

Original Article

Isolation and bioactivity of an angiogenesis inhibitor extracted from the cartilage of *Dasyatis akajei*

Hongyu Luo MAppSci, Jiajing Xu MAppSci and Xinwei Yu MAppSci

Department of Ocean Science & Technology, Zhejiang Ocean University, Zhoushan 316004, China

The aim of this study is to isolate, characterize, and identify the bioactivity of an angiogenesis inhibitor derived from the *Dasyatis akajei* cartilage. By guanidine hydrochloride extract, refrigerated centrifugation, ultrafiltration, 20%-80% acetone precipitation, dialysis and refrigerated desiccation, the *DCGE* (*Dasyatis akajei* cartilage guanidine hydrochloride extract) of molecular weights from 3 kDa ~300 kDa was obtained from the *Dasyatis akajei* cartilage. *DCAI* (*Dasyatis akajei* cartilage angiogenesis inhibitor) from 20%-30% acetone precipitation of *DCGE* was found to have the strongest angiogenesis inhibitory effect. Protein quantification of *DCAI* shows the main components were proteins with about 73.5% weight of the total *DCAI*. The bioactivity of the *DCAI* was investigated by inhibiting the formation of the blood vessels of the chick embryo chorioallantoic membrane (CAM). The ratio between the vascular area and the non-vascular area (VA/NVA) in sampling point was used for quantitative analysis of the inhibitory effect of *DCAI*, which can be obtained by computer image analysis system (CIAS). Compared with the control group, the sampling point of the *DCAI* group had significantly lower VA/NVA. A large area of blood vessels in sampling point was significantly faded and the vascular structure was blurred with broken branches accompanied by the decreased density of vessels. The inhibitory effect with same dosage of *DCAI* is about ten times higher than that in the positive control group (chondroitin sulphate group) and the effect increases with the concentration of *DCAI*. The results from the present study indicates that the *DCAI* from *Dasyatis akajei* cartilage has an angiogenesis inhibitory effect, and there is also a positive correlation between the concentration and the inhibitory effect.

Key Words: *Dasyatis akajei* cartilage, isolation, angiogenesis inhibitor, chick embryo chorioallantoic membrane

Introduction

The development of a system of tumor blood supply is a key step in the malignant transformation. The interaction between the tumor vascularization and their malignant transformation has been shown for various tumors. Therefore, tumor angiogenesis, i.e., the generation of new capillaries from the preexisting blood vessels, has been actively studied during the subsequent years. Shark cartilage has been revealed to contain a protein, an angiogenesis inhibitor that significantly inhibits the development of blood vessels that nourish solid tumors by Brem and Folkman¹, in 1975. Since then, the angiogenesis inhibitors with different molecular weight have been partially purified from shark cartilage extracts, including the 10-14kDa U-995², 18 kDa SCAF-1³, and the 10 kDa SCF2⁴ and so on. Although cartilage is a potential source of angiogenesis inhibitors, but only relatively small quantities are present in shark with 6% to 8% of the total weight. In contrast, cartilage represents about 17% of the total weight of *Dasyatis akajei*, and there is plenty of *Dasyatis akajei* source, making *Dasyatis akajei* an ideal source of cartilage in the search for tumor angiogenesis inhibitors. It's important to isolate and study the antiangiogenesis effect of *Dasyatis akajei* cartilage. However, to our knowledge no one has yet successfully isolated an angiogenesis inhibitor from the cartilage of *Dasyatis akajei*. In this report we report in detail the isolation and characterization of an angiogenesis inhibitor from this species.

Materials and methods

Raw materials

Dasyatis akajei were obtained from the local market (Zhoushan, China)

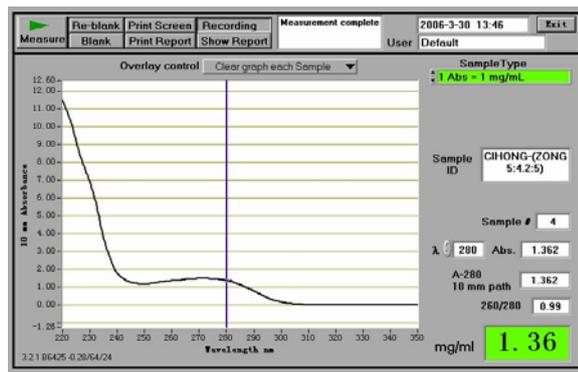
Reagent

Guanidine hydrochloride (A.R), MES (A.R), EDTA (A.R), sodium azide (A.R), acetone (A.R), MC-450(A.R), Tris (A.R), chondroitin sulphate (A.R) were obtained from Sangon (Shanghai, China)

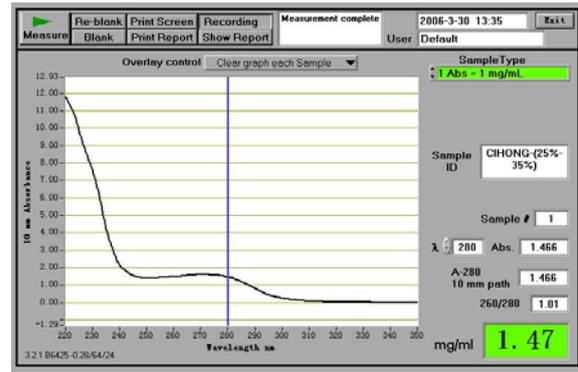
Preparing *Dasyatis akajei* cartilage guanidine hydrochloride extract (DCGE)

Dasyatis akajei cartilage was extracted in 1 mol/L guanidinium hydrochloride and 0.02 mol/L MES solution (contain 0.02% EDTA) (48 h) at 28 °C. The extract was then centrifuged at 5000×g for 30 min. The sediment was discarded and the supernatant was collected, and subsequently centrifuged at 12,000×g for another 30min. The supernatant was combined and followed by an ultrafiltration procedure that results in a fraction containing a variety of molecules with molecular weights from 3 kDa~300 kDa.

Corresponding Author: Associate Professor Hongyu Luo, Dept of Ocean Science & Technology, Zhejiang Ocean University, Zhoushan 316004, China
Tel: 13506607900; Fax: 86-580-2554818
Email: Lisa8919@163.com



(B) The protein concentration of *Dasyatis akajei* cartilage angiogenesis inhibitor (DCAI)



(A) The protein concentration of *Dasyatis akajei* cartilage from the guanidine hydrochloride extract (DCGE)

Figure 1. The results of protein quantification assay

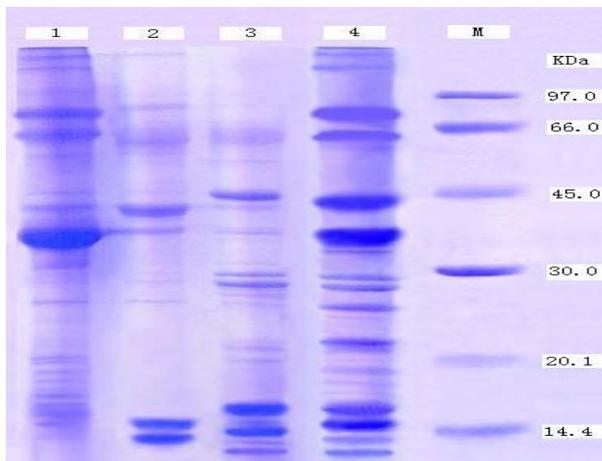


Figure 2. SDS-PAGE of the production of acetone-fractioned precipitation of the DCGE. lane 1, DCAI; lane 2, sediment of 40% acetone-fractioned precipitation; lane 3, sediment of 50%-80% acetone-fractioned precipitation; lane 4, DCGE; Lane M, standard molecular weight markers

The concentrated cartilage extract was dialyzed extensively (membrane molecular weight cutoff 3 kDa) in 0.02 mol/L Tris-HCl (pH 7.6), containing 0.05 mol/L NaCl for 24 h, leading to the *Dasyatis akajei* cartilage guanidine hydrochloride extract (DCGE).

Prepare *Dasyatis akajei* cartilage angiogenesis inhibitor (DCAI)

The obtained DCGE was fractioned through precipitation with 20%-80% acetone and centrifugation. The sediment of 20%-30%, 40%, 50%-80% were collected and lyophilized, respectively. The desired DCAI was the sediment from the 20%-30% acetone-fractioned precipitation, which shows the highest angiogenesis inhibitory effect (Fig 3)

Protein concentration quantification

2 mg of lyophilized DCGE and DCAI were mixed with 2 mL of dd water, respectively. The protein concentrations of the DCGE and DCAI was determined by NanoDrop ND-1000 Spectrophotometer with bovine serum albumin as a standard (Fig 1)

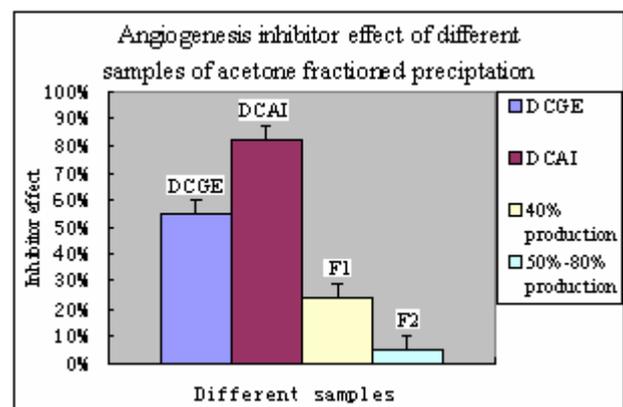


Figure 3. Angiogenesis inhibiting activity of different productions of the acetone-fractioned precipitation of the DCGE. The concentration of each sample was 1 mg.mL⁻¹. 10% MC for negative control and chondroitin sulphate for positive control. The inhibitory effect was determined by the CAM assay. Each bar represents the mean ±SEM.

SDS-PAGE analyses

The products of the acetone-fractioned precipitation were analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis⁵ (SDS-PAGE) (Fig 2), and detected by colloidal Coomassie blue staining. The molecular mass markers used in SDS-PAGE was rabbit muscle phosphorylase (M r 97400), bovine serum albumin (M r 66200), hen egg albumin (M r 42700), bovine carbonicanhydrase (M r 31000), soybean trypsin inhibitor (M r 21 500), and hen egg lysozyme (M r 14400).

Activity studies

Fertilized chicken eggs were incubated at 37.6 °C under 75% relative humidity throughout the experiment. Well-developed eggs were divided into five groups with five eggs for each one: the negative control group (10% MC), the positive control group (1mg/mL chondroitin sulphate CS), and the other three DCAI groups. For the three DCAI groups, the following concentration of DCAI was prepared by mixing lyophilized DCAI with 10% MC solution, 10 mg/mL, 1 mg/mL, and 0.1 mg/mL. 10μL of each samples after filtration was sterilized by an vacuum driven filtration systems with PES filter (0.2 μM) on the surface of CAM. After sampling, the chicken eggs were incubated

for another 24 h to observe the growth of capillary vessels in the sampling point (Fig 4, Fig 5).

Evaluation Method

Computer image analysis system (CIAS) was also used to quantify antiangiogenesis effect of *DCAI* by the ratio between the vascular area and the non-vascular area (VA/NVA) in the sampling point. The images of this experiment were processed by the computer program according to the principle of Y G Fan's image analysis software.

Basic Principle

The images of the experiment are high-resolution JPEG files. The program would load them to the workplace first. After converting to the gray image, the image would be changed to B/W ones by finding the threshold of depth and then binarize it into the B/W image. The ratio between the VA/NVA (black area to white area) in the sampling point is then calculated as shown in Fig 4.

Results and discussion

Protein concentration quantification assay

The results show that the protein concentration of the *DCGE* and the *DCAI* were about 1.36mg/mL (Fig1A),

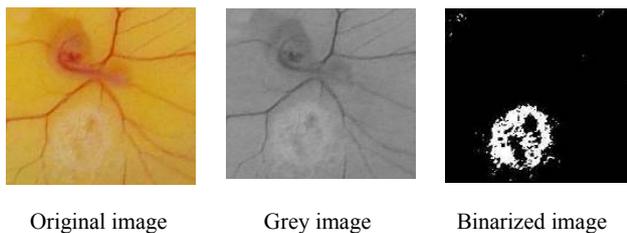


Figure 4. Procedure of the treatment of sampled CAM images



Figure 5. The relationship between the *DCAI* concentration and the inhibitory effect. The samples of *DCAI* were diluted to different concentrations in 10% MC

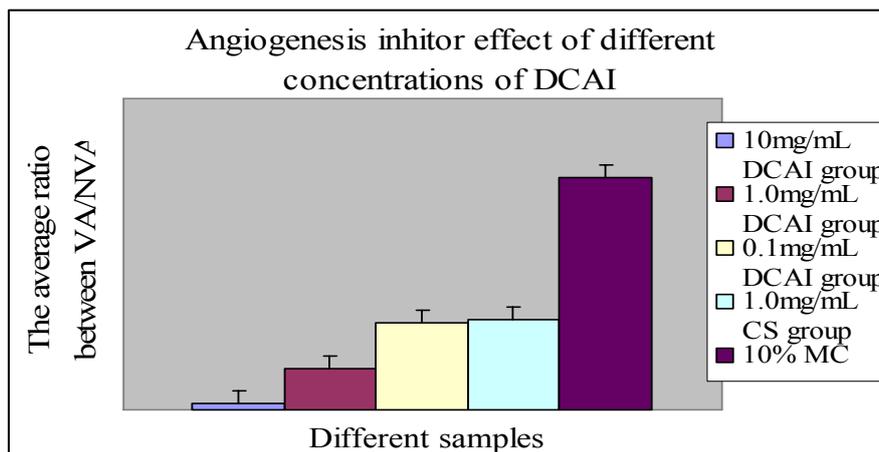


Figure 6. Concentration-dependent inhibitory effect of *DCAI*. Samples of *DCAI* were diluted to different concentrations in 10% MC. 10% MC for negative control and 1mg.mL⁻¹ chondroitin sulphate for the positive control group. The inhibitory effect was determined by the CAM assay. Each bar represents the mean±SEM

1.47 mg/mL (Fig1B). The protein in *DCAI* is higher than that in *DCGE*, and about 73.5% weight of total *DCAI*. The main components of *DCAI* are proteins.

Acetone-fractioned precipitation assay

The three *DCGE* fractions were obtained from acetone precipitation. Figure 3 shows that *DCAI* containing about 50% weight of the *DCGE* has the highest bioactivity; 15% weight of the *DCGE* is in F1 (the sediment of 40% acetone-fractioned precipitation) and it has about 24% angiogenesis inhibitory ratio (inhibited eggs / total eggs); the other 35% weight of the *DCGE* is in F2 (the sediment of 50%-80% acetone) and it has 5% angiogenesis inhibitory ratio, with no antiangiogenesis effect. Therefore the *DCAI* studied here has the strongest effect on angiogenesis inhibition. The activity in *DCAI* may be due to the aggregation of the proteins with molecular weights from 30 kDa~100 kDa (Fig 2).

Inhibitor effect Analysis of *DCAI*

As shown in Fig 5, the bioactivity of an angiogenesis inhibitor was confirmed by that the fact that a large area of blood vessels in sampling point of significantly *DCAI* group was faded, the vascular structure blurred with broken branches accompanied by the decreased density of vessels. In the control group, the leaflike vascular net was clear and radiative. As shown in Fig 6, the ratio between VA/NVA index of *DCAI* groups in sampling point were significantly lower than that of the control group. The inhibitory effect increases with the concentration of *DCAI*. When the concentration of *DCAI* is 10 mg/ml, the inhibitory effect reaches its maximum compared with other concentration of *DCAI*. The effect with same dosage of *DCAI* is about ten times higher than that in positive control group (chondroitin sulphate group).The results show *DCAI* has a positive correlation between the concentration

and the inhibitory effect and it is more effective than chondroitin sulphate.

It has been proposed that a tumour growth is limited to $1\pm 2\text{ mm}^2$ by the diffusion of nutrients unless it recruits additional blood vessels and, hence, an 'angiogenic switch' is a critical step in tumorigenesis.⁵ A large number of angiogenesis inhibitors have been identified by looking for the inhibitory effect of new vessels in a variety of in vivo assays, including cell factor, hormone, antibiotics, enzyme inhibitor and so on. Some of them have been applied to clinic, but most of them have demonstrated new and unusual toxicity profiles including dose limiting arthritis as well as unexpected bleeding and thrombotic complications. The angiogenesis inhibitor from *Dasyatis akajei* cartilage is a natural source of material with strong antiangiogenesis activity, and the inhibitory effect was positively associated with the *DCAI* concentration. Therefore it is hoped that this angiogenesis inhibitor may provide a novel treatment for patients with malignancies, and perhaps even for those with non-malignancies. It must be recognized, however, that our knowledge of the molecular mechanisms of this angiogenesis inhibitor from *Dasyatis akajei* remains limited, and further studies are undergoing.

Acknowledgements

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