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Original article

Recombinant Russell's viper venom-factor X activator (RVV-X)-specific antibody: neutralization and cross-reactivity with *Cryptelytrops albolabris* and *Calloselasma rhodostoma* venoms

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Background: Russell's viper venom-factor X activator (RVV-X) is a major procoagulant in Russell's viper venom, and is composed of a heavy chain (RVV-XH) and two light chains (RVV-XL). It directly activates factor X in the final common coagulation pathway, which leads to rapid formation of blood clots.
Objective: Produce rabbit anti-recombinant protein antibodies and identify their cross-reactivity with two viperine snake venoms.
Methods: cDNA clones encoding RVV-XH and one of the light chains (RVV-XL; LC1) were recombinantly expressed in *E. coli* BL21 and used as antigens for rabbit immunization. The cross-reactivity of these anti-recombinant protein antibodies with two viperine snake venoms was determined using Western blot analysis.
Results: rRVV-XH was more immunogenic than rRVV-XL. Rabbit anti-rRVV-XH and rRVV-XL IgG antibodies bind specifically to RVV-X, but they do not neutralize purified RVV-X. In addition, rabbit anti-rRVV-XH IgG antibody also bind to an 18-kDa protein in *C. rhodostoma* venom, and many proteins in *C. albolabris* venom. Rabbit anti-rRVV-XL IgG antibody recognized protein bands of crude venoms of *C. rhodostoma* and *C. albolabris* at about 25-kDa and 23-kDa, respectively.
Conclusion: Rabbit anti-rRVV-XH and rRVV-XL IgG antibodies cross-reacted with molecules in other viperine venoms, which could have molecules with similar antigenic determinants. These antibodies could be useful to purify snake venom molecules by affinity chromatography as the first step in purification of factor X activator and other cross reacting molecules.

Keywords: Anti-rRVV-XH IgG antibody, anti-rRVV-XL IgG antibody, cross-reactivity, *Daboia russellii siamensis*, Russell's viper venom-factor X activator (RVV-X)

The immunological cross-reactivity of snake venoms have been widely studied to promote the development of anti-venom for snake bite treatment [1-3], diagnosis [4-6], and indicating the intraspecific snake venom variations [2, 7]. Previous studies have reported that toxic fragment-specific antibody neutralized the toxicity of crude venom [2, 8, 9] and cross-reacted with venom components of the distinct venom species [2, 3]. In addition, to raise antitoxin safely, some toxins such as tetatus need to be treated to become less toxic.

Russell's viper venom-factor X activator (RVV-X) present in Russell's viper venom was selected as a key toxin because it is a major procoagulant in this venom. RVV-X is a glycoprotein containing 13% carbohydrate with molecular mass of approximately 93 kDa [10]. It is composed of two interdisulfide bonded domains, a heavy chain of molecular mass 58 kDa and two light chains of heterogenous molecular mass 19 and 16 kDa. The heavy chain of RVV-X contains metalloproteinase, disintegrin (platelet aggregation inhibitor)-like and cysteine-rich domains. The light chains resemble the

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C-type (calcium-dependent) lectins. This protein (RVV-X) activates coagulation factor X by cleaving a specific peptide bond (Arg_{52}-Ile_{53}) of the heavy chain of the clotting factor, and requires calcium ion for the proteolytic activity [11, 12]. As a result, the final common coagulation pathway is induced, which leads to rapid formation of blood clots. Thus, RVV-X should be a major lethal factor in Russell's viper venom.

Quite recently, we established a novel technique to obtain cDNAs encoding RVV-XH and RVV-XL (unpublished data). In this study, we developed this method further and produced recombinant RVV-XH (rRVV-XH) and recombinant RVV-XL (rRVV-XL) proteins in *E. coli* BL21. Using these obtained recombinant proteins, we investigated how the rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies react with RVV-X activation factor and cross-reacted with two viperine snake venoms using Western blot analysis.

Materials and methods

Snake venom proteins

Lyophilized crude venom samples from Russell's viper (*D. r. siamensis*), *Cryptelytrops albolabris* (formerly *Trimeresurus albolabris*), and *Calloselasma rhodostoma* were obtained from the Queen Saovabha Memorial Institute (QSMI) at the Thai Red Cross Society, Bangkok, Thailand. Venoms from the same species were pooled from an underdetermined number of snakes. RVV-X was purified from crude Russell's viper venom (cRVV).

Antibody

Horse whole blood containing polyvalent and commercial horse anti-cRVV F $(ab')_2$ anti-venom was obtained from the QSMI. Horse anti-cRVV IgG antibody was purified from horse whole blood by ammonium sulfate precipitation and gel filtration chromatography, which was used as the positive control in enzyme-linked immunosorbent assay (ELISA). Mouse anti-His antibody was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody was purchased from DAKO (California, USA). HRP-conjugated goat anti-rabbit antibody was purchased from Zymed Laboratories (California, USA). HRP-conjugated sheep anti-horse antibody was provided by Dr. Kavi Ratanabanangkoon, Mahidol University, Bangkok, Thailand

Animals

Groups of two adult female New Zealand white rabbits (*Oryctolagus cuniculus* weighing two kg were obtained from the National Laboratory Animal Center (NLAC), Mahidol University, Nakornpathom, Thailand. All procedures were approved by the Committee on Animal Care of Chulalongkorn University.

Expression of rRVV-XH and rRVV-XL in E. coli

cDNAs encoding RVV-XH and RVV-XL were obtained from our previous study (unpublished data). These genes were cloned into pTrcHisα-A vector and transformed in *E. coli* DH5α. The expression of rRVV-XH and rRVV-XL proteins was performed in *E. coli* BL21. Expressed recombinant proteins were purified using immobilized metal affinity chromatography (BD Biosciences Clontech, California, USA). rRVV-XH and rRVV-XL proteins were used as antigens for rabbit anti-recombinant proteins antibodies production.

Rabbit immunization

For primary immunization, a 250 μg of rRVV-XH or rRVV-XL proteins was diluted to 0.5 mL with sterile normal saline and combined with 0.5 mL of the complete Fruend's adjuvant (CFA). One milliliter of each sample was injected intradermally into 20 sites with 0.05 mL per site on the shaved back of two adult female New Zealand white rabbits. Three weeks later, a 100 μg of rRVV-XH or rRVV-XL proteins were separately mixed with incomplete Freund's adjuvant (IFA) and injected in the similar manner. Subsequent injection was performed after a three-week rest period. From each rabbit, 50 mL of blood was drawn from the central ear artery with a 20-gauge 3/4" long needle at three-week intervals. Blood was collected by gravity into a 50 mL plastic test tube. The collected blood was store in 4°C for 24 hours. Then, coagulate blood was centrifuged for 20 minutes at 2,000 g. The sera were removed. Serum samples were pooled and stored at -80°C. ELISA was used to measure the antibody titer of the serum.

Enzyme-linked immunosorbent assay

The titers of crude and purified rabbit anti-rRVV-XH and anti-rRVV-XL antibodies were determined by an ELISA-modified procedure reported by Rungsiwongse and Ratanabanangkoon [13]. The polyvinyl microtiter plate (NUNC™, New York, USA)

was coated with 50 μL/well of 5 μg/mL of purified RVV-X in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6 at 4°C for 18 hours. The coated plate was washed four times for three minutes intervals with washing buffer (0.05% Tween-20 in normal saline), and blocked with blocking buffer (0.15 M phosphate buffer saline (PBS), pH 7.4 containing 0.5% bovine serum albumin (BSA)). Appropriate starting dilutions of individual sera from immunized animals and purified rabbit anti-recombinant proteins IgG antibodies including positive and negative controls were diluted in 0.15 M PBS, pH 7.4 containing 0.05% Tween-20 and 0.5% BSA. A 50 μL of sample dilution was added to each well and incubated for one hour at room temperature. After a washing step, 50 μL/well of 1:10,000 diluted HRP-conjugated goat anti-rabbit antibody (Zymed Laboratories, California, USA) was added and incubated for one hour at room temperature. After four washes with washing buffer, 100 μL/well of freshly prepared substrate solution (0.01% tetramethylbenzidine and 3% H_2O_2 in 0.075 M citrate-phosphate buffer, pH 5.0) was added. The plate was incubated in the dark for 30 minutes at room temperature. The reaction was stopped by adding 25 μL of 4 N sulfuric acid. The absorbance of sample was monitored at 405 nm using an ELISA reader (Multiskan EX, Thermo Labsystems, California, USA). A horse anti-cRVV IgG antibody was used as the positive control, which was included in every plate to correct for day-to-day or plate-to-plate variations.

Purification of rabbit anti-recombinant proteins IgG antibodies

Sera of rabbits containing the highest titers of rRVV-XH- and rRVV-XL-specific antibodies were purified by protein G column affinity chromatography using the protein G agarose kit (KPL, Maryland, USA) according to the manufacturer's instructions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was carried out in 8% resolving gel and 4% of stacking gel according to LaemmLi (1970) [14] under reducing and non-reducing conditions at 20 mA (constant). Gel was stained with 0.25% brilliant blue Coomassie G250 in acetic acid:methanol:water (10:45:45, *v/v*) for one hour. Gel was then destained in acetic acid:methanol:water (10:30:60, *v/v*).

Chromogenic substrate assay

A 30 μL of rabbit anti-rRVV-XH IgG antibody, anti-rRVV-XL IgG antibody, anti-rRVV-XH and anti-rRVV-XL IgG antibodies, or commercial horse anti-cRVV $F(ab')_2$ anti-venom (QSMI, Bangkok, Thailand) at various concentrations were pre-mixed with 30 μL of 2 μg/mL of purified RVV-X at 37°C for 30 minutes. Effects of rabbit anti-recombinant proteins IgG antibodies in neutralizing factor X activator activity of purified RVV-X were tested using a factor Xa-specific chromogenic substrate S-2765 as described previously [15].

Western blot analysis

A 5 μg of crude venom samples of *D. r. siamensis*, *C. albolabris*, *C. rhodostoma*, purified RVV-X (250 ng), bovine serum albumin (BSA; 2 μg), and mouse serum (5 μg) were separated by 12% non-reduced SDS-PAGE. Following electrophoretic separation, gels were electroblotted onto 0.45 μm nitrocellulose membrane (Whatman® GmbH, Dassel, Germany) using Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, California, USA). The nitrocellulose membrane was incubated in blocking solution (5% *w/v* skim milk in PBS, pH 7.4) for one hour with gentle agitation and then was washed three times with PBS, pH 7.4 for three minutes each time. The membrane was incubated with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody, or a 1:1,000 dilution of rabbit anti-rRVV-XL IgG antibody in blocking solution for one hour at room temperature. The membrane was washed three times with PBS, pH 7.4 for three minutes each time. After that, the membrane was then incubated with a 1:5,000 dilution of HRP-conjugated goat anti-rabbit antibody (Zymed Laboratories, California, USA) in blocking solution for one hour at room temperature. The protein bands were visualized by adding the visualizing solution (1.66 mM diaminobenzidine, 8% $NiCl_2$, and 30% H_2O_2).

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). Their significance was analyzed by the student's t-test. The level of significance was at $p < 0.05$. *P*-values were compared with the negative control.

Results

rRVV-XH and rRVV-XL were expressed with approximately 0.300 mg/L culture and 0.250 mg/L

culture, respectively. These recombinant proteins were used as antigen for rabbit immunization.

Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies were purified from serum of immunized rabbits by a protein G affinity column with approximately 4% and 5% yield, respectively (**Table 1**). The antibody titer of rabbit anti-rRVV-XH IgG antibody was approximately five times higher than that of rabbit anti-rRVV-XL IgG antibody.

Purified rabbit anti-rRVV-XH- and anti-rRVV-XL IgG antibodies showed a broad band at about 150 kDa using SDS-PAGE under non-reducing conditions (lane 5-6, **Figure 1**).

Horse anti-cRVV $F(ab')_2$ anti-venom at the concentration of 31.25 μg/mL completely neutralized factor X activator activity of a 0.125 μg/mL of purified RVV-X but rabbit anti-rRVV-XH antibody, anti-rRVV-XL IgG antibody, or the mixture of rabbit anti-rRVV-XH and anti-rRVV-XL antibodies were unable to neutralize factor X activator activity of purified RVV-X (**Figure 2**).

Table 1. Purification of rabbit anti-recombinant proteins IgG antibodies from rabbit anti- recombinant proteins serum.

Sample	Volume (mL)	Protein concentration (mg/mL)[a]	Total protein (mg)[b]	Total activity (U)[c]	Specific antibody activity (U/mg)[d]	Recovery of protein (%)[e]	Purification (fold)[f]
Rabbit anti-rRVV-XH serum	10	59.34	593.40	6200	10.45	100	1.00
Rabbit anti-rRVV-XH IgG antibody	5.64	4.19	23.63	10000	423.16	3.98	40.50
Rabbit anti-rRVV-XL serum	10	51.06	510.60	1500	2.94	100	1.00
Rabbit anti-rRVV-XL IgG antibody	6.36	4.14	26.33	2300	87.35	5.16	29.73

[a]Protein concentration was determined by BCA method. [b]The total protein (mg) was calculated by multiplying (total volume; mL) x (venom protein concentration; mg/mL). [c]The highest dilution giving an absorbance reading of two was regarded as the end point titer. Total activity (U) was calculated by dividing protein concentration (mg/mL) by the end point titer concentration (mg/mL). [d]Specific activity (U/mg) was calculated by total activity (U) by total protein (mg) in each sample. [e]Recovery of protein was defined as the total protein recovered of at each step of purification. [f]Purification factor was the number of times that specific antibody activity increased over crude rabbit serum immunized with recombinant proteins.

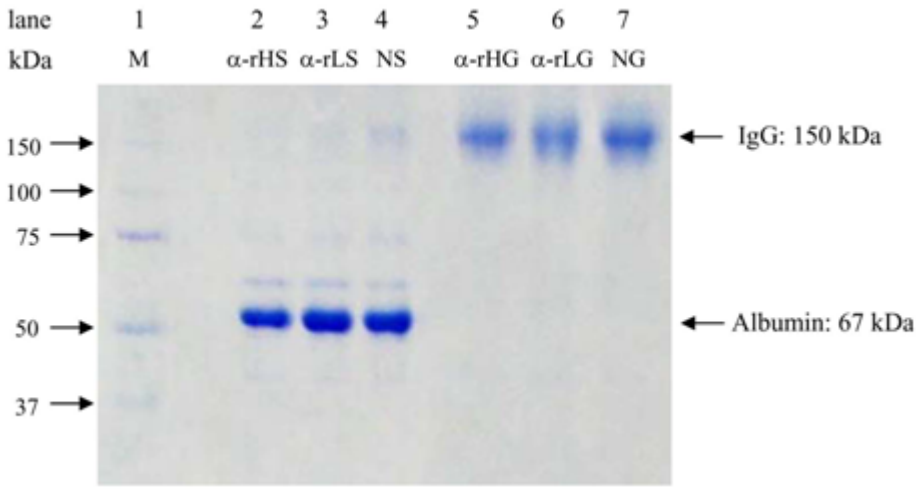

Figure 1. SDS-PAGE analysis of purified recombinant proteins-specific IgG antibody from rabbit anti-recombinant proteins serum. Samples (5 μg) were run on 8% Bis-Tris gel under non-reducing conditions at 20 mA (constant). Gel was stained with Coomassie blue G250 and destained. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: rabbit anti-rRVV-XH serum (α-rHS); lane 3: rabbit anti-rRVV-XL serum (α-rLS); lane 4: rabbit normal serum (NS); lane 5: rabbit anti-rRVV-XH IgG antibody (α-rHG); lane 6: rabbit anti-rRVV-XL IgG antibody (α-rLG); lane 7: rabbit normal IgG antibody (NG).

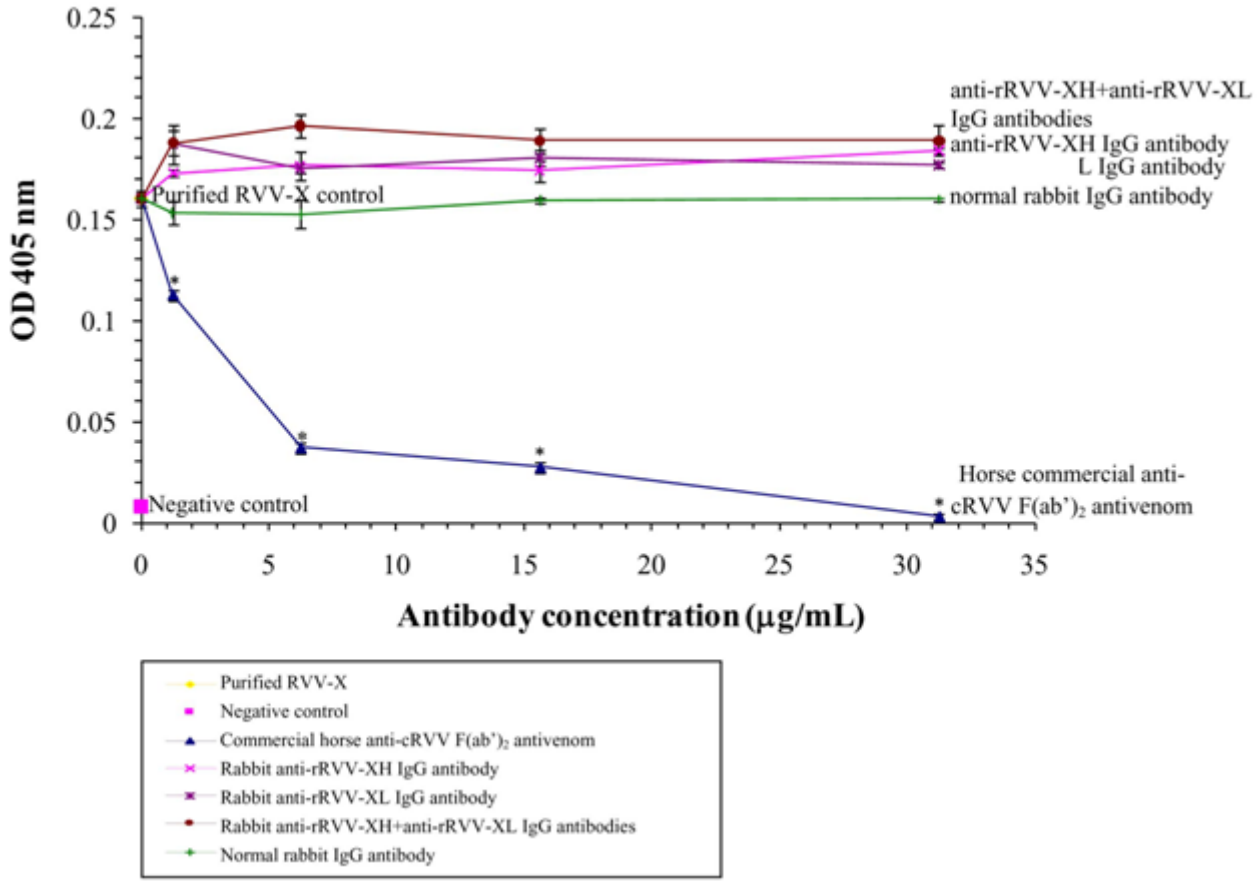

Figure 2. Effects of rabbit anti-recombinant proteins IgG antibodies in neutralizing factor X activator activity of purified RVV-X. A factor Xa-specific chromogenic substrate S-2765 was used to measure the decrease in factor X activator activity of purified RVV-X. The negative control was normal citrated human plasma reconstituted with only $CaCl_2$. Venom control was normal citrated plasma activated with purified RVV-X and 0.025 M $CaCl_2$. Antibody control was normal citrated plasma activated with purified RVV-X pre-mixed with commercial horse anti-cRVV $F(ab')_2$ anti-venom and 0.025 M $CaCl_2$. The mean and SD was calculated (n = 3). *statistical significance ($p < 0.05$) compared with the purified RVV-X control.

Figure 3 shows cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies against *C. rhodostoma* and *C. albolabris* venoms. Purified RVV-X with molecular mass of 90 kDa from crude venom was recognized by both purified rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies using Western blot analysis. In addition, rabbit anti-rRVV-XH IgG antibody was able to bind to a 18–kDa protein band of *C. rhodostoma* venom (lane 4, **Figure 3A**), and different protein bands with a molecular mass range between 28-70 kDa of *C. albolabris* venom (lane 5 in **A**). Rabbit anti-rRVV-XL IgG antibody recognized protein bands of crude venoms of *C. rhodostoma* and *C. albolabris* at about 25 kDa (lane 4 in **B**) and 23 kDa (lane 5 in **B**), respectively. Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies were unable to bind to bovine serum albumin (lane 6 in **A** and **B**), and normal mouse serum (lane 7 in **A** and **B**).

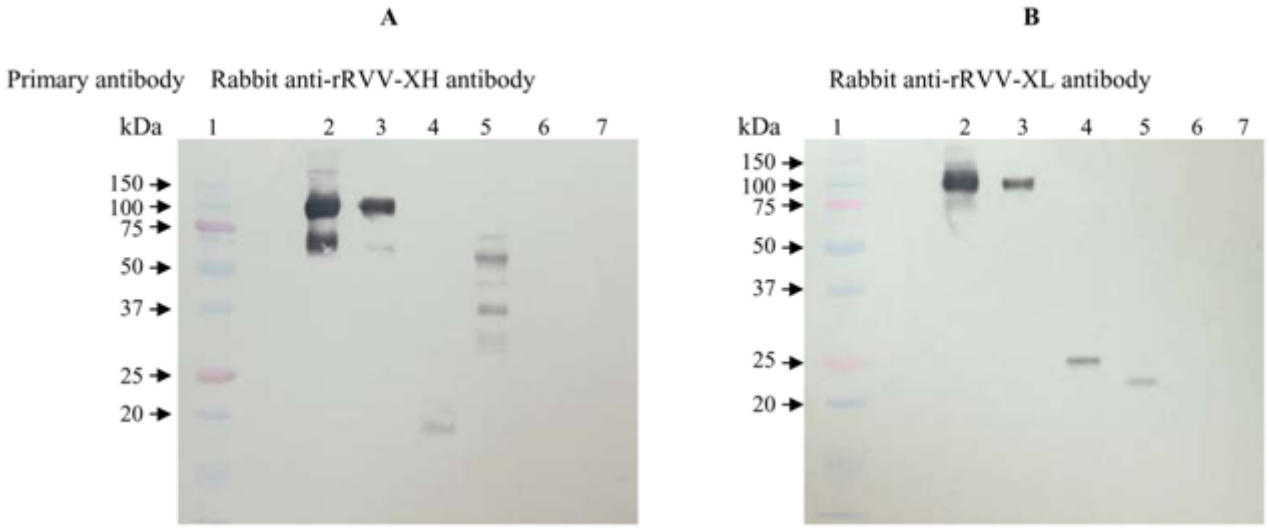

Figure 3. Cross-reactivity of rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies against *C. rhodostoma* and *C. albolabris* venoms using Western blot analysis. (**A**) Western blotting developed with a 1:1,500 dilution of rabbit anti-rRVV-XH IgG antibody. (**B**) Western blotting developed with a 1:1,000 dilution of rabbit anti-rRVV-XL IgG antibody. Lane 1: Precision Plus Protein™ standards (dual color, Bio-Rad Laboratories); lane 2: cRVV (5 μg); lane 3: purified RVV-X (250 ng); lane 4: crude *C. rhodostoma* venom (5 μg); lane 5: crude *C. albolabris* venom (5 μg); lane 6: bovine serum albumin (2 μg); lane 7: normal mouse serum (5 μg).

Discussion

In this study, we separately expressed recombinant RVV-X heavy chain and a light chain proteins in *E. coli* BL21 and used these recombinant proteins to produce rabbit anti-recombinant proteins antibodies. The antibody specific activities for purified rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies increased by a factor of 40 and 30, respectively when purified. This demonstrates the ability of rRVV-XH and rRVV-XL to produce antibody response in rabbits. It is not surprising that rRVV-XH had a much higher specific activity antibody response than rRVV-XL by a factor 4.8, since the molecular weight of rRVV-XH is approximately three times higher than rRVV-XL. Higher molecular weight antigens have more antigenic determinant sites and would produce higher titers of antibodies [16].

These rabbit anti-recombinant antibodies IgG antibodies individually or mixtures of both failed to neutralize the factor X activator activity of purified RVV-X, as shown in **Figure 2**. This suggests that at least antibody was produced to antigenic site for both recombinant proteins but neutralization of RVV-X might require correct conformation of the whole RVV-X molecule. Morita [17] suggested that the two light chains of RVV-X serves as an exosite by which RVV-X recognizes and binds to the Gla domain of factor X. In our study, only one light chain of RVV-X (LC1) was expressed and immunized in rabbit. The construction of three chains of RVV-X in one expression cassette might be needed to produce a neutralizing antibody for RVV-X. Rabbit purified anti-rRVV-XH IgG antibody recognized the same protein bands with molecular weight of about 55 and 90 kDa of cRVV and purified RVV-X using Western blot analysis, as shown in **Figure 3**. The protein band at 90 kDa is the intact protein of RVV-X. The protein band at 55 kDa is likely degraded heavy chain of RVV-X, as the light chains of RVV-X might be cleaved off due to the long-term storage. Kroon et al. [18] have demonstrated that the monoclonal antibody OKT3 stored at 2-8°C for nine months was degraded by the oxidation of cysteine and several methionines.

The fact that rabbit anti-recombinant proteins IgG antibodies cross-reacted with *C. albolabris* and *C. rhodostoma* venoms is not surprising as snake venom metalloproteinase (SVMP) [19-21] and C-type lectin [22, 23] are common venom components of most vipers. Rabbit anti-rRVV-XH IgG antibody was able to bind to an 18-kDa protein in *C. rhodostoma* venom and a number of proteins in *C. albolabris* venom. A snake venom metalloproteinase, kistomin (25 kDa),

has been identified in *C. rhodostoma*, can cleave platelet glycoprotein VI and impairs platelet functions [24]. A 60-kDa metalloproteinase alborhagin, platelet membrane glycoprotein VI (GPVI) agonist, was also found in *T. albolabris* venom [25, 26]. In addition, previous studies have reported that *C. rhodostoma* venom had factor X activator activity [27, 28]. Factor X activators are present in the snake venoms of genera Bothrops [29, 30], Vipera [31, 32], and Cerastes [33, 34]. These molecules vary widely in molecular weights from 12 to 102 kDa. Presence of metalloproteinases and/or factor X activator could explain cross-reactivity of RVV-XH-specific antibody with other venoms.

Rabbit anti-rRVV-XL IgG antibody can bind low molecular weight proteins in *C. rhodostoma* and *C. albolabris* venoms. Previous studies have reported that *C. rhodostoma* venom contains a C-type lectin aggretin (28 kDa), which is a potent platelet aggregation inducer [35, 36]. The venom C-type lectin with similar function, a 25-kDa alboaggregin-B, was also found in *C. albolabris* venom [37-39]. These venom components with immunological cross-reactivity to anti-rRVV-XL IgG antibody could be the C-type lectin molecule.

In conclusion, the recombinant proteins (RVV-XH and RVV-XL) were used to raise antibodies in rabbit. These rabbit anti-recombinant proteins antibodies did not inhibit purified RVV-X as measured in a chromogenic substrate assay. Rabbit anti-rRVV-XH and anti-rRVV-XL IgG antibodies specifically bind to purified RVV-X and no other molecules in crude Russell's viper venom. However, these same antibodies cross-reacted with molecules in other venoms (*C. albolabris* and *C. rhodostoma*). Other venoms have molecules with similar antigenic determinants. Despite non-specificity of anti-rRVV-XH and anti-rRVV-XL IgG antibodies, these antibodies could potentially be used in affinity chromatography to purify RVV-X molecules from crude venom. These antibodies are potentially useful to identify factor X activator-like, metalloproteinase, or C-type lectin molecules from other snake venoms.

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