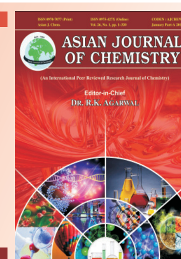
Asian Journal of Chemistry; Vol. 26, No. 7 (2014), 2073-2076

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.15900



An Anticoagulant Peptide from *Whitmania pigra*

SHAN ZHONG[1,2], YUHUA YIN[1], WEIMING CHENG[3,*] and DEPO YANG[1]

[1]School of Pharmaceutical Science, Sun Yat-sen University, No.132 East Outer Ring Road, Guangzhou University City, 510006 Guangzhou, Guangdong Province, P.R. China

[2]State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University (Guangzhou Medical University), No 151 YanJiang Road, Yuexiu Dist. 510120, Guangzhou, Guangdong Province, P.R. China

[3]Zhejiang Institute for Food and Drug Control, No. 86 Lane 1, Jichang Road, Jianggan Dist. 310004, Hangzhou, Zhejiang Province, P.R. China

*Corresponding author: Tel: +86 18058705320; E-mail: weiming_cheng@hotmail.com

Received: 12 June 2013; *Accepted*: 10 September 2013; *Published online*: 22 March 2014; AJC-14964

This work reports the isolation, purification and characterization of the anticoagulant peptides from *W. pigra*. The cold extraction solvent system used for the total bioactive components in *W. Pigra* was acetone-water (10:90, v/v), the extracts were then subjected to gel permeation chromatography on Sephadex LH-20, Sephadex G-50 and Sephadex G-25 columns successively, affording a fraction with potent anticoagulant activity. The fraction was then undergone further chromatographic procedures. An anticoagulant named Whitmanin B was then isolated and purified from the above bioactive fraction by RP-HPLC and its molecular weight was determined as 1574.7 Da by ESI ionization time-of-flight mass spectrometry (ESI-TOF-MS) and its partial amino acid sequence were CNQSQY (I/L) R by high-resolution liquid-mass spectrometry. The isolation and purification procedure could maintain the bioactivity of the product, therefore providing an effective method for the further isolation of components for the bioactive experiments.

Keywords: Leech; *Whitmania pigra*, Whitmanin B, Sephadex LH-20, Sephadex G-25, Sephadex G-50.

INTRODUCTION

The dried whole body of *Whitmania pigra* is a kind of traditional Chinese medicine (TCM), which could promote blood circulation and remove blood stasis, but there were only a new pteridinedione[1] and two heterocyclics[2] were isolated from *W. pigra* in recent years. Previous authors[3-5] have done some research on *W. pigra* and reported a new anticoagulant peptide called Whitmanin with 8608 Da at earlier stage[6]. In our new study, another peptide with anticoagulant activity was found from *W. pigra*, with 1574.7 Da. All of our research results might provide basic data and theoretical support for the anticoagulant material basis to clarify the leech *W. pigra*.

EXPERIMENTAL

Dried leech was purchased from Changsha Medicinal Materials Market (Changsha, China), identified as *W. pigra* by Professor Depo Yang, School of Pharmaceutical Science, Sun Yat-Sen University and a voucher specimen (20100302) was retained in the School of Pharmaceutical Science, Sun Yat-sen University, for future identification and use. The Sephadex LH-20, Sephadex G-25 and Sephadex G-50 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The ECOSIL C18 column (250 × 4.6 mm i.d., 5 µm, 100 Å) was purchased from Greenherbs Company (Beijing, China). For pH measurements, a pHS-25C pH meter (Shanghai, China) was used. The freeze-drying system was Free Zone Traid 2.5 L (7400030) made by LABCONCO (USA). Centrifugation was carried out in J2-HS centrifuge (Beckman, Germany). The high performance liquid chromatograph was produced by SHIMADZU Co. (Kyoto, Japan).

The molecular weight determination was performed with Bruker micrOTOFQ 125 (Bruker Ltd., USA) equipped with an electrospray interface and micro spring pump. The electrospray source was composed with dual nebulizers-one nebulizer for the sample eluent and the other for the external reference solution. The reference standards was standard peptide Glu-Fibrino peptide B, introduced into the TOF-MS with an automated calibrant delivery system and the accurate mass measurements of the components were obtained with this calibrant delivery system and thus achieved with this on-line prompt calibration.

TOF-MS condition was positive (ESI^+) ion mode under the following operation parameters: Capillary voltage 4000 V; drying gas 4 L/min; nebulizer 1.0 psig; gas temp 210 ºC; fragmentor voltage 175V (ESI^+); skimmer voltage 60 V; octopole dc1 33.3 V (ESI^+); octopole RF 250 V. The full-scan carried out by LC/TOF-MS was recorded across the mass range

50-2000 *m/z* and the elemental composition of every peak was calculated by TOF software with the accuracy error threshold set as 5 ppm for a strict criterion.

Acetonitrile (ACN, chromatographic grade) was procured from Burdic & Jackson Company (USA) and all other chemicals and reagents used were of analytical grade purchased from Damao Chemical Company (Tianjin, China).

Coagulation assay: The anti-coagulant activities of all fractions and samples were evaluated by measuring their plasma recalcification time (PRT)[7]: The whole blood, phle-botomized from the vein of a rabbit mixed with 3.8 % sodium citrate at a ratio of 9:1 (v/v), was centrifuged at 1000 rpm for 10 min to get the plasma. 100 µL normal saline (NS) or sample was added into 100 µL plasma and incubated at 37 ºC for 1 min in a 96-well microtiter plate and then 100 µL calcium chloride (0.025 M) was added into these wells and started timing until fibrin was detected by a probe. This procedure was repeated five times and the mean value was taken as the final plasma recalcification time.

Extract solvent and methods screening: Thirteen solvent systems and extract methods were screened to extract the dried body of *W. pigra*, respectively: NS at 4 ºC, water at boiling state, water at 4 ºC, acetone-water (2:98, 5:95, 10:90, 20:80, 40:60, 60:40, v/v) at 4 ºC, ethanol-water (20:80, 40:60, 60:40, 80:20, 95:5, v/v) at boiling state, ethanol-water (20:80, v/v) at 4 ºC.

The method of cold soak was as the following: The fine powder of *W. pigra* was soaked by solvent (1:4, m/v) at 4 ºC for 24 h with continuous stirring and extracted 4 times. The method of boiling extraction was followed as: The fine powder of *W. pigra* was immersed by solvent (1:4, m/v) with reflux condensation for 1 h and extracted 3 times. All the extract solution was combined and concentrated under reduced pressure at 40 ºC until the relative density reached 1.13 and then lyophilized.

The anticoagulant activity of each extract was evaluated by plasma recalcification time (Table-1) and acetone-water (10:90, v/v) at 4 ºC was found to be the most appropriate solvent system.

Solvent extraction: Dried leeches ground into a powder were extracted with cold acetone-water (10:90, v/v), with the extraction solution volume 4 times as the quantity of the powder, for 24 h and this processing was repeated for 4 times. All the extract solution was combined and concentrated under reduced pressure at 40 ºC until the relative density reached. The concentration was lyophilized and its anticoagulant activity was then evaluated by measuring plasma recalcification time (Table-2).

TABLE-1
PRT AND YIELDS OF EXTRACTS WITH DIFFERENT SOLVENT SYSTEM ($n = 5$)

Group	Concentration (v/v)	Yields (%)	PRT (s)
0.9 % NaCl			289.60 ± 22.109
The Extracts			
NS at 4 ºC		7.10	389.60 ± 34.653[aa]
Water at boiling state		6.60	298.00 ± 22.989
Ethanol-water at 4 ºC	20 %	8.0	389.80 ± 59.856[aa]
Water at 4 ºC		7.8	401.20 ± 57.269[a]
Ethanol-water at boiling state	20 %	10.2	396.80 ± 28.752[aa]
	40 %	9.0	381.00 ± 7.036[aa]
	60 %	7.2	363.40 ± 43.855[aa]
	80 %	3.1	342.80 ± 29.508[aa]
	95 %	2.2	309.60 ± 40.599[a]
Acetone-water at 4 ºC	2 %	10.4	436.00 ± 17.161[aa]
	5 %	9.2	443.00 ± 5.099[aa]
	10 %	11.7	449.60 ± 14.673[aa]
	20 %	9.1	399.20 ± 43.448[aa]
	40 %	6.6	365.60 ± 23.839[aa]
	60 %	5.2	331.00 ± 52.115[aa]

Values are $\bar{x}$ ± SD.
[a]: $p < 0.05$, [aa]: $p < 0.001$, compared with NS (treated group)

TABLE-2
THE RESULT OF DETERMINING PRT IN EACH STEP ($n = 5$)

Group	PRT (s)	Relative PRT	Activity recovery
0.9 % NaCl	249.0 ± 3.9	1.000	
Starting material	321.1 ± 2.5	1.289	100 %
Purification process			
Sephadex G-25			
Fr.I	250.2 ± 6.3	1.005	
Fr.II[a]	369.7 ± 4.7	1.485	72.8 %
Fr.III[a]	289.6 ± 2.1	1.163	
Fr.IV	252.2 ± 5.4	1.013	
Sephadex LH-20			
Fr.II-1	249.6 ± 5.2	1.002	
Fr.II-2	253.8 ± 3.4	1.020	
Fr.II-3[a]	356.0 ± 2.8	1.430	69.2%
Sephadex G-50			
Fr.II-3-1	254.4 ± 3.2	1.022	
Fr.II-3-2	252.7 ± 3.1	1.015	
Fr.II-3-3[a]	384.4 ± 5.2	1.544	64.8 %
RP-HPLC			
Fr.II-3-3-1[a]	392.7 ± 2.2	1.577	59.9 %
Fr.II-3-3-2*	256.5 ± 2.4	1.047	

Values are $\bar{x}$ ± SD.
[a]: $p < 0.05$, [aa]: $p < 0.001$, compared with NS (treated group)
*Fractions from RP-HPLC expected Fr.II-3-3-1were collected as Fr.II-3-3-2

Purification of the anticoagulant peptide

Sephadex G-25 chromatography: The lyophilized powder of 1.4 was dissolved in 2 mL of 0.01 M ammonia and the suspension was centrifuged at 3000 rpm/h for 10 min. The clear supernatant was then applied to a Sephadex G-25 column (Pharmacia, 100 × 20 mm i.d.), which was pre-equilibrated and eluted with 0.01 M ammonia at a flow rate of 50 mL/h. Fractions (6 mL/tube) were collected, with UV absorbance monitored at 280 nm. Fractions corresponding to different absorption peaks were collected and concentrated (Fig. 1) and their activities were tested, data shown in Table-2. The active fractions, namely Fr.II and Fr.III were lyophilized and the result showed that the amount of Fr.III was too little for the next isolation (Table-3), so only Fr.II was performed the continuous isolation in the next research.

Sephadex LH-20 chromatography: Fr.II was dissolved in 3 mL of water and the suspension was centrifuged (3000 rpm, 10 min). The clear supernatant was applied to a Sephadex LH-20 column (Pharmacia, 100 × 20 mm i.d.), which was

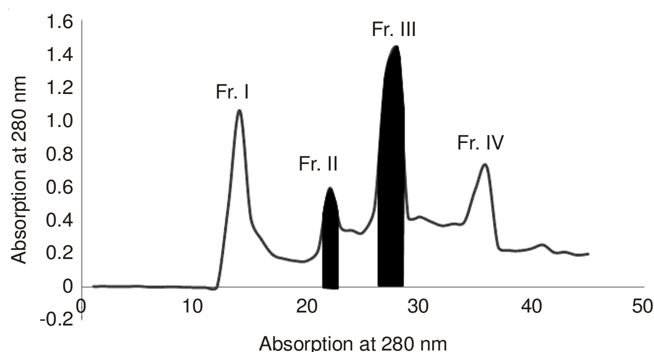


Fig. 1. Gel permeation chromatography of the extracts on a sephadexG-25 column

TABLE-3
PURIFICATION AND YIELDS OF *Whitmania pigra*

Purification steps	Yield		Content of protein[a]	
	Total	Yield (%)	Total	Content (%)
Medicinal materials	988 g	100	40.60 g	4.11
Crude extract	115.6 g	11.7	33.49 g	28.97
1.Sephadex G-25 (Fr. II)	23.75 g	2.49	19.26 g	81.1
2.Sephadex LH-20 (Fr. II-3)	2.98 g	0.30	2.69 g	90.2
3.Sephadex G-50 (Fr. II-3-3)	845 mg	0.008	788.39 mg	93.3
4.RP-HPLC (Fr.II-3-3-1)	101.6 mg	0.00102	98.75 mg	97.2

[a] Determined with the method of Folin-hydroxybenzene

pre-equilibrated and eluted with 0.01 M ammonia at a flow rate of 40 mL/h. Fractions (4 mL/tube) were collected and the absorption of the fractions was monitored at 280 nm. Fractions corresponding to different absorption peaks were collected, freeze-dried and the activity was then tested. The fraction with activity was named Fr.II-3 and lyophilized (Fig. 2).

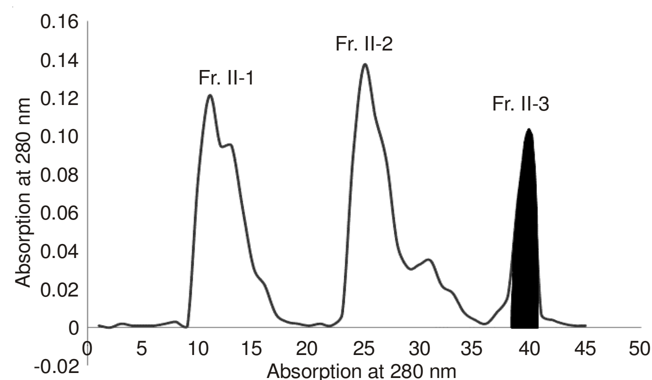


Fig. 2. Gel permeation chromatography of Fr.II on a SephadexLH-20 column

Sephadex G-50 chromatography: Fr.II-3 from the Sephadex LH-20 chromatography was dissolved in water at a concentration of 9 mg/mL and then the suspension was centrifuged (3000 rpm/h, 10 min). The clear supernatant was applied to a Sephadex G-50 column (Pharmacia, 100 × 20 mm i.d.), which was pre-equilibrated and eluted with water at a flow rate of 40 mL/h. Fractions (3 mL/tube) were collected, with UV absorbance monitored at 280 nm, which finally yielded Fr.II-3-3, showing potent anticoagulant activity (Table-2).

Preparative C18 RP-HPLC: The last purification step was carried out on a HPLC system with a ECOSIL C18 column (250 × 4.6 mm i.d., 5 µm, 100 Å). Fr.II-3-3 was dissolved in 3 mL of the mobile phase of CH_3CN-H_2O (2:98, v/v) and filtered with filtration membrane (0.45 µm). 20 µL filtrate was loaded onto the chromatographic column and eluted at a flow rate of 1 mL/min at room temperature and UV absorbance was monitored at 280 nm. Peak fractions with a retention time of 18.5 min named Fr.II-3-3-1 showed potent anticoagulant activity (Table-2) and gave a positive ninhydrin test for polypeptide.

Identification and sequence analysis of Fr.II-3-3-1

ESI-TOF-MS: The molecular weight of Fr.II-3-3-1 was identified by ESI-TOF-MS. The sample was dissolved in 60 % (v/v) CH_3CN-0.1 % (v/v) formic acid at a concentration of 2 mg/mL and then the solution was directly injected into the ESI-TOF- MS instrument for the component analysis.

LC-HRMS Analysis of Fr.II-3-3-1: The standard peptide Glu-Fibrino peptide B was used as the external calibration standard for the mass spectrometer and the mass data generated peaklist (pkl) files by Masslynx 4.0 software for the local Mascot 2.0 IPI database to query the protein database for the protein identification.

RESULTS AND DISCUSSION

To extract anticoagulant from the dried body of leeches, 13 solvent systems widely used were tested. At the first, ethanol-water at boiling state (40, 60, 80 and 95%, v/v), NS at 4 ºC, water at boiling state, acetone-water at 4 ºC (20:80, 40:60, 60:40, v/v) were tested and the results showed that solvent with lower degree of organic solvent was better than those with high degree of organic solvent in extracting anticoagulant (Table-1). So the following groups were tested: ethanol-water (20 %, v/v) at boiling state and at 4 ºC, water at 4 ºC, acetone-water at 4 ºC (10:90, v/v). The activity of acetone-water at 4 ºC (10:90, v/v) was much more obvious, but with the acetone ratio decreased, activity was likely to continuously to increase (Table-1). The last group was proved that the activity changed to be in apparent when acetone concentration decreased to a certain extent (5:95, 2:98, v/v, Table-1). Therefore acetone-water at 4ºC (10:90, v/v) was selected for extraction.

Purification of the extract included 4 steps summarized in Tables 2 and 3. Firstly, salts and other substances with low molecular weights were removed by elution with 0.01 M ammonia on Sephadex G-25 and afforded 4 fractions Fr. I-IV (Fig. 1). The main parts with anticoagulant activity of *W. pigra* were Fr.II and Fr.III (Table-2), but the quality of Fr.II was more than Fr.III, therefore only the former was analyzed here.

Sephadex LH-20 and Sephadex G-50 were selected as the stationary phases for the second and third purification steps, respectively. Because sephadex G-50 is suitable for the separation of polypeptides with low molecular weight (Da: 1500-30000). Sephadex LH-20 is much more suitable for the separation because of its polarity distribution and molecular weight distribution (Da: 1000-5000) and this report was for the molecular weight distribution mainly. Fr.II (Fig. 2) was separated

on Sephadex LH-25 using pure water, with obviously different molecular weights. Fr.II-3 showed remarkable anticoagulant activity (Table-2).

Fr.II-3 was subjected to Sephadex G-50 column chromatography and eluted with pure water, this yielded three fractions: Fr.II-3-1, Fr.II-3-2 and Fr.II-3-3 (Fig. 3). Only Fr.II-3-3 showed remarkable activity.

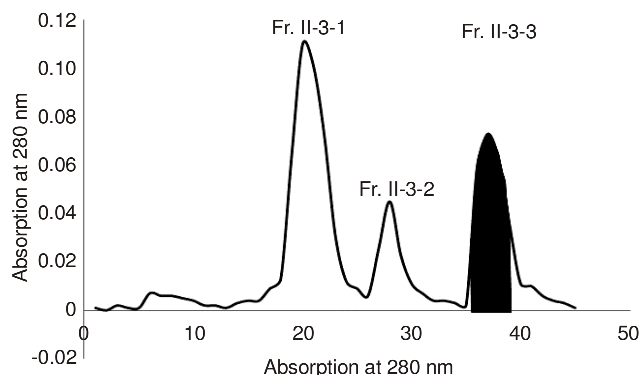


Fig. 3. Gel permeation chromatography of Fr. II-3 on a sephadexG-50column

The final purification step was carried out on RP-HPLC. Combining bioassay experiments, several kinds of mobile phases were tested to isolate anticoagulants from Fr.II-3-3 and finally the solvent system was selected as CH_3CN-H_2O (2:98, v/v). As shown in Fig. 4, Fr.II-3-3 showed a single peak at 18.5 min called Fr.II-3-3-1 by HPLC and this substance was collected. It was confirmed by the bioassay that this substance had pronounced anticoagulant activity (Table-2).

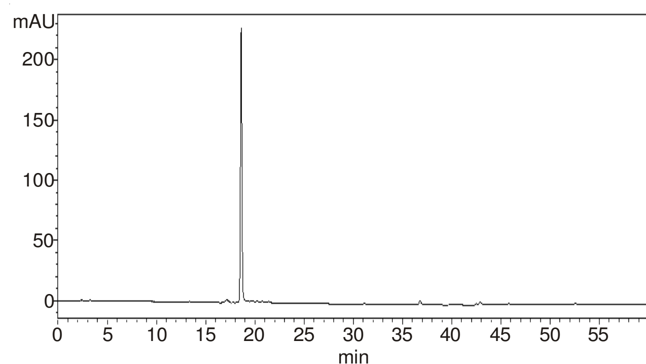


Fig. 4. Preparative HPLC spectrum of Fr.II-3-3

From the MS spectrum, it could be concluded that the component at *m/z* 788 was a peptide, which exhibited peptide-specific MS characteristic, namely double ions contained in one molecule, showing 0.5 ion step between two isotopic peaks. Therefore, after the deconvolution, the real molecular weight of Fr.II-3-3-1 could be deduced as 1574.7 Da (Fig. 5). According to this result, MS2 analysis was performed towards this peptide, shown in Fig. 6, the Bruker peptide analysis software extracted the differences among the fractional *m/z* and according to the accuracy limit, generated the partial amino acid sequence of Fr.II-3-3-1 as cystein-asparagine-glutamine-serine-glutamine-tyrosine-(isoleucine/leucine)-arginine [CNQSQY (I/L) R]. From which protein was this bioactive peptide split and what enzyme did the clearage work was still performed in our laboratory.

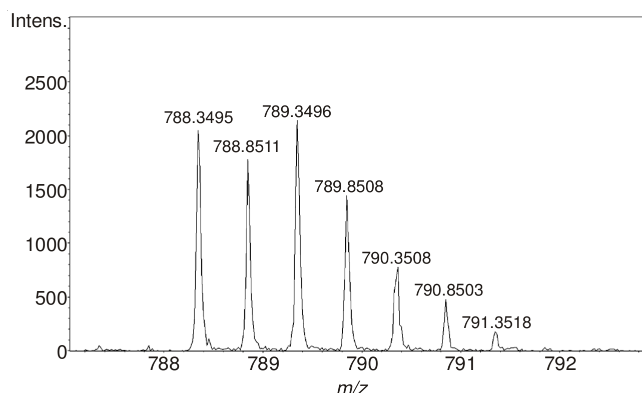


Fig. 5. ESI-TOF-MS of Fr.II-3-3-1

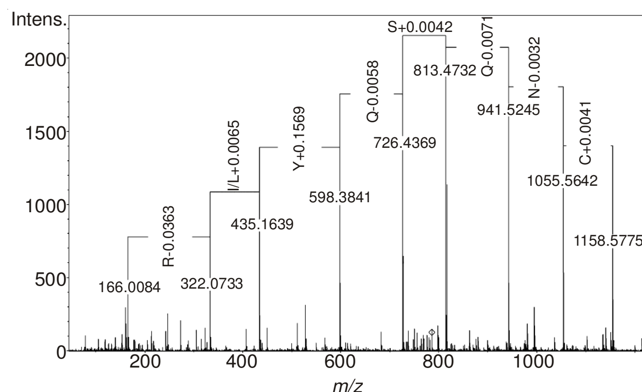


Fig. 6. ESI-FTICR mass spectrum of Fr.II-3-3-1

Fr.II-3-3-1, with anticoagulant activity, was named as whitmanin B and it is supposed that it would be one of the potential bioactive components of *W. pigra*.

ACKNOWLEDGEMENTS

This work was funded by the National Natural Science Foundation of China (Grant no. 30901951).

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