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Ancordin, the major rhizome protein of madeira-vine, with trypsin inhibitory and stimulatory activities in nitric oxide productions

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ABSTRACT

Anredera cordifolia (Ten.) Steenis, or the synonymous name of *Boussingaultia baselloides* or *Boussingaultia gracilis* var. *pseudobaselloides*, is a South American species of ornamental succulent vine, commonly known as the madeira-vine. The fresh leaves of madeira-vine are frequently used as vegetables. *A. cordifolia* is an evergreen climber that grows from fleshy rhizomes. The rhizome contained one major (23 kDa) protein band under non-reducing condition in the SDS-PAGE. The first 15 amino acids in the N-terminal region of the major protein band (23 kDa), named tentatively ancordin, were KDDLLVLDIGGNPVV which were highly homologous to sequences of winged bean seed protein ws-1, *Medicago truncatula* proteinase inhibitor, soybean trypsin inhibitor, and sporamin. By using activity stains, the ancordin showed trypsin inhibitory activity in the SDS-PAGE gel which was found not only in rhizomes but also in aerial tubers, but few in fresh leaves. The crude extracts from rhizomes of madeira-vine were directly loaded onto trypsin–Sepharose 4B affinity column. After washing with 100 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl, the ancordin was eluted directly by 0.2 M KC1–HC1 buffer (pH 2.0). In calculation, the purified protein exhibited 0.0428 μg trypsin inhibition/μg ancordin (corresponding to 0.53 unit of TPCK-treated trypsin inhibited/μg ancordin). The purified ancordin was used to evaluate the nitric oxide productions in RAW264.7 cells in the presence of polymyxin B (poly B, 50 μg/ml) to eliminate the lipopolysaccharide (LPS) contaminations. It was found that ancordin (1.25–5 μg/ml) could dose-dependently ($R = 0.954$) stimulate the nitric oxide (NO) productions (expressed as nitrite concentrations) in RAW264.7 cells without significant cytotoxicity, and kept the similar effects in NO production in 6.25 μg/ml ancordin.

1. Introduction

Madeira-vine, *Anredera cordifolia* (Ten.) Steenis, (aka *Boussingaultia baselloides* or *Boussingaultia gracilis* var. *pseudobaselloides*) [14], is a South American species of ornamental succulent vine. The fresh leaves of madeira-vine are frequently used as vegetables in Taiwan and also as folk medicines for analgesics and stomachics [15]. The ethanolic extracts of *B. gracilis* var.

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pseudobaselloide were reported to have inhibitory activities against spasmogen-induced contractions of the isolated gastric fundus of rats [21] and ethanol-induced gastric lesions in rats [20]. The hot-water extracts of *B. gracilis* var. *pseudobaselloide* were reported to have antiviral activities against Herpes simplex virus and adenovirus-3 [3]. It was also reported that the butanolic fraction from *B. gracilis* exhibited an anti-diabetic effects in alloxan-induced mice [22].

Shewry [28] reviewed recently the biological functions of different tuber storage proteins including patatin from potato tubers, sporamin from sweet potato roots, and dioscorin from yam tubers. Patatin, the potato tuber storage protein, has been demonstrated to have lipid acyl hydrolase and acyltransferase activities that are involved in tuber tissue response to wounding [2] and exhibited to have antioxidant activities *in vitro* [23]. The root storage protein of sweet potato not only have trypsin inhibitory (TI) activity, but also have both dehydroascorbate reductase and monodehydroascorbate reductase activities [5], glutathione peroxidase-like activities [10], and antioxidant activities [11]. The tubers of yam storage protein, dioscorin, exhibited carbonic anhydrase and trypsin inhibitor activities [7], and dehydroascorbate reductase and monodehydroascorbate reductase [8]. It was also reported that yam tuber storage protein of dioscorin and its peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activities *in vitro* [13] and antihypertensive activities on spontaneously hypertensive rats *in vivo* [19].

In this study, it was found that the rhizome of *A. cordifolia* contained one major (23 kDa) and one minor (32 kDa) protein bands under non-reducing condition in the SDS-PAGE. The N-terminal amino acid sequences of the major protein band (23 kDa), named tentatively ancordin, were highly homologous to winged bean seed protein ws-1, *Medicago truncatula* proteinase inhibitor, soybean trypsin inhibitor, and sporamin. The ancordin showed the TI activities and dose-dependently stimulatory activity in nitric oxide productions in RAW264.7 cells in the presence of polymyxin B (10 μg/ml).

2. Materials and methods

2.1. Materials

Fresh leaves, aerial tubers, and rhizomes of madeira-vine, *A. cordifolia*, were purchased from a local farm in Yangmingshan, Taipei. The voucher specimen of *A. cordifolia* (AC-001) is deposited in Graduate Institute of Pharmacognosy, Taipei Medical University. Electrophoresis grade of acrylamide, *N*,*N*′-methylene-bis-acrylamide (Bis), *N*,*N*,*N*′*N*′-tetramethyl-ethylenediamine (TEMED), ammonium persulfate, and *N*-benzoyl-L-arginine-4-nitroanilide were from Merck Inc. (Darmstadt, Germany); trypsin (TPCK-treated, from bovine pancreas, 9940 units/mg solid, T1426), Coomassie brilliant blue R-250, polyvinylpyrrolidone, polymyxin B (poly B), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO). The CNBr-activated Sepharose 4B were purchased from Amersham Biosciences (Uppsala, Sweden). The See Blue™ pre-stained standard kit for SDS-PAGE was from Invitrogen (Carlsbad, CA, USA). The kit contains myosin (250 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), lysozyme (16 kDa), aprotinin (6 kDa), and insulin B chain (4 kDa).

2.2. Purification of ancordin from rhizomes of A. cordifolia

Extraction and purification processes were modified from those of TI purification from sweet potato [5,10,11]. After washing and peeling, the rhizomes of madeira-vine were cut into pieces and extracted with four volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.9) containing 1% ascorbate and 1% (w/v) polyvinylpyrrolidone in a homogenizer for 30 s (four times). The homogenates were filtered through four layers of cheesecloth and centrifuged twice at 12,000 × *g* for 30 min. The supernatants were saved as crude extracts. For purification, the crude extracts were loaded directly onto a self-prepared trypsin–Sepharose 4B affinity column (1.0 cm × 10 cm) to absorb ancordin and were eluted by changing pH value with 200 mM KC1 buffer (pH 2.0). The self-prepared trypsin–Sepharose 4B affinity gels were according the manufacturer's instructions using CNBr-activated Sepharose 4B gels. The purified ancordin was dialyzed against distilled water overnight and then lyophilized and stored at −18 °C for further use.

2.3. Electroblotting and protein sequencing

The purified ancordin was subjected to 15% SDS-PAGE according to Laemmli [17]. After electrophoresis, gels were equilibrated in Tris–glycine (pH 8.3) and then transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA). Ancordin band was excised with a sharpe razor blade and then for protein sequencing. Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid phase Model 470A/900A sequencer with an on-line Model 120A phenylthiohydantoin–amino acid analyzer [12].

2.4. Determination of trypsin inhibitory (TI) activity and TI stains in SDS-PAGE gels

The TI activity determination of ancordin was according to the method of Lee and Lin [18] by the inhibition of trypsin-catalyzed hydrolysis of *N*-benzoyl-L-arginine-4-nitroanilide at 0.1 M Tris–HCl buffer (pH 8.2). Different amounts of purified ancordin (20, 40, 60, 80 μg) were preincubated with 7.5 μg trypsin at room temperature for 15 min, and then the substrate was added for additional 20 min. The absorbance at 405 nm was determined. Three determinations were averaged for TI activity and expressed as μg trypsin inhibited. The TI stains in the SDS-PAGE gels were performed according to the method of Hou and Lin [6]. Four parts of samples were mixed with one part of sample buffer, namely 60 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue without 2-mercaptoethanol for TI activity stains at 4 °C overnight. Coomassie brilliant blue R-250 was used for protein staining [27]. After electrophoresis, 12.5% gels were washed with 25% isopropanol in 10 mM Tris–HCl buffer (pH 7.9) for 10 min twice to remove SDS [6] and then for TI activity stain. The gel was dipped in trypsin solution at 37 °C for 30 min. After rinsing with 10 mM Tris–HCl buffer (pH 7.9),

the gel was incubated in the dark at 37 °C for at least 30 min with 80 ml of the substrate–dye solution immediately prepared before use. The substrate–dye solution consisted 20 mg N-acetyl-phenylalanine (β-naphthyl ester in 10 ml of N,N-dimethylformamide that was brought to 80 ml with 70 ml of O-dianisidine were dissolved. The blank zones (white ones) showed positions where the trypsin inhibitory activities locate.

2.5. Cell culture and treatments

RAW 264.7 cells were cultured in Dulbecco's modified eagle medium (DMEM, GibcoBRL, USA) supplemented with 10% fetal calf serum (FCS), 10,000 I.U./ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin, and 1% L-glutamate. The cell number was adjusted to 4×10^5 cells/ml. Cell suspension (1 ml) were seeded onto a 24-well microtiter plate and various concentrations of ancordin (1.25, 2.5, 3.75, 5, 6.25 μg/ml) or LPS (600 ng/ml) were added in the presence of polymyxin B (50 μg/ml, poly B) and cultured in 5% CO_2 humidified incubator at 37 °C for 24 h. The cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide staining [1] and expressed as relative cell viability (%). The poly B only group was recognized as 100% relative cell viability. The cultured plate was centrifuged at 1500 rpm for 10 min, and supernatants were collected for determinations of nitric oxide (NO) productions. Each of the 100 μl of cultured supernatant was added onto a 96-well micotiter plate. The 100 μl of Griess reagent was added to each well and stand for 15 min at room temperature. The absorbance at 530 nm was measured and sodium nitrite (0–500 microM) was used to plot the standard curve [16].

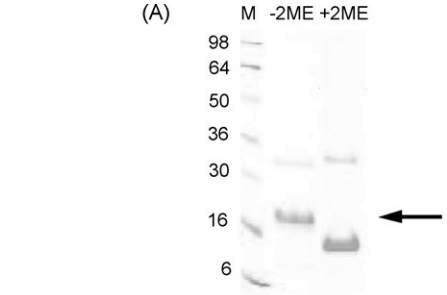
2.6. Statistics

Mean ± S.D. of triplicates were measured ($n = 3$). Student's t-test was used for comparison between two treatments. A difference between the group of (LPS + poly B) and each treatment was considered statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

3. Results and discussion

3.1. The major protein in the rhizome of A. cordifolia

The fresh rhizomes were extracted with four volumes (w/v) of 100 mM Tris–HCl buffer (pH 7.9) containing 1% (w/v) ascorbate and 1% (w/v) polyvinylpyrrolidone to get the crude extracts. The protein patterns in crude extracts were directly analyzed by 12.5% SDS-PAGE gels with or without 2-mercaptoethanol addition (Fig. 1A). It was found that the rhizome contained one major (23 kDa) and one minor (32 kDa) protein bands under non-reducing condition, however, the major (12 kDa) and the minor (33 kDa) protein bands were found under reducing condition in the SDS-PAGE gels. In the present data, it was difficult to interpret the molecular changes in the reducing or the non-reducing condition, however, it might be proposed that the major band might be composed by homodimers. Sporamin, the storage proteins of sweet potato, was showed two protein bands (22 kDa and 31 kDa) without dithiothreitol treatment while one protein band with the intermediate molecular weight (25 kDa) in the presence of dithiothreitol [25] in the SDS-PAGE gels. Dioscorin, the yam tuber storage protein, was showed two protein bands (28 kDa and 82 kDa) in SDS-PAGE gels without 2-ME, but only one band (32 kDa) was found with 2-ME treatment [7]. The arrow indicated the



(B)

	4	5	6	7	8	9	10	11	12	13	14	15
Ancordin (this study)	L	L	V	L	D	I	G	G	N	P	V	V
Winged bean protein☒	E	D	V	L	D	I	G	G	N	P	L	L
Medicago truncatula PI#	E	Q	V	L	D	I	N	G	N	P	I	F
Soybean TI*	D	F	V	L	D	N	E	G	N	P	L	E
Sporamin ##	T	P	V	L	D	I	N	G	D	E	V	R

☒ accession number A61491; # accession number AF526372;
* accession number X64447; ## accession number U17334

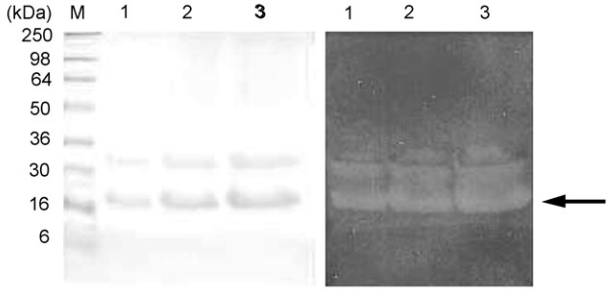


Fig. 1 – (A) The protein patterns of crude extracts of the rhizome of *A. cordifolia* were directly analyzed by 12.5% SDS-PAGE gels with or without 2-mercaptoethanol addition and were stained with Coomassie brilliant blue R-250. The 10 μg protein was loaded in each well. The arrow indicated one major (23 kDa) protein band, named tentatively ancordin, was electroblotted onto PVDF membrane and subjected to N-terminal amino acid sequencing. (B) Sequence alignment of the first 4–15 amino acids of ancordin in the N-terminal region with wingbean protein (A61491), medicago TI (AF526372), soybean TI (X64447), and sporamin (U17334). The black shading shows the identical amino acids. The protein stains (C), and the trypsin inhibitory stains (D) of the different amounts of crude extracts (lanes 1–3 were 10, 15, and 20 μg, respectively) from rhizomes of *A. cordifolia* in the 12.5% non-reduced SDS-PAGE gels. The arrow indicated 20 kDa protein of ancordin.

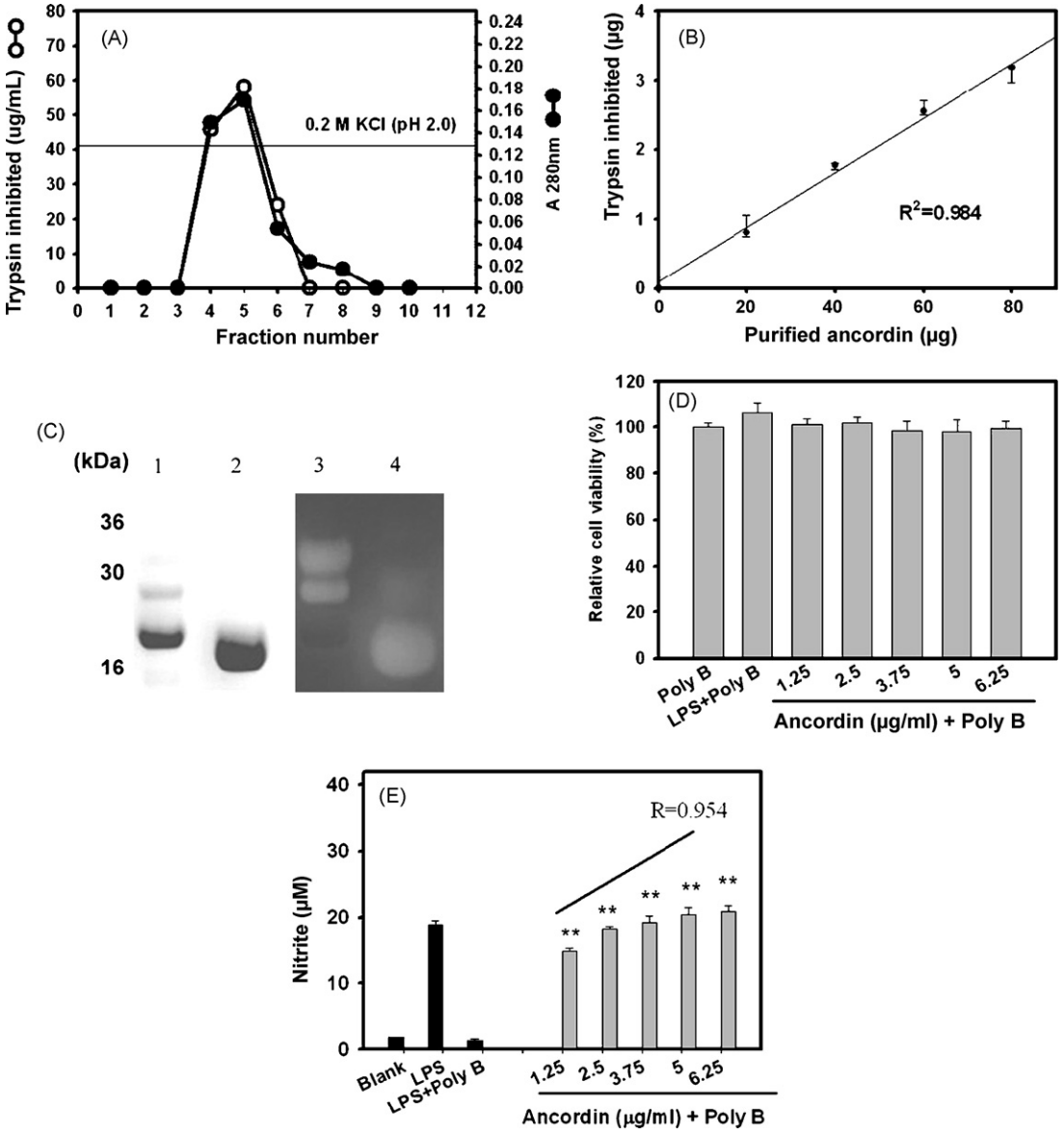


Fig. 2 – (A) The chromatogram of crude extracts from the rhizome of *A. cordifolia* on the trypsin affinity column (1.5 cm × 20 cm). After washing with 100 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl, the absorbed ancordin were eluted by changing pH value with 200 mM KC1 buffer (pH 2.0). (B) The trypsin inhibitory activities of ancordin (20, 40, 60, and 80 μg) were determined by the inhibition of N-benzoyl-L-arginine-4-nitroanilide hydrolysis in the presence of 7.5 μg trypsin. (C) The protein stains (lanes 1 and 2) and TI activity stains (lanes 3 and 4) of purified sporamin from sweet potato (lanes 1 and 3) and ancordin from madeira-vine rhizomes (lanes 2 and 4) analyzed by 12.5% SDS-PAGE gels without 2-mercaptoethanol addition. (D) Effects of ancordin (1.25, 2.5, 3.75, 5, and 6.25 μg/ml) and LPS (600 ng/ml) on the cytotoxicity and (E) the stimulatory nitric oxide productions (expressed as nitrite) in RAW264.7 cells in the presence of polymyxin B (50 μg/ml). The regression constant of NO productions among 1.25–5 μg/ml ancordin is 0.954. A difference between the (LPS + polymyxin B) and each treatment was considered statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

major (23 kDa) protein band, named tentatively ancordin, was electroblotted onto PVDF membrane and subjected to N-terminal amino acid sequencing. The first 15 amino acids in the N-terminal region were KDDLLVLDIGGNPVV. The Fig. 1B showed the sequence alignment of the first 4–15 amino acids of ancordin in the N-terminal region with wingbean seed protein (A61491), medicago TI (AF526372), soybean TI (X64447), and sporamin (U17334). It was found that the N-terminal amino acid sequences of ancordin were highly homologous to sequences (the black shading shows the identical amino acids) of winged bean seed protein ws-1, *Medicago truncatula* proteinase inhibitor, soybean trypsin inhibitor, and sporamin. From sequence alignments, it was found that the major rhizome protein of madeira-vine, ancordin, was closely related to proteinase inhibitors, soybean TI and root storage protein of sweet potato, sporamin, which was reported to have TI activity [31]. Therefore, the trypsin inhibitory activity of ancordin will be tested in the following section.

Table 1 – The purification of ancordin from crude extracts of madeira-vine rhizomes by trypsin affinity column

Procedure	Protein (mg)	Activity (mg trypsin inhibited)	Specific Activity (mg trypsin inhibited/mg protein)	Fold	Recovery (%)
Crude extracts[a]	162.79	5.762	0.0354	1	100
Trypsin affinity column	3.65	0.894	0.245	6.92	15.52

[a] The 14.5 g fresh rhizomes of madeira-vine was extracted with 100 mM Tris–HCl buffer (pH 7.9) containing 1% ascorbate and 1% (w/v) polyvinylpyrrolidone in a homogenizer for 30 s (four times). The homogenates were filtered through four layers of cheesecloth and centrifuged twice at 12,000 × *g* for 30 min. The supernatants were saved as crude extracts.

3.2. *TI stains of ancordin in SDS-PAGE gels*

It was previously reported that the reduced TI might lost its inhibitory activity against trypsin [6,9,30]. Therefore, the different amounts of crude extracts (10, 15, and 20 μg) of madeira-vine rhizome were analyzed by 12.5% non-reduced SDS-PAGE gels for protein staining (Fig. 1C) and TI stains (Fig. 1D). It was found that the ancordin showed trypsin inhibitory activity (arrow indicated, Fig. 1D) in the dose-dependent matter in the SDS-PAGE gel. The blank zones (white ones) against the purple-reddish background showed positions where the trypsin was inhibited. It was also found that the minor protein band (32 kDa, Fig. 1D) were also with minor trypsin inhibitory activities. It was the first report that the major rhizome protein of madeira-vine exhibited TI activities. By using TI staining method, it was found that the aerial tuber of madeira-vine also contained ancordin which was also exhibited TI activity but the fresh leaves of madeira-vine did not found (data not shown).

3.3. *Purification of ancordin from rhizomes of A. cordifolia*

It was reasonable to design the trypsin affinity column [5,10,11] for ancordin purification for its TI activity as above-mentioned. The 50 ml crude extracts of madeira-vine rhizomes were directly loaded onto trypsin affinity column. After washing with 100 mM Tris–HCl buffer (pH 7.9) containing 100 mM NaCl, the absorbed ancordin were eluted by changing pH value with 200 mM KC1 buffer (pH 2.0) (Fig. 2A). The purification table was showed at Table 1. The purification of ancordin is calculated 6.92-fold in one step of trypsin affinity column. The protein curve was matched with the trypsin inhibitory activities determined by the inhibition of *N*-benzoyl-L-arginine-4-nitroanilide hydrolysis in the presence of trypsin. The active fractions were collected, readjusted pH to 7.0, dialyzed against distilled water overnight with water changes twice, and then lyophilized for further uses. It was calculated that the purified ancordin exhibited 0.0428 μg trypsin inhibition/μg ancordin (corresponding to 0.53 unit of TPCK-treated trypsin inhibited/μg ancordin, $R^2 = 0.984$) (Fig. 2B). Using sporamin from sweet potato as positive controls (Fig. 2C, lanes 1 and 3), it was found that the purified ancordin (23 kDa) from trypsin affinity column was single band (Fig. 2C, lane 2) and with trypsin inhibitory activity (clear zone, Fig. 2C, lane 4).

3.4. *Effects of ancordin on the NO productions in RAW 264.7 cells*

The purified ancordin (1.25–6.25 μg/ml) was used to test the cytotoxicity toward RAW264.7 cells (Fig. 2D) in the presence of polymyxin B. It was found that under the tested concentrations, the ancordin showed little cytotoxic activities toward RAW264.7 cells (Fig. 2D). The nitrite in the cultured medium was detected by the Griess reagent, an indirect method to measure the release of NO. It was found that ancordin (1.25–5 μg/ml) could dose-dependently ($R = 0.954$) stimulate the NO productions (expressed as nitrite concentrations) in RAW264.7 cells without significant cytotoxicity, and kept the similar effects in NO production in 6.25 μg/ml ancordin. The stimulatory NO productions by ancordin showed significantly differences with the (LPS + polymyxin B) group ($p < 0.01$, Fig. 2E). LPS (600 ng/ml) was used as a control in the presence or absence of polymyxin B (50 μg/ml) in the cultured medium. It was found that the presence of polymyxin B could eliminate the effects of LPS in the NO productions of RAW264.7 cells (Fig. 2E, column 3), but have few effects on ancordin for the stimulations of NO productions. This result suggested that ancordin could stimulate RAW264.7 cells to produce NO, not in the way of LPS contaminations. The protein of smilaxin, isolated from *Smilax glabra* rhizomes with molecular mass of 30 kDa, could stimulate NO productions in murine peritoneal macrophages [4], and the recombinant transferring could stimulate NO responses in goldfish and murine macrophages [29]. Macrophages released several mediators, including inflammatory cytokines and NO [26]. These mediators induced the activation and differentiation of lymphocytes, and the proliferation of granulocytes, support enhanced cytotoxicity against tumor cells and accelerate immunoreactivity *in vivo* [24].

In conclusions, the purified ancordin with trypsin inhibitory activities and stimulatory effects on NO productions in RAW 264.7 cells. The gene cloning and overexpression of ancordin are currently performing. Several cell model systems for cytokine productions and other biological activities will be further investigated in the future.

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