5 and H-6, coupling constants (*J*) are the value of each of the 6.30 Hz. The H-6 triplet appears down field of the H-5 triplet because of the deshielding influence of the nitrogen. Two aromatic protons (H-11 and H-12) shows a doublet-doublet pattern. The H-11 doublet and H-12 doublet appear at δ 7.98 and 8.11, respectively. The coupling constants (*J*) are the value of each of the 9.00 Hz.

The ^{13}C NMR. spectrum showed a tetra substituted with a methylenedioxy group at C2 and C3, and two methoxy groups at C9 and C10.

4. The molecular formula

When these ^{1}H NMR, ^{13}C NMR, and ESI-MS data were compared to those reported in the literature (Lee et al. 2005; Min & Cho 2008; Deevanhxay et al. 2009; Hu et al. 2009; Ma et al. 2009), compounds IIb was identified as berberine ($C_{20}H_{18}NO_4$: molecular weight 336.1184) in Cortex Phellodendri. The pharmacological actions of berberine has been reported to have a variety of biological activities such as anticancer, anti-inflammatory, immunomodulation, inhibition of oxidative stress and weight control effect (Garcia et al. 2006; Chen et al. 2010).

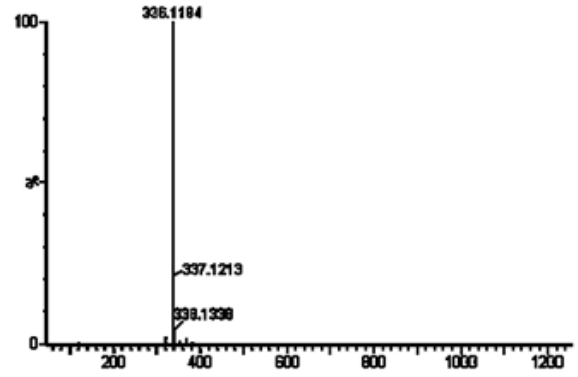

Fig. 3. Electrospray ionization mass spectroscopy (ESI-MS) chromatograms of berberine (IIb).

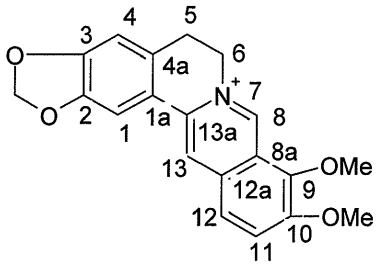

Fig. 4. The structures of berberine (IIb).

SUMMARY

Cortex Phellodendri (CP) is derived from the dried bark of *Phellodendron amurense*. It has been widely used as a drug in traditional Korea medicine for treating diarrhea, jaundice, swelling pains in the knees and feet, urinary tract infections, and infections of the body surface. Many analytical methods have been used to study oriental herbal medicines, such as thin-layer chromatography, column liquid chromatography, and high performance liquid chromatography (HPLC). In this study, preparative centrifugal partition chromatography (CPC) was successfully carried out in order to separate pure compounds from a CP methanol extract. The optimum two-phase CPC solvent system was composed of n-butanol: acetic acid: water (4:1:5 v/v/v). The

flow rate of the mobile phase was 3 mL/min in ascending mode with rotation at 1,000 rpm. The CPC-separated fraction and purification procedures were carried out by preparatory HPLC. The ^{1}H NMR spectrum revealed that the resonances at δ 4.10 and 4.20 ppm corresponded to three protons ($-OCH_3$), whereas those at δ 6.10 ppm corresponded to two protons ($-OCH_2O-$). Further, two aromatic protons (H-11 and H-12) conveys a doublet-doublet pattern. The H-11 doublet and H-12 doublet appear at δ 7.98 and 8.11, respectively. The ^{13}C NMR. spectrum showed a tetrasubstituted with a methylenedioxy group at C2 and C3, and two methoxy groups at C9 and C10. The chemical structure of the berberine was identified by ^{1}H, ^{13}C-nuclear magnetic resonance and electrospray ionization-mass spectroscopy spectral data analysis.

References

Chen ML, Xian YF, Ip SP, Tsai SH, Yang JY, Cde CT. 2010. Chemical and biological differentiation of Cotex *Phellodendri chinensis* and Cortex *Phellodendri amurensis*. *Planta Med* 76:1530-1535

Deevanhxay P, Suzuki M, Maeshibu N, Li H, Tanaka K, Hirose S. 2009. Simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *Coscinium fenistratum* by liquid chromatography hybrid ion time-of-flight mass spectrometry. *J Pharm Biomed Anal* 50: 413-425

Garcia GE, Nicole A, Bhaskaran S, Gupta A, Kyprianou N, Kumar AP. 2006 Aktand CREB-mediated prostate cancer cell proliferation inhibition by Nextrutine, a *Phenllodndron amurense* extract. *Neoplasia* 8:523-533

Hu YM, Su GH, Sze SCW, Ye W, Tong Y. 2010. Quality assessment of Cotex Phellodendri by high performance chromatography couled with electrospray ionization mass spectrometry. *Biomed Chromatogr* 24:438-453

Jeon JS, Kim CY. 2013. Preparative separation and purification of flavonoids and stilbenoids from *Parthenocissus tricuspidata* stems by dual-mode centrifugal partition chromatography. *Sep Purif Technol* 105:1-7

Kim CY, Ahn MJ, Kim JW. 2006. Preparative isolation of mangiferin from *Anemarrhena asphodeloides* rhizomes by centrifugal partition chromatography. *J Sep Sci* 29:869-875

Kim CY, Kim JW. 2007. Preparative isolation and purification of geniposide from gardenia fruits by centrifugal partition chromatography. *Phytochemical Analysis* 18:115-117

Kim JB, Shin WS, Kwon YI, Bang BH. 2013. Isolation and purification of antibacterial components in Cortex Phellodendri. *Korean J Food & Nutr* 26:547-552

Kim JB. 2011. Isolation of berberine from the rhizome of *Coptis chinensis* by centrifugal partition chromatography. *Korean J Food & Nutr* 24:617-621

Kim SM, Sang YF, Um BH. 2010. Preparative separation of chlorogenic acid by centrifugal partition chromatography from highbush blueberry leaves. *Phytochemical Analysis* 21:457-462

Lee JH, Ko JY, Samarakoon K, Oh JY, Heo SJ, Kim CY, Nah JW, Jang MK, Lee JS, Jeon YJ. 2013. Preparative isolation of saragachromanol E from *Sargassum siliquastrum* by centrifugal partition chromatography and its anti-inflammatory activity. *Food and Chemical Toxicology* 62:54-60

Lee JH, Lee BW, Moon YH, Yang MS, Jang KC, Park KH. 2005. Phytochemical constituents from the stem bark of *Phellodendron amurense* Rupr. *Agric Chem Biotechnol* 48: 93-96

Ma CH, Li ZX, Wang LX, Tang YH, Xiao HB, Huang CG. 2009. Identification of major alkaloids in rat urine by HPLC/DAD/ESI-MS/MS method following oral administration of Cortex Phellodendri decoction. *Helvetica ACTA* 92:379-398

Min BS, Cho JS. 2008. Quantitive determination of protoberberine from the roots of *Coptis chinensis*. *Natural Product Scinces* 14:68-72

Osamu S, Kumi N, Setsuko S, Noriko K, Masanori K, Makoto I, Sanae o, Mitsuyasu U. 2008. Preparative separation of the saponin lancemaside A from *Codonopsis lanceolate* by centrifugal partition chromatography. *Phytochemical Analysis* 19:403-410

Poucault AP. 1994. Centrifugal Partition Chromatography. pp 51-239. Marcel Dekker. New York

Seneviratne CJ, Wong RWK, Samaranayake LP. 2007. Potent anti-microbial activity of traditional Chinese medicine herbs against Candida species. *Mycoses* 51:30-34

Yang Q, Zhang F, Gao SH, Sun LA, Chen WS. 2010. Determination of bioactive compounds in Cortex Phellodendri by high performance liquid chromatography. *J AOAC Inter* 93:855-861

Yin L, Lu B, Lu Y, Xu L, Han X, Xu Y, Peng J, Sun C. 2009. Simultaneous determination of 11 active components in two well-known traditional Chinese medicines by HPLC coupled

with diode array detection for quality control. *J Pharm Biomed Anal* 49:1101-1108

Zhang S, Wang W, Wang C. 2011. Preparative separation and purification of alkaloids from *Rhizoma coptidis* by high speed counter-current chromatography. *Sep Purif Technol* 76:428-431

Zhu SL, Dou SS, Liu XR, Liu RH, Zhang WD, Huang HL, Zhang Y, Hu YH, Wang SP. 2011. Qualitative and quantitive analysis of alkaloids in Cortex Phellodendri by HPLC-ESI-MS/MS and HPLC-DAD. *Chem Res Chinese Universities* 27:38-44

접 수 : 2014년 4월 25일
최종수정 : 2014년 6월 2일
채 택 : 2014년 6월 17일