Fish & Shellfish Immunology 35 (2013) 625–631

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Isolation and characterization of a novel antibacterial peptide derived from hemoglobin alpha in the liver of Japanese eel, *Anguilla japonica*

Dong Ling Zhang [a,b,c,d], Rui Zhang Guan [a,b,d,*], Wen Shu Huang [b,d], Jing Xiong [b,d]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, Hubei Province, China
[b] College of Fisheries, Jimei University, Xiamen 361021, Fujian Province, China
[c] Graduate School, Chinese Academy of Sciences, Beijing 100049, China
[d] Engineer Research Center of Eel Modern Industry Technology, Ministry of Education, China

ARTICLE INFO

Article history:
Received 24 May 2012
Received in revised form
9 August 2012
Accepted 17 August 2012
Available online 27 August 2012

Keywords:
Antibacterial peptide
Hemoglobin alpha chain
Japanese eel
Anguilla japonica
Liver

ABSTRACT

We isolated and characterized a novel antibacterial peptide, AJHbα, derived from hemoglobin alpha in the liver of Japanese eel, *Anguilla japonica*. It with concentration of 11.30 μM exhibited stronger antibacterial activity against pathogenic bacterium 1×10^6 cell ml^{-1} *Edwardsiella tarda* than other two bacteria. The extraction procedure for AJHbα included extraction with acetate acid, ultrafiltration, cation-exchange chromatography on HiTrap™ CM FF, reverse-phase liquid chromatography on Source 5R RPC and C18 RP-HPLC. MALDI-TOF MS suggested that the peptide had an observed molecular weight of 2388.05 Da. Its amino acid sequence determined by Edman degradation was similar to those of hemoglobin alpha chain in other fish by BLAST analysis. A complete N-terminal amino acid sequence of the AJHbα was FAHWPDLGPGSPSVKKHGKVIM corresponding to the cDNA sequence by RACE amplification. Its synthetic peptide had strong antibacterial activities against ten Gram-positive or negative bacteria. To our knowledge, AJHbα was the first identified fragment of hemoglobin alpha chain with strong antibacterial activity in fish.

1. Introduction

Antimicrobial peptides (AMPs) are distributed in most living organisms as a key component of the innate immune system, ranging from bacteria to plants and animals [1]. These peptides exhibit a wide range of activities against numerous microbes including Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites, while with little or no toxicity to host cells [2]. In addition, some AMPs have ability to inhibit the growth of tumor, to regulate iron balance, or function as immunity regulator and vaccine adjuvant [3]. To date, 3782 AMPs sequences have been characterized (http://www.bicnirrh.res.in/antimicrobial/), with a large number of them isolated from mammals, birds, amphibians and insects. Although fish are the most diversified group of vertebrates till now, relatively few AMPs have been isolated from them, which reflected presumably a simple lack of attention to this potentially rich source of AMPs.

Japanese eel, *Anguilla japonica*, is one of catadromous fish, distributed widely in coastal countries in the west of Pacific Ocean [4]. It is also one of the most important aquaculture species in the region including China where the fish has high annual yield. The most significant factor that affects Japanese eel culture is the incidence of various diseases, especially those caused by bacterial pathogens [5–7]. Studies on the prevention and cure of its diseases have focused on the isolation of pathogens and medical treatment [8,9]. However, few attentions have been paid on immune factors of eels. Only a few AMPs or antimicrobial substances have been identified from the skin mucus of the fish. Thus far, two types of lectins, AJL-1 and AJL-2 with antimicrobial activity, have been isolated from skin mucus of the Japanese eel, with the former showing agglutinating activity against *Streptococcus difficile*, and the latter AJL-2 against *Escherichia coli* [10,11]. An AMP, Parasin I has been identified in skin mucus of Japanese eel [12]. We have previously identified an AMP, named as AJN-10, and characterized partially, which exhibited antibacterial activity against *Aeromonas hydrophila*, but its amino acid sequence was not determined [13]. Moreover, only a 45 kDa glycoprotein was identified in skin mucus of other eels, i.e. from the European eel, which inhibited *A. hydrophila* with a minimal inhibitory concentration (MIC) of

* Corresponding author. College of Fisheries, Jimei University, Xiamen 361021, Fujian Province, China. Tel.: +86 592 6188973; fax: +86 592 6180973.
E-mail addresses: rzguan@jmu.edu.cn, zhangdongling@jmu.edu.cn (R.Z. Guan).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2012.08.022

l μg ml^{-1} [14]. AMPs or antimicrobial substances have not been reported from other parts of eel. It seems worthwhile to identify novel AMPs or antimicrobial substances from other parts of eel, and to investigate their possible antibacterial activities.

To the best of our knowledge, liver plays important roles in immunity. It is the main site for differentiation of T-cells besides the thymus, and some immune-relevant (including acute phase) genes and AMPs have been identified in the liver of some fish [15–17]. However, AMPs have not been characterized in the liver of Japanese eel till now. In our previous studies, antibacterial activities of acetic acid extract were determined from different tissues or organs, including skin mucus, liver, gill, spleen, bile, kidney and serum of Japanese eel, and antibacterial activity of liver extract was found to have the highest antibacterial activity against all tested bacteria, including several bacterial pathogens of eels, such as *Edwardsiella tarda* (B09), *Aeromonas* sp. (B18) and *A. hydrophila* (B27) [18]. In this report, a novel AMP was isolated from the liver of Japanese eel, which exhibited strong antibacterial activity against bacterial pathogen of eels, such as *E. tarda* (B09). Characterization of the AMP showed that it was derived from hemoglobin alpha chain (Hba). Isolation of the AMP and determination of its antibacterial activity were presented.

2. Materials and methods

2.1. Preparation of sample

Ice-cooled livers of healthy Japanese eel were obtained from Putian Dong-Yuan Roast Eel Ltd. (Putian, Fujian Province, China). They were collected to laboratory within approximately 2.5 h after dissection, and immediately frozen in −80 °C for further use.

Frozen livers weighting 150 g were cut into shards and mixed with 900 ml 10% acetic acid. Samples were then homogenized and stirred for 24 h at 4 °C, with the supernatant collected by centrifugation at 4 °C and 10,000 *g* for 35 min to prepare acidic extract of the liver. The acidic extract was subjected to boiling water bath for 10 min with continuous agitation, and cooled immediately in ice. The supernatant was lyophilized for further use after centrifugation at 4 °C and 10,000 *g* for 35 min. The lyophilized extract was dissolved in sterile water, and the antibacterial activity was determined.

Acidic extract of liver with antibacterial activity was further screened through 10 kDa Midgee™/MidGee Hoop by ultrafiltration system (GE Healthcare Life-Sciences). Protein samples of MW < 10 kDa was collected, lyophilized, and the antibacterial activity was again determined.

2.2. Cationic-exchange chromatography purification

The lyophilized powder was reconstituted in sterile water and loaded onto HiTrap™ CM FF 5 ml column (GE Healthcare Life-Sciences) connected to an ÄKTA Purifier 100 (GE Healthcare Life-Sciences). The column was equilibrated with 0.1 M sodium acetate (pH 7.4). Elution was then performed at 2 ml min^{-1} with a linear gradient (0–100%) of 4.0 M ammonium acetate (NH_4Ac) for 25 min. The elution was monitored at 280 nm, and 2 ml fractions were collected. Each fraction was lyophilized, reconstituted in 0.1 ml sterile water for antimicrobial activity determination.

2.3. Reverse-phase chromatography purification

Fractions with NH_4Ac concentration between 1.27 and 2.08 M were pooled and applied onto Source 5R RPC-ST-4.6/150 column (GE Healthcare Life-Sciences) connected to an ÄKTA Purifier 100 (GE Healthcare Life-Sciences). Elution was performed at 1 ml min^{-1} with a gradient from 0.1% (v/v) trifluoroacetic acid (TFA) in 2% acetonitrile (ACN) to 50% ACN for 50 min. The absorbance was monitored at 280 nm, and 1 ml fractions were collected. Each fraction was lyophilized, reconstituted in 0.1 ml sterile water for antimicrobial activity determination.

The active fractions were denoted by the bar in Fig. 2A, the 39th fraction was pooled from each time of Source 5R RPC-ST-4.6/150 column elution, lyophilized and reconstituted in sterile water. An aliquot (0.1 ml) was subjected to C18 reverse-phase high-performance liquid chromatography (RP-HPLC) on an analytical Stable Bond 300SB column (particle size 5 mm, 4.6 mm × 250 mm, Agilent, USA) equilibrated with 0.1% TFA in 2% ACN. The concentration of ACN in the eluting solvent was raised to 17% over 5 min, 17–27% over 31 min and 27–100% over 5 min with linear gradients. Absorbance was monitored at 280 nm, and fractions were collected from each peak. Each fraction was lyophilized, reconstituted in sterile water for antibacterial activity determination.

The active fractions eluted were pooled, lyophilized, reconstituted in sterile water and further purified by a second round of C18 RP-HPLC on the same column with a lower linear gradient (0–19.5% over 5 min/19.5–24.5% over 50 min/24.5–100% over 5 min). Fractions were collected, lyophilized, and antibacterial activity was determined.

2.4. Bacterial strains

E. tarda (B09), *Aeromonas caviae* (B14), *Aeromonas* sp. (B18), *A. hydrophila* (B27), *A. jandaei* (B29) and *A. veronii* (B69) were isolated and identified from diseased eel in our laborotory. *Staphylococcus aureus* (1.879), *Micrococcus lysodeikticus* (1.634), *Vibrio alginolyticus* (1.18333), *V. parahaemolyticus* (1.164) and *V. harveyi* (1.1600) were obtained from the China General Microbiological Culture Collection Center (Beijing, China). *S. aureus* (1.879) was inoculated on normal nutrient agar for 18 h at 37 °C. Other bacteria were inoculated on normal nutrient agar for 18 h at 28 °C. *E. tarda* (B09), *Aeromonas* sp. (B18), *A. hydrophila* (B27) were used to detect antibacterial activity of the purified peptide. All bacteria were used to detect antibacterial activity of the synthetic peptide exception *Aeromonas* sp. (B18).

2.5. Antibacterial assay

Inhibition zone assay was used to determine the antibacterial activity of acidic extract and protein sample of MW < 10 kDa. 20 ml of normal nutrient agar was seeded with 1×10^5 bacterial cells and poured into Petri dishes. Four 3-mm diameter wells were punched into the agar in the Petri dishes, and 8 μg protein samples were added into three wells, 16 μl sterile water was added into the fourth well as blank control. Samples were incubated for 24 h at 28 °C, radius of inhibition zones were then measured and to indicate antibacterial activity.

Antimicrobial activity of the peptide from chromatograph was determined by the liquid growth inhibition assay as described by Anderson and Beaven [19]. For sample experimental set, 20 μl peptide sample was mixed with isopyknic bacterial suspension (1×10^6 cell ml^{-1}) from a log-phase culture, and a sample blank, 20 μl peptide sample was mixed with isopyknic physiological saline; for bacterial set, 20 μl bacterial suspension was mixed with isopyknic physiological saline, and a bacterial blank, 40 μl physiological saline. After 3 h incubation at 28 °C, 50 μl Mueller-Hinton Broth was added into each well for further 21 h incubation at 28 °C. The numbers of variable bacteria was determined by measuring the change of OD_{620} value on a microtiter plate reader (Bio-Tek Synergy 4, USA). The experiment was repeated twice. corrAMP = OD_{620} of sample + bacteria corrected by subtraction of

sample blank value, and corrAB = OD_{620} of bacteria + physiological saline corrected by subtraction of bacterial blank value. A killing index (Ki%) was calculated by following formula:

$$Ki\% = [1 - corrAMP/corrAB] \times 100\%$$

Antimicrobial activity of synthetic peptide (CL Bio-Scientific Co. Ltd) was determined by the same procedure as described above, except that the concentration of all test bacteria were diluted into 0.1 OD_{620} value, and the concentrations of synthetic peptide were designed as 83.72 μM, 41.86 μM, 20.93 μM, and 10.47 μM respectively.

2.6. Protein quantification

sProtein concentration of samples was estimated by the method of Bradford using bovine serumalbumin (Amresco,USA) as standard [20].

2.7. MALDI-MS

The molecular weight, purity of the isolated antimicrobial peptide were determined by a matrix assisted laser desorption ionization tandem time-of-flight mass spectrometer (MALDI-TOF-MS) instrument (Bruker) operated in linear mode at Xiamen University in Xiamen of China.

2.8. N-terminal sequence and homology analyses

Amino acid sequence of the homogeneous peptide was determined by automated Edman degradation using an automatic peptide sequencer (ABI PROCISE™492cLC) at Genecorn Co. Ltd (Shanghai, China). The kinds of amino acids were determined according to the chromatogram of standard amino acids.

Peptide sequences obtained were aligned with homologous sequences from the National Center for Biotechnology Information databases, using blast programs and searching for short, nearly exact matches.

2.9. 3′-RACE and 5′-RACE amplification

3′-RACE were performed with the 3′-Full RACE Core Set kit 2.0 (TaKaRa, Santa Ana, CA, USA), following the manufacturer's instructions. All primers are listed in Table 1. PCR were performed with 10 μM outer primer and 10 μM gene specific primer (S1), Tm was 56 °C.

5′-RACE was performed with the SMARTer™ RACE cDNA Amplification Kit (Clontech), following the manufacturer's instructions. All primers were also listed in Table 1, Nested gradient PCR were performed. The first round of PCR reaction mixture contained first strand cDNA, 0.4 μM 10× Universal Primer A Mix (UPM), 10 μM gene specific primer (A2), Tm was 65 °C. The second round of PCR reaction mixture contained the first PCR mixture diluted 50 times, 10 μM Nested Universal Primer A(NUP) and gene specific primer (A1), PCR was performed with the following conditions: 94 °C for 2 min, 5 cycles at 94 °C for 30 s, 72 °C for 2 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 2 min; 20 cycles at 94 °C for 30 s, 69 °C for 30 s, 72 °C for 3 min. The final extension step was performed at 72 °C for 5 min. PCR products were sequenced by the Dragon Genomics Center (Invitrogen).

Table 1
Primers listed for 3′-RACE and 5′-RACE.

Primers	Sequence (5′–3′)	Purpose
Adaptor primer(Ap)	Containing the dT region designed by TaKaRa and Adaptor primer part	3′ Race cDNA
Out primer	TACCGTCGTTCCACTAGTGATTT	3′ Race
Gene-specific primer (S1)	CTCCCTCYGTCAAGAAGCAYGG	3′ Race
SMARTer IIA oligo primer	AAGCAGTGGTATCAACGC AGAAGTAC××××	5′ Race cDNA
5′-RACE CDS Primer A	$(T)_{25}$VN	5′ Race cDNA
10× Universal Primer A Mix (UPM)	Long: CTAATACGACTCA CTATAGGGC AAGCAGTGGTATCAACGCAGAGT Short: CTAATACGACTCA CTATAGGGC	5′ Race
Nest primer	AAGCAGTGGTATCAACGCAGAGT	5′ Race
Gene-specific primer (A1)	CAGCGTCTTGAAGTTGCCAGGGTCG	5′ Race
Gene-specific primer (A2)	GCACCCAGAGCTGCAAGGAATTTAT	5′ Race

2.10. Sequence analysis

The cDNA sequence was blasted by searching for short and nearly exact matches program at NCBI (National Center for Biotechnology Information).

3. Results

3.1. Purification of antibacterial peptide from the liver of Japanese eel

Nine hundred milliliter of acidic extract, containing 4.76 g dry substance, was obtained from 150 g frozen liver tissue. Some deposit was removed from acidic extract by boiling water bath and ultrafiltration, protein sample of MW < 10 kDa was then found to have strong antibacterial activity. The radius of inhibition zone was listed in Table 2.

In cation-exchange chromatography, fractions eluted from 28th to 32th tubes, corresponding to concentration of ammonium acetate between 1.27 M and 2.08 M, had antibacterial activity against *E. tarda* (B09), *Aeromonas* sp. (B18) and *A. hydrophila* (B27) (Fig. 1). These fractions were pooled, lyophilized and dissolved in sterile water, and applied to a Source 5R RPC-ST-4.6/150 column. In this column, fractions with antimicrobial activity were predominantly seen in the elution profile (Fig. 2), and the 39th fraction had strong antibacterial activity to B09, B18 and B27.

All 39th fractions was pooled, lyophilized, reconstituted in sterile water as loading sample, and the C18 RP-HPLC was performed for twenty-one times. The elution profile of each time had three active peaks, eluting from 24.5 to 25.3 min, 25.3 to 26.2 min and 26.2 to 27.1 min (Fig. 3, labeled peak1, peak2 and peak3).

In the present study, we focused on the peak labeled peak3. Fractions at peak3 from 21 times performance were collected, a total volume of 18.9 ml was then obtained. The sample was lyophilized before reconstituted in 1.0 ml sterile water for the

Table 2
Antibacterial activities of acidic extract, heated protein sample and protein sample of MW < 10 kDa (radius of inhibition zone, mm).

Bacterial strains	Radius of inhibition zone(mm)		
	Acidic extract	Heated protein sample	Protein of MW < 10 kDa
E. tarda (B09)	3.00 ± 0.57	6.50 ± 1.98	11.00 ± 0.85
Aeromonas sp. (B18)	8.30 ± 1.56	4.00 ± 0.42	13.20 ± 1.27
A. hydrophila (B27)	9.00 ± 1.70	10.00 ± 1.41	15.50 ± 3.11

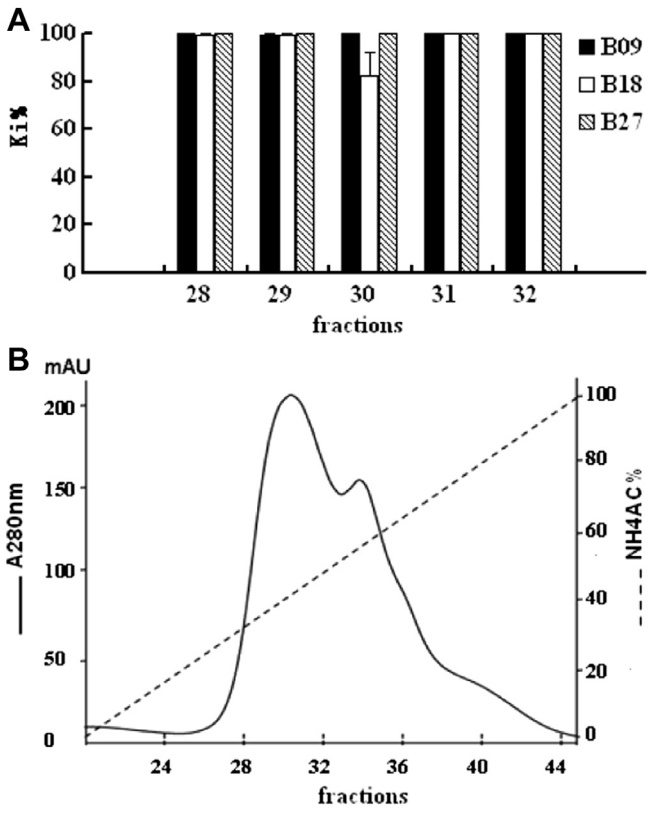

Fig. 1. Elution profile for the cation-exchange chromatography on proteins of MW < 10 kDa from the liver of Japanese eel. A: Antibacterial activity of the 28th to 32nd tubes on cation-exchange chromatography; B: Cation-exchange chromatography on peptide of MW < 10 kDa. The sample was loaded onto HiTrap™CM FF 5 ml column pre-equilibrated with sodium acetate (solvent A), and fractions were eluted with a linear gradient of 0–100% 4.0 M ammonium acetate (solvent B) at a flow rate of 2 ml min^{-1} for 25 min. The effluent was monitored at 280 nm.

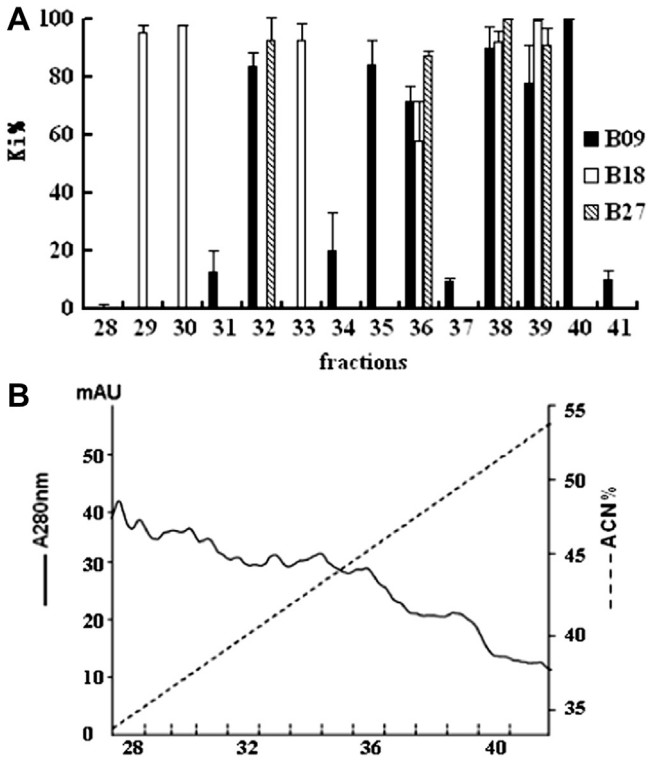

Fig. 2. Elution profile for reversed phase chromatography of the active fractions obtained from cation-exchange chromatography. A: Antibacterial activity of the 28th–41st tubes on reversed phase chromatography; B: the 28th to 32nd tubes of cation-exchange chromatography was subjected to a Source 5R RPC-ST-4.6/150 column. The column was pre-equilibrated with 0.1% TFA in 2% ACN (solvent A), and fractions were eluted with a linear gradient of 0–100% 50% ammonium acetate (solvent B) at a flow rate of 1 ml min^{-1} for 50 min. The effluent was monitored at 280 nm.

second loading sample. The second round of C18 RP-HPLC was performed for ten times with a slower water/acetonitrile gradient. A single active peak with a retention time of between 26 and 28 min was yielded each time from the elution profile (Fig. 4). A total of 20 ml active fractions, eluted for 10 times, were pooled, lyophilized, reconstituted in 0.5 ml of sterile water. The ultimately pure peptide solution was submitted for further chemical characterization and antibacterial spectrum assay.

3.2. Structural characterization

The pure sample was subjected to exact molecule mass measuring by MALDI-TOF MS and N-terminal amino acid sequencing. The result of MALDI-TOF MS showed that the pure peptide had an observed molecular mass of 2388.05 Da (Fig. 5). The sequence of the antibacterial peptide was FAHWPDLGPGSPSVKKHG according to the results of N-terminal amino acid sequencing. According to the results of 3′-RACE and 5′-RACE amplification, the complete sequence of the peptide was FAHWPDLGPGSPSVKKHGK-VIM, with a theoretic molecular mass of 2388.8 Da. It was well consistent with the observed molecular mass of MALDI-TOF MS. Analysis by the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi-tool.html) showed that pI of the peptide was 9.70.

3.3. Antimicrobial activity

The peptide at concentration of 11.30 μM purified from chromatography had much higher antibacterial activity to *E. tarda* (B09) than other two bacteria (Table 3). Synthetic peptide (FAHWPDLGPGSPSVKKHGKVIM) at concentration of 83.72 μM had strong antibacterial activity to all 10 tested bacteria including Gram-positive and Gram-negative bacteria, and to 6 Gram-negative bacteria including *A. caviae*, *A. hydrophila*, *A. jandaei*, *A. veronii*, *V. alginolyticus*, *V. parahaemolyticus* at concentration of 41.86 μM. However, Synthetic peptide had only weak antibacterial activity to *V. parahaemolyticus*, *V. alginolyticus* at concentration of 20.93 μM or 10.47 μM (Fig. 6).

3.4. 3′-RACE and 5′-RACE amplification

The nucleotide sequence of gene and the amino acids sequence of peptide were shown in Fig. 7 obtained from 3′-RACE and 5′-RACE amplification. The sequence analysis revealed that the amplified cDNA sequence encodes the complete sequence of an ORF of 429 bp. The deduced amino acids comprises 143 residues, being similar to Hb alpha of *Anguilla anguilla*, *Oncorhynchus mykiss*, *Danio rerio*, *Salmo salar* (GenBank accession Nos: AAB35054, ACO08763, CAE30442 and ACN10007 respectively). The nucleotide sequence of Japanese eel Hba has been deposited in the GenBank under accession number JN558592. Thus, the pure peptide was named as AJHbα.

4. Discussion

Here we report the isolation and characterization of AJHbα, a 22-amino acid-residue AMP, from the liver of Japanese eel, *A. japonica*.

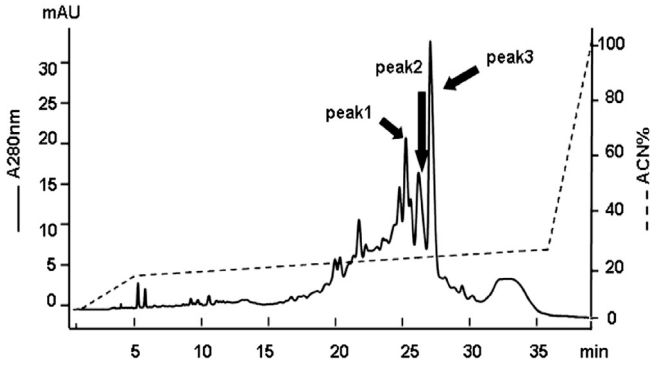

Fig. 3. Purification profile of the pooled 39th fractions by the first RP-HPLC. The RP-HPLC was performed on an analytical 300SB C18 column. Elution was executed at 25 °C with a gradient of 0.1% TFA in 2% ACN (solvent A) and 100% ACN (solvent B) at a flow rate of 1 ml min^{-1} as follows: washing with solvent B 0–17% over 5 min, 17–27% over 31 min and 27–100% over 5 min. The effluent was monitored at 280 nm. The fractions from peak3 denoted by the arrow were pooled, and subjected to further purification.

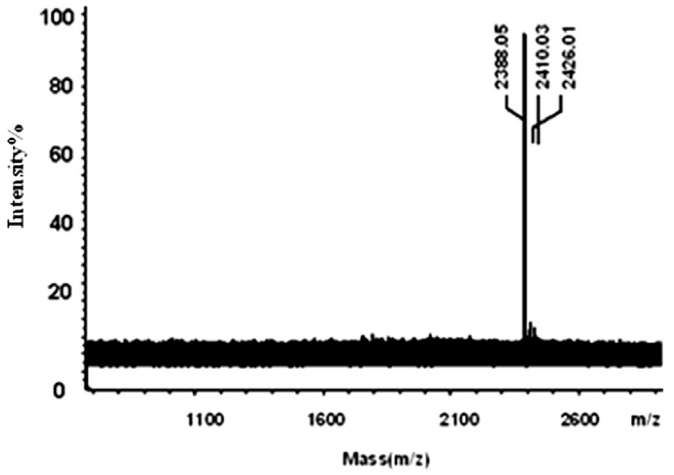

Fig. 5. Mass spectrogram obtained by MALDI-TOF-MS analysis of the purified antibacterial peptide from Japanese eel liver. Values are indicated in *m/z*. The transformed spectrum shows a mass (MHþ) of 2388.05 Da.

Synthetic and natural AJHba were active against a wide range of Gram-positive and Gram-negative bacteria from both fish pathogenic and non-aquatic species, implying a broad antibacterial spectrum. Activity could be detected down to AJHba concentrations of 41.86 μM against *S. aureus* and *M. lysodeikticus*, concentrations of 10.47 μM against *V. parahaemolyticus* and *V. harveyi*. Other studies on hipposin MIC was 40–80 μM against 1×10^5 cells ml^{-1} *S. aureus*, pleurocidin MIC was 5 μM against the same bacterial suspension [21]; two cathelicidins in Rainbow Trout MIC was 0.5–4 μM against 1×10^5 cells ml^{-1} *V. parahaemolyticus* [22]; hepcidin from the head kidney of large yellow croaker had antibacterial activity concentration 38 μM against 110^6 cells ml^{-1} *S. aureus*, *M. lysodeikticus*, *V. parahaemolyticus* and *V. harveyi* [23]. This indicates that Japanese eel, like several other fish possess an innate peptide-dependent host defense system to combat microbes.

In fish, most AMPs were isolated from skin, mucus and gill, although these AMPs were gradually proved to be existent in many tissues or organs by RT-PCR, Dot-blot, immunohistochemistry and in situ hybridization [24–27]. Perhaps, skin, mucus and gills constitute the frontier of defense against pathogens, so various AMPs were isolated from these sites. In this research, liver as original material for purifying AMPs from Japanese eel, we also found that there were many antibacterial fractions in the liver of Japanese eel (Figs. 1A, 2A and 3). Only AJHbα was characterized, and some other unidentified peptides might also have antibacterial activity or might be active against other pathogens, which would need further investigation. In addition, AMPs may be highly synergistic [28], including Hb-derived AMPs [29]. Perhaps, there are some other AMPs derived from Hb being existent in Japanese eel, and they are synergistic to various pathogene. At present, we only analyzed antibacterial activity of AJHbα, although Hb-derived AMPs are inhibitory to bacterial, fungi and parasites. To other chemical characterization of AJHbα, we would analyze latter.

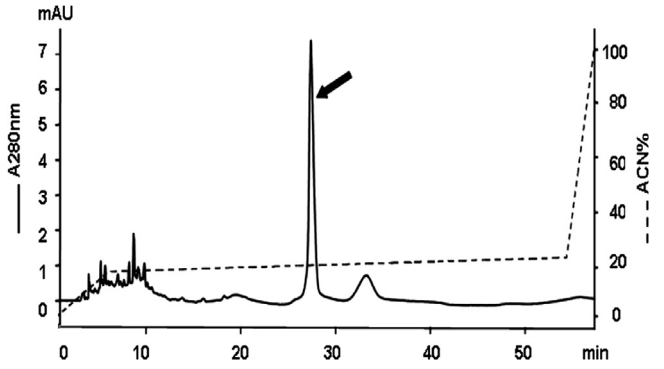

Fig. 4. Purification profile of the pooled peak3 fractions by the second round RP-HPLC. It was performed on the same column with a slower line gradient. Elution was executed at 25 °C with a gradient of 0.1% TFA in 2% ACN (solvent A) and 100% ACN (solvent B) at a flow rate of 1 ml min^{-1} as follows: washing with solvent B 0–19.5% over 5 min, 19.5–24.5% over 50 min and 24.5–100% over 5 min. The effluent was monitored at 280 nm. The fractions denoted by the arrow were pooled and submitted for further chemical characterization and antibacterial spectrum assay.

In this report, we have isolated an AMP, AJHbα, which is derived from Hbα. Hb as heme protein is present in all groups of organisms, even those without a bloodstream. It has multiple biological functions including transport of oxygen between tissues, hormone releasing, immunomodulatory, and hemopoietic, coronaroconstrictory, antigonadotropic, opioid-like activities [30]. In fact, Hb is also a potent antibacterial protein. The first report documenting Hb bactericidal properties appeared in 1958 [31], however, this finding was questioned immediately. Some researchers believed that the hemoglobin might promote bacterial growth through interfering with leukocyte oxygen metabolism under some conditions [32,33]. To mid-twentieth century, growing evidence suggested that Hb-derived peptides should be the host defense peptides, and they were named as hemocidins [34,35]. Hemocidins exhibited a broad-spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi. Various AMPs derived from Hbα or Hbβ were distributed in human, cattle, rabbit, with minimal inhibitory concentration (MIC) of 1 μg ml^{-1}. Furthermore, intact Hbα or Hbβ of human, horse, alligator and snake had antibacterial activity [36]. In fish, three peptides were derived from β-subunits of channel catfish (*Ictalurus punctata*). Hbβ could kill trophont stage of *Ichthyophthirius multifiliis*, and Hbβ-1 had antibacterial activity against Gram-negative and Gram-positive bacteria [37]. In addition, antimicrobial activity has been identified in rainbow trout erythrocytes, although the chemical identity of this activity has not been determined [38].

Table 3
Antibacterial spectra of AJHbα isolated from the liver of Japanese eel, *A. japonica*.

Bacterium	Identification code	Gram reaction	Ki%
E. tarda	B09	Negative	88.64% ± 3.91%
Aeromonas sp.	B18	Negative	17.57% ± 5.75%
A. hydrophila	B27	Negative	8.06% ± 7.30%

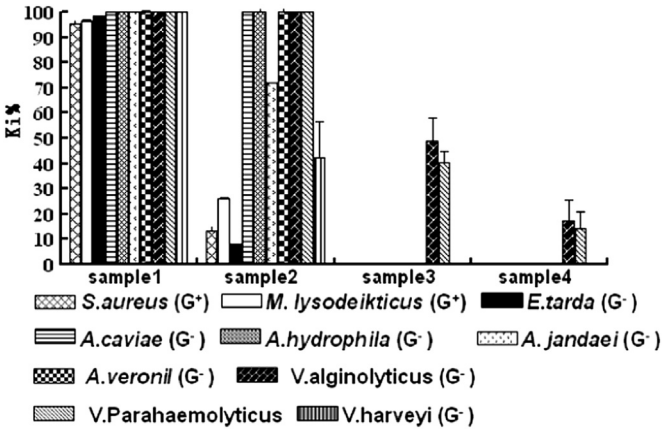

Fig. 6. Antimicrobial assays of synthetic peptide against ten bacterial strains. Sample1: synthetic peptide (83.72 μM); Sample2: synthetic peptide (41.86 μM); Sample3: synthetic peptide (20.93 μM); Sample4: synthetic peptide (10.47 μM).

AMPs can be generated via proteolysis from a number of functional proproteins, such as hemocyanin, lactoferricin, H2A, lysozyme, ubiquitin and complement [39–42]. AMPs derived from Hb support this proteolysis. 30 antimicrobial peptides were obtained from peptic hemoglobin hydrolysate, 24 peptides were derived from α chain of hemoglobin and 6 peptides were derived from β chain of hemoglobin [43]. Hb could give rise to relatively long peptides containing ca. 30 amino acid residues inside red blood cells naturally and secreted outside the cells. Further, these relatively long peptides were degraded into shorter peptides by proteases, forming "hemoglobin peptide library" with biological effect [44]. Thus, it is likely that AJHbα is a proteolysis product of Hba. Peptide Cutter tools (http://au.expasy.org/tools/peptidecutter/) analysis result shows that chymotrypsin can identify Y-X and M-X (Fig. 6) protease hydrolysis site, AJHbα (FAHWPDLGPGSPSVKKHGKVIM) is then hydrolyzed exactly from Hba by chymotrypsin.

Vertebrate Hb has been thought traditionally to be exclusively present in erythrocytes circulating in blood. Recent studies have

```
  1 TGCCTGCAGGTCGACGATTAAGCAGTGGTATCAACGCAGAGTACATGGGGGGTTCTCTTC 60
 61 TCAGTCGTTCTAAGAGGACAAGAGTTACAAAGCAGCTAAAAatgagtctgaccgcaaagg 120
  1                                          M  S  L  T  A  K  D  7
121 acaagagcttggtaacgggattctggcagaagatctccagcaaggctgatgagctcggag 180
  8   K  S  L  V  T  G  F  W  Q  K  I  S  S  K  A  D  E  L  G  A 27
181 ccgatgctctgtcaagaatgctcatagtcttccctgctacgaaaacatactttgctcact 240
 28   D  A  L  S  R  M  L  I  V  F  P  A  T  K  T  Y  F  A  H  W 47
241 ggcctgaccttggacctggctctccctccgtcaagaaacatggcaaggtcatcatgacag 300
 48   P  D  L  G  P  G  S  P  S  V  K  K  H  G  K  V  I  M  T  A 67
301 ctgttggcgatgctgtcggcaaaatggataaccttgtcggtggactaagtgcactcagtg 360
 68   V  G  D  A  V  G  K  M  D  N  L  V  G  G  L  S  A  L  S  D 87
361 acctgcatgcttccaaacttcgcatcgaccctggcaacttcaagacgctgtcccacaaca 420
 88   L  H  A  S  K  L  R  I  D  P  G  N  F  K  T  L  S  H  N  I 107
421 tcctggtggcttgcgcggtcaacttcccggctgatttcaccgctgaggtgcatgtggcaa 480
108   L  V  A  C  A  V  N  F  P  A  D  F  T  A  E  V  H  V  A  M 127
481 tggataaattccttgcagctctgggtgcagctctgtcagacaaataccgaTAAGATGTCA 540
128   D  K  F  L  A  A  L  G  A  A  L  S  D  K  Y  R  *          143
541 TCCACTGGCAGCTTTGTTAAATGCCGTCTGCCAAGCAAACTCAATTCAAAGAAACGAACA 600
601 AAAATAAAAGTTCTTTCCAAAAAAAAAAAAA 631
```

Fig. 7. Nucleotide and deduced amino acid sequence of a-chain of hemoglobin from Japanese eel, *A. japonica*. The start codon (ATG) is boxed. The stop codon (TAA) is asterisk. The polyadenylation signal motif (AATAAA) is bold italic. The underlined indicates the protein sequence of AJHbα as described in report, and the other residues are deduced from the nucleotide sequence.

subverted this notion, which demonstrated that Hb is expressed in a variety of nonhematopoietic tissues and cell lines. Liu et al. found that β-globin gene was induced in murine macrophages by the treatment of lipopolysaccharide (LPS) and interferon-γ (IFN-γ) [45]. Nishi et al. demonstrated that Hb was expressed by mesangial cells in the kidney [46]. Two groups of scientists also independently reported the expression of Hb in alveolar epithelial type II cells of lung [47,48]. In addition, Hb has been also found in brain, including cerebral neurons and neuronal [49,50]. The role of Hb in these sites is not clear, but the function of the Hb is also predictably different from the protein in erythrocytes. In this study, we have isolated an antibacterial Hb fragment from the liver in Japanese eel. Thus, fish Hb may also expressed in cell of liver, with the function distinct from the Hb in erythrocytes.

In conclusion, a heat stable peptide with strong antibacterial activity against *E. tarda* (B09) was purified from the liver of Japanese eel, *A. japonica*. The amino acid sequence of the peptide was FAHWPDLGPGSPSVKKHGKVIM with a molecular mass of 2388.05 Da. The amino acid sequence has 100% identity with partial peptide sequence of Hb, named as AJHbα. The synthetic AJHbα had strong antibacterial activities against ten Gram-positive or negative bacteria.

Acknowledgments

This work was supported by grants from Agricultural eel Public Welfare research project under contract (nyhyzx07-043), the key project of Department of Education of Fujian Province (JA09157), Hall of Fujian Province Science and Technology platform construction project (2009N2003-1), Fujian Province Science and Technology project (2010N0022).

References

[1] Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sönksen CP, Ludvigsen S, et al. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 2005;437:975–80.
[2] Hancock RE, Scott MG. The role of antimicrobial peptides in animal defenses. PNAS 2000;97:8856–61.
[3] Rajanbabu V, Chen JY. Applications of antimicrobial peptides from fish and perspectives for the future. Peptides 2011;32:415–20.
[4] Ohta H, Kagawa H, Tanaka H, Okuzawa K, Iinuma N, Hirose K. Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol Biochem 1997;17:163–9.
[5] Cao HP, He S, Wei RP, Diong M, Lu L. *Bacillus amyloliquefaciens* G1: a potential antagonistic bacterium against eel-pathogenic *Aeromonas hydrophila*. eCAM 2011;82104:1–7.
[6] Guan R, Xiong J, Huang W, Guo S. Enhancement of protective immunity in European eel (*Anguilla anguilla*) against *Aeromonas hydrophila* and *Aeromonas sobria* by a recombinant *Aeromonas* outer membrane protein. Acta Biochim Biophys Sin 2011;43:79–88.
[7] Joh SJ, Kim MJ, Kwon HM, Ahn EH, Jang H, Kwon JH. Characterization of *Edwardsiella tarda* isolated from farm-cultured eels, *Anguilla japonica*, in the Republic of Korea. J Vet Med Sci 2011;73:7–11.
[8] Fan HP, Wu B, Zeng ZZ, Zhong QF, Zhang XY, Xin ZM, et al. Isolation and identification of bacteriosis pathogen from *Anguilla japonica* ulceration of skin and preparation of its monoclonal antibody. Fish Sci China 2009;2:295–301 [in Chinese].
[9] Ling MH, Liu CH, Chen DY, Huang DB, Sun BX. Acute toxicity test of Japanese eel and bactericidal property test of tetrakis hydroxymethyl hosphonium sulfate. Anhui Agric Sci 2011;6:3647–8 [in Chinese].
[10] Tasumi S, Yang WJ, Usami T, Tsutsui S, Ohira T, Kawazoe I, et al. Characteristics and primary structure of a galectin in the skin mucus of the Japanese eel, *Anguilla japonica*. Dev Comp Immunol 2004;28:325–35.
[11] Tasumi S, Ohira T, Kawazoe I, Suetake H, Suzuki Y, Aida K. Primary structure and characteristics of a lectin from skin mucus of the Japanese eel, *Anguilla japonica*. J Biol Chem 2002;30:27305–11.
[12] Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 2002;16:429–31.
[13] Liang Y, Guan RZ, Huang WS. Isolation and identification of a novel inducible antibacterial peptide from the skin mucus of Japanese eel, *Anguilla japonica*. Protein 2011;30:413–21.
[14] Ebran N, Julien S, Orange N, Auperin B, Molle G. Isolation and characterization of novel glycoproteins from fish epidermal mucus: correlation between their

pore-forming properties and their antibacterial activities. Biochi Biophys Acta 2000;1467:271–80.
[15] Bo J, Giesy JP, Ye R, Wang KJ, Lee JS, Au DW. Identification of differentially expressed genes and quantitative expression of complement genes in the liver of marine medaka *Oryzias melastigma* challenged with *Vibrio parahaemolyticus*. Comp Biochem Physiol Part D Genomics Proteomics 2012;3:1–10.
[16] Guo M, Wei J, Huang X, Huang Y, Qin Q. Antiviral effects of β-defensin derived from orange-spotted grouper (*Epinephelus coioides*). Fish Shell Immunol 2012; 32:828–38.
[17] Bayne CJ, Gerwick L, Fujiki L, Nakao M, Yano T. Immune-relevant (including acute phase) genes identified in thelivers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. Dev Comp Immunol 2001;25:205–17.
[18] Xu TL, Guan RZ, Lang Y, Huang WSH. Antibacterial activities in different tissues or organs of Japanese eel, *Anguilla japonica*. J Jimei Univ 2010;15:172–8 [in Chinese].
[19] Anderson RS, Beaven AE. Antibacterial activities of oyster (*Crassostrea virginica*) and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquat Living Resour 2001;14:343–9.
[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[21] Birkemo GA, Lüders T, Andersen Ø, Nes IF, Nissen-Meyer J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). Biochim Biophys Acta 2003;1646:207–15.
[22] Chang CI, Zhang YA, Zou J, Nie P, Secombes CJ. Two cathelicidin genes are present in both rainbow trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*). Antimicrob Agents Chemother 2006;50:185–95.
[23] Zhang JJ, Yan QP, Ji RX, Zou WZ, Guo GJ. Isolation and characterization of a hepcidin peptide from the head kidney of large yellow croaker, *Pseudosciaena crocea*. Fish Shell Immunol 2009;26:864–70.
[24] Silphaduang U, Noga EJ. Peptide antibiotics in mast cells of fish. Nature 2001; 414:268–9.
[25] Bergsson G, Agerberth B, Gudmundsson GH. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). FEBS J 2005;272:4960–9.
[26] Mason AJ, Bertani P, Moulay G, Marquette A, Perrone B, Drake AF, et al. Membrane interaction of chrysophsin-1, a histidinerich antimicrobial peptide from red sea bream. Biochemistry 2007;46:15175–87.
[27] Chen SL, Xu MY, Ji XS, Yu GC, Liu Y. Cloning, characterization, and expression analysis of hepcidin gene from red sea bream (*Chrysophrys major*). Antimicrob Agents Chemother 2005;49:1608–12.
[28] Yan H, Hancock REW. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob Agents Chemother 2001;45:1558–60.
[29] Mak P, Wojcik K, Wicherek L, Suder P, Dubin A. Antibacterial hemoglobin peptides in human menstrual blood. Peptides 2004;25:1839–47.
[30] Mak P, Wójcik K, Silberring J, Dubin A. Antimicrobial peptides derived from heme-containing proteins: hemocidins. Antonie Van Leeuwenhoek 2000;77: 197–207.
[31] Hobson D, Hirsch JG. The antibacterial activity of hemoglobin. J Exp Med 1958; 107:167–83.
[32] Welch WD, Rose DM, Carlson R. Reduced hemoglobin as an inhibitor of human polymorphonuclear leukocyte bacterial killing. Role of hemoglobin oxygen tension in polymorphonuclear function. Surgery 1982;91:75–80.
[33] Hand WL. Inhibition of cell-free oxidative bactericidal activity by erythrocytes and hemoglobin. Infect Immun 1984;44:465–8.
[34] Liepke C, Baxmann S, Heine C, Breithaupt N, Ständker L, Forssmann WG. Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. J Chromatogr B 2003;791:345–56.
[35] Patgaonkar M, Aranha C, Bhonde G, Reddy KVR. Identification and characterization of antimicrobial peptides from rabbit vaginal fluid. Vet Immunol Immunop 2011;139:176–86.
[36] Parish CA, Jiang H, Tokiwa Y, Berova N, Nakanishi K, McCabe D, et al. Broad-spectrum antibacterial activity of hemoglobin. Bioorg Med Chem 2011;9: 377–82.
[37] Ullal AJ, Wayne Litaker R, Noga Edward J. Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev Comp Immunol 2008;32:1301–12.
[38] Fernandes JMO, Smith VJ. Partial purification of antibacterial proteinaceous factors from erythrocytes of *Oncorhynchus mykiss*. Fish Shell Immunol 2004;16:1–9.
[39] Fernaud S, Evans RW. Lactoferrin – a multifunctional protein with antimicrobial properties. Mol Immunol 2003;40:395–405.
[40] Fernandes JMO, Kemp GD, Molle G, Smith VJ. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Biochem J 2002;368:611–20.
[41] Alonso S, Pethe K, Russell DG, Purdy GE. Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy. Proc Natl Acad Sci USA 2007;104:6031–6.
[42] Nordahl EA, Rydengård V, Nordahl EA, Rydengård V, Nyberg V, Nitsche DP, et al. Activation of the complement system generates antibacterial peptides. Proc Natl Acad Sci USA 2004;101:16879–84.
[43] Nedjar-Arroume N, Dubois-Delval V, Adje EY, Traisnel J, Krier F, Mary P, et al. Bovine haemoglobin: an attractive source of antibacterial peptides. Peptides 2008;29:969–77.
[44] Ivanovv VT, Karelin AA, Philippova MM, Nazimov IV, Pletnev VZ. Hemoglobin as a source of endogenous bioactive peptides: the concept of tissue-specific peptide pool. Biopolymers 1997;43(2):171–88.
[45] Liu L, Zeng M, Liu Limin, Zeng M, Stamler JS. Hemoglobin induction in mouse macrophages. Proc Natl Acad Sci USA 1999;96:6643–7.
[46] Nishi H, Inagi R, Kato H, Tanemoto M, Kojima I, Son D, et al. Hemoglobin is expressed by mesangial cells and reduces oxidant stress. J Am Soc Nephrol 2008;19:1500–8.
[47] Bhaskaran M, Chen H, Chen Z, Liu L. Hemoglobin is expressed in alveolar epithelial type II cells. Biochem Biophys Res Commun 2005;333:1348–52.
[48] Newton DA, Murali Krishna Rao K, Dluhy RA, Baatz JE. Hemoglobin is expressed by alveolar epithelial cells. J Biol Chem 2006;281:5668–76.
[49] Schelshorn DW, Schneider A, Kuschinsky W, Weber D, Krüger C, Dittgen T, et al. Expression of hemoglobin in rodent neurons. J Cereb Blood Flow Metab 2009;29:585–95.
[50] He YD, Hua Y, Lee JY, Liu WQ, Keep RF, Wang MM, et al. Brain alpha- and beta-globin expression after intracerebral hemorrhage. Transl Stroke Res 2010;1: 48–56.