CHAPTER V

CONCLUSION AND DISCUSSION

1. Isolation of full length cDNA encoding Serine beta-fibrinogenase (SBF) from Russell's viper (RV)

Many of cDNA clones obtained from our cDNA library were truncated at 5' end, especially the clones with the cDNA insert of over 1.0 kb. The full length Serine beta-fibrinogenase homolog (RV SBF) thought to be approximately 1.6 kb in length. The RVV141 clone containing 0.8 kb of RV SBF was identified as a partial SBF gene fragment as it lacked of about 0.8 kb from 5'end of full length gene. To get the full length SBF cDNA sequence, we used two methods, plaque lift hybridization and 5'RACE. Unfortunately, we could not get the full length SBF cDNA clone from plaque lift hybridization. The chemiluminescent signal could neither be detected from the plaque lifted membrane nor in the control which was dot blot with SBF DNA fragment. We hypothesized that there might be something wrong in North2South HRP hybridization such as we were prepared missing some step in probe labeling procedure or reagents since it had more loger on the day we performed experiment. Therefore, we try the other method to get the RV SBF full length by 3' and 5'RACE. Gene specific primers were designed to amplify the unknown sequence as the recommendation of the kit. The 3' and 5' RACEs PCR products were cloned and sequenced. Finally, the full length RV SBF homolog sequence was obtained after sequence assembly. The full length RV SBF homolog comprises of 1,582 bp and encodes for 258 amino acids, including 24 amino acids of signal peptide. The signal peptide is highly conserved as it is identical to many signal peptides from several snake toxins. The active protein encoded by RV SBF homolog shows 80% amino acid identical to the most closely related SBF protein from Middle East Asian viper, *Macrovipera lebetina*. RV SBF homolog also contains the major SBF signatures such as having conserved catalytic triad (His57, Asp102, Ser195) and 12 conserved cysteines.

2. Expression of RV SBF in *Escherichia coli*

To express the active SBF protein, the mature cDNA encoding RV SBF was amplified from RV mRNA by RT-PCR using degenerate primers designed from consensus sequences flanking 3' and 5'ends. The expected PCR product was 702 bp encoding 234 amino acids of SBF active protein. The obtained PCR product was then ligated into pGEM®-T easy vector and screened for the recombinant clones. From 10 recombinant pGEM®-T easy clones, W001 clone was selected to perform further subcloning into pTrc-His A and pET32 expression vector. The subcloning of W001 to expression vector was successful for both pTrc-His A and pET32 vectors. Before performing protein expression, the recombinant pTrc-His A and pET32 clones were sequenced to ensure the correction in protein translation. Unfortunately, we found one nucleotide deletion at 5'end of cDNA insert which located in the primer region. This one nucleotide deletion leads to mis-frame in protein translation of recombinant RV SBF. Although it is unusual that the primer sequence is mis-synthesized, as it is degenerate primer, this may enhance the error rate when it is synthesized. Anyway, we tried to subclone another mature SBF clone, W004, which was confirmed containing the correct sequence. With some unknown technical problems, up to now, the recombinant clones of SBF in pTrc-His and pET32 expression vector couldn't be obtained yet.

3. Sequences alignment and Bioinformatics study

The open reading frame of Serine beta-fibrinogenase homolog (RV SBF) from *Daboia russellii siamensis* (258 amino acids) comprises 24 amino acids of prepropeptide (18 amino acids of signal peptide and 6 amino acids of the activation peptide) followed by 234 amino acids of the secreted enzyme sequence. The calculated molecular weight for SBF homolog is 25529.1 and the classical isoelectric point is 6.68. The SBF homolog primary sequence contains four putative glycosylation sites, NXS/T (Asn-44 were formed NDSN, Asn-154 were formed NETY, Asn-170 were formed NYTV and Asn-253) but not all of them need to be glycosylated. Therefore, molecular mass of the native enzyme should be more than 30 kDa according to glycosylation process like other serine protease.

On the other hand, The nearest homologous of RV SBF is VLBF are *Macrovipera lebetina* serine beta-fibrinogenase precursor (AF536235) (80%), *Gloydius ussuriensis* thrombin-like serine protease (AAL68708) 74%, VSP3_BOTJA Venom serine proteinase A precursor (Q9PTU8) 74%, *Macrovipera lebetina* serine alpha-fibrinogenase precursor: VLAF (AAM96674) 74%, respectively. SBF Homolog shows greatest homology with VLBF is typical arginine esterases devoid of caseinolytic activity. In addition, the molecules of RV SBF comprises 24 amino acids of prepropeptide was similar to and VLAF. Moreover RV SBF, VLBF and VLAF were contained the same amino acids forming catalytic triad – His57, Asp102 and Ser195 [chymotrypsinogen numbering (Wang *et al.*, 1985)] and highly conserved flanking sequences. The twelve cysteine residues are all in strongly conserved positions predicted to form six disulfide bridges but not only for SBF homolog. We noticed that RV SBF were contained glycosyl chains less than VLBF. The VLBF should contain 6 glycosyl chains than RV SBF. Actually the deduced protein sequence exhibits 5 putative N-glycosylation sites at the residues Asn36, Asn94, Asn109,

Asn147, Asn245. Potential O-glycosylation site is located at the position Ser125. Indicated that the discrepancies probably resulted from glycosylation at specific Asn residues and geographical variation between the venom protein and the venom glands used (Siigur *et al.*, 2003).

Furthermore, RV SBF and both enzymes belong to the clan SA where the order of the catalytic triad is His, Asp, Ser and the tertiary structure consists mainly of ß-sheet (Barrett and Rawlings, 1995). On the basis of the evolutionary markers RV SBF VLAF and VLBF are the members of the S1 (chymotrypsin) subfamily (Krem and Di Cera., 2001). Ser195 and Ser214 are both encoded by TCT, and the position 225 is occupied by proline. It has been shown already by Itoh (Itoh *et al.*, 1988) that snake venom serine esterases are members of the trypsin-kallikrein family, on the basis of gene (exon/intron) organization. Wang et al. (Wang *et al.*, 2001) compared venom proteases according to subtypes and found some regularities in the sequences. They noticed that three regions (residues 82-99, 192-193, and 217-219) bear unique substitutions that might be subtype-specific. These regions happen to be the known substrate-binding subsites. In almost all of the active serine proteinases, the N-terminal residue (Val16 in RV SBF, VLBF and VLAF) forms an internal salt bridge between its amino group and the side chain carboxyl group of Asp194 (Wang *et al.*, 1985). Moreover, RV SBF had apart from Asp102 and Asp194 there is the third essential aspartate in the molecule of trypsin-like serine proteinases – Asp189 which is located in the bottom of the primary specificity pocket near the active site and forms a salt bridge with the basic residue of the scissile bond (Janin and Chothia, 1976). However, In the case of VLAF and VLBF Asp189 is replaced by Gly189 that points to the possible lack of the trypsin-type substrate specificity for basic amino acids at the P1 position. This replacement deprives trypsin of the arginine specificity and turns it into nonspecific proteolytic enzyme (Czapinska and Otlewski, 1999) like it has happened with VLAF. At the same time, SV SBF might be like VLBF to behave like an arginine

esterase although the activity against arginine esters is remarkably lower than that of trypsin (Siigur *et al.*, 1991). In this case perhaps some other yet unknown factors contribute to the redesigning of the S1 pocket. The feature distinguishing VLAF difference from RV SBF and VLBF. VLAF is the sequence –RRR- (aa 185-187) in the vicinity of the putative S1 pocket that may cause steric and electrostatic resistance to the positively charged substrates.

By the way, the phylogenetic tree (Figure 21) reveals the independent evolution of four major enzyme subtypes, the beta-fibrinogenase, the coagulating enzymes (CL), the plasminogen activators (PA) and the kininogenases (KN). Deshimaru et al. (Deshimaru *et al.*, 1996) used the mature proteincoding DNA sequences of eight serine-protease cDNA sequences from two Asian pit vipers to construct a tree that also revealed parallel evolution of several enzyme subtypes. The classification of the four functional subtypes might find support from the literature, as foilows. (1) ß-Fibrinogenase is a typical thermostable arginine esterase that hydrolyzes esters and amides of arginine and attacks the ß -chain of fibrinogen (2) Most of the well-known venom-clotting enzymes that convert fibrinogen into a fibrin clot, e.g. batroxobin, ancrod, crotalase and acutobin, are linked together in the CL subtype and are characterized by being Aa fibrinogens and highly glycosylated. (3) More than half of the enzymes of the KN subtype had been characterized as weakly clotting or non-clotting, but might cleave Bb or both the Aa and Bb fibrinogen chains. Many of them have been characterized as being capable of cleaving kininogen to produce bradykinin (Teng *et al.*, 1992; Serrano *et al.*, 1998; Matsui *et al.*, 1998), leading to a hypotensive effect. (4) At least two of the enzymes (TSV-PA and Haly- PA) of the PA subtype have been confirmed to have the role of a plasminogen activator and to facilitate the solubilization of fibrin clots, as their names imply (Zhang *et al.*, 1995; Park *et al.*, 1998). Members of the KN and the PA subtypes usually are less or not glycosylated, so their molecular masses are lower than those of the CL subtype.

The cDNA sequence encoding mature RV SBF was constructed the phylogenetic tree against RV SBF from several snakes. RV SBF is grouped with VLBF from *Macrovipera lebetina*, these snakes also belong to *Viperidae* family found in North Africa and Middle East Asia. The high degree of diversity of the amino acid sequence has been found in the mature proteins of serine proteinase from snake venom. Therefore, the SBF homolog and other fibrinoginase enzyme might be evolved from another coagulant enzyme to be the way to generate new functional protein.

Since the RV SBF was classified in serine protease and showed greatest homology with VLBF, it might have similar function with VLBF. However, the function and the role in pathogenesis in snake bite of this protein should be clarified.