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A novel heme-containing protein with anti-HIV-1 activity from skin secretions of *Bufo andrewsi*

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Abstract

A novel protein, named BAS-AH, was purified and characterized from the skin of the toad *Bufo andrewsi*. BAS-AH is a single chain protein and the apparent molecular weight is about 63 kDa as judged by SDS-PAGE. BAS-AH was determined to bind heme (0.89 mol heme/mol protein) as determined by pyridine haemochrome analysis. Fifty percentage cytotoxic concentration (CC_{50}) of BAS-AH on C8166 cells was 9.5 μM. However, at concentrations that showed little effect on cell viability, BAS-AH displayed dose dependent inhibition on HIV-1 infection and replication. The antiviral selectivity indexes (CC_{50}/EC_{50}) were 14.4 and 11.4, respectively, corresponding to the measurements of syncytium formation and HIV-1 p24 antigen expression. BAS-AH also showed an inhibitory effect on the activity of recombinant HIV-1 reverse transcriptase ($IC_{50}=1.32$ μM). The N-terminal sequence of BAS-AH was determined to be NAKXKADVIGKISILLGQDNLSNIVAAM, which exhibited little identity with other known anti-HIV-1 proteins. BAS-AH is devoid of antibacterial, proteolytic, trypsin inhibitory activity, L-amino acid oxidase activity and catalase activity.

Keywords: Toad; Skin secretion; HIV-1; Heme; *Bufo andrewsi*

1. Introduction

Amphibian skin is a morphologically, biochemically and physiologically complex organ, which fulfills a wide range of functions necessary for the amphibian survival, including respiration, water regulation, anti-predator, antimicrobial defense, excretion, temperature control, etc. (Clarke, 1997). Many types of molecules with interesting biological functions are produced by amphibian skin. They include amines, lipophilic alkaloids, indolic alkaloids, toxic steroids of the bufadienolide-class, peptides and proteins (Daly, 1995). Peptides/proteins from amphibian skin showed various biological activities such as antimicrobial, hemolytic, platelet aggregation activating, bradykinin-like and insulin-releasing activities (Apponyi et al., 2004; Conlon et al., 2004; Lee et al., 2005; Marenah et al., 2004; Zhang et al., 2005).

Bufo andrewsi is a common toad distributed widely in west China, living in very harsh environments. The toad skins have been widely used in Chinese traditional medicine

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and the dried skin secretions, known as Chansu, have the functions of detoxification, detumescence and pain relief. Previously, we have studied the bioactivity of skin extract of *Bufo andrewsi* and found that it showed strong lethal toxicity and possessed proteolytic, trypsin inhibitory, cytotoxic and antimicrobial activities (Lai et al., 2002b). Here we present the isolation and characterization of an anti-HIV-1 protein from *Bufo andrewsi* skin.

2. Materials and methods

2.1. Materials

Adult specimens of *Bufo andrewsi* of both sexes were collected in Qujing county, Yunnan province of China. Bovine pancreatic trypsin (EC 3.4.21.4), bovine serum albumin (BSA), SCHIFF'S reagent, periodic acid, pyridine, $K_3Fe(CN)_6$, sodium dithionite, 3′-azido-3′-deoxythymidine (AZT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were from Sigma (St. Louis, USA). Mouse anti-HIV-1 p24 antigen monoclonal antibody was produced by Laboratory of Molecular Immunopharmacology, Kunming Institute, Chinese Academy of Sciences (CAS). Human polyclonal anti-HIV-1 serum was kindly donated by Dr Hiroo Hoshino (Department of Virology and Preventive Medicine, Gunma University School of Medicine, Japan). Chromogenic substrate, H-D-Pro-Phe-Arg-*p*NA (S-2302) was from Kabi Vitrum (Stockholm, Sweden). DEAE-Sephadex A-50, Sephadex G-75 (superfine) and Q-Sepharose (high performance) were from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein concentration of the final product was determined by a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. C8166 cell and HIV-1_{IIIB} strain were kindly donated by Medical Research Council (MRC), AIDS Reagent Project, UK. The cell and HIV-1 virus were cultured as described previously (Zhang et al., 2003). All other reagents were of the highest purity available.

2.2. Purification of BAS-AH

Adult specimens of *Bufo andrewsi* of both sexes ($n=50$; weight range 80–120 g) were used. The toads were electrically stimulated at 24 V by a transformer and washed in 0.9% NaCl. The secretions were filtered and the insoluble material was eliminated by centrifugation at 10,000*g* at 4 °C for 30 min. The supernatant was filtered again, lyophilized and stored at -80 °C until use. The lyophilized skin secretions were dissolved in 50 ml of 0.05 M Tris–HCl, pH 7.8 buffer, dialyzed against the same buffer and then applied on a DEAE-Sephadex A-50 ion exchanger chromatography column (3.5×50 cm), equilibrated with the same buffer. The elution was first performed with two column volumes of the same buffer without NaCl gradient. Eluted proteins were monitored at 280 nm. The fractions containing anti-HIV-1 activity from the DEAE-Sephadex A-50 column were pooled and concentrated, then applied to a Sephadex G-75 gel filtration column (2.6×100 cm) equilibrated with 0.05 M Tris–HCl, pH 7.8 buffer, containing 0.1 M NaCl. Fractions with anti-HIV-1 activity were concentrated and dialyzed against 0.05 M Tris–HCl, pH 8.8 buffer, then further purified by a Q-Sepharose (high performance) column (1×20 cm). The column was pre-equilibrated with the same buffer.

2.3. SDS-polyacrylamide gel electrophoresis

Ten percentage of non-continuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the apparent molecular weight of BAS-AH according to the method of Laemmli (1970). Proteins were mixed with 6× reducing loading buffer (1% SDS, 30% glycerol, 0.28 M Tris–HCl, pH 6.8, 0.001% bromphenol blue, 0.5 M DTT) or 6× non-reducing loading buffer (same as reducing loading buffer, but without DTT). The gel was stained with Coomassie brilliant R-250 (Sigma). Periodic acid-Schiff's staining was performed according to the protocols provided by the manufacturer.

2.4. Automatic amino acid sequence determination

The amino acid sequence of N-terminal was determined by automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer (model 476A).

2.5. Determinaton of bound heme

The assay was performed according to the method of Berry and Trumpower (1987). Briefly, 0.5 ml of a stock solution containing 200 mM NaCl and 40% (v/v) pyridine and 3 μl $K_3Fe(CN)_6$ were placed in a 1 ml cuvette. A 0.5 ml aliquot of BAS-AH (10 μM) was added with total mixing, and the oxidized spectrum was recorded from 350 to 700 nm. To record the reduced spectrum, 1.5 mg of solid sodium dithionite was added.

2.6. Determination of BAS-AH cytotoxicity

The cellular toxicity of BAS-AH on C8166 cells was assessed by MTT colorimetric assay as described previously (Zheng et al., 1999). Briefly, 100 μl of 3×10^4 cells was seeded on a microtiter plate and then 100 μl of various concentrations of BAS-AH was added. The plate was incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 72 h. After discarding the supernatant, MTT reagent was added and incubated for 4 h, and then 100 μl of 50% *N*,*N*-dimethylformamide-10% SDS was added. After the formazum was dissolved completely, the plate was read on an enzyme-linked immunosorbent assay (ELISA) reader (eLx 800, Bio-Tek Instruments, Winooski, VTUSA) at 595 nm/