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Comparative of Recombinant *Vespa Affinis* Hyaluronidase Expressed in Different Cloning Vectors and their Biological Properties

Piyapon Janpan
Faculty of Pharmaceutical Sciences,
Khon Kaen University, Khon Kaen, Thailand

Yutthakan Saengkun
Faculty of Pharmaceutical Sciences,
Khon Kaen University, Khon Kaen, Thailand

Prapenpuksiri Rungsa
Faculty of Pharmaceutical Sciences,
Protein and Proteomics Research Center
for Commercial and Industrial Purposes
Khon Kaen University, Khon Kaen, Thailand

Mongkol Vesaratchavest
Research and Development Center
Betagro Public Company Limited
Klong Nueng, Klong Luang, Pathumthani

Tewa Upathanpreecha
Research and Development Center,
Betagro Public Company Limited,
Klong Nueng, Klong Luang, Pathumthani

Patthana Tastub
Research and Development Center,
Betagro Public Company Limited,
Klong Nueng, Klong Luang, Pathumthani

Sakda Daduang *
Faculty of Pharmaceutical Sciences
Protein and Proteomics Research Center for Commercial and Industrial Purposes
Khon Kaen University, Khon Kaen, Thailand

Abstract: Cloning and expression of recombinant *Vespa affinis* hyaluronidase (*rVesA2*) were successfully expressed in *Escherichia coli* system. The VesA2 gene was cloned into pET-17b and pET-32a cloning vectors with a molecular weight of 41.71 and 59.0 kDa, respectively. The recombinant plasmid of pET-17b was composed of 1.08 kDa his-tag at the N-terminal. The 17.14 kDa of fusion tag, thioredoxin tag, histidine tag, and S-tag, was found in pET-32a. The verified expression conditions of *rVesA2* induced under the conditions of 0.1 mM IPTG at 37°C for 4 hrs gave the highest quantity of protein expression. The colony PCR and sequencing analysis were used to verify the *rVesA2*. The positive clones were detected the hyaluronidase activity by a zymographic gel. Recombinant proteins from both cloning vectors were insoluble. However, the recombinant form pET-32a showed higher solubility than pET-17b after dissolving in a 4 M urea solution. This result suggests that the fusion tag increases protein solubility.

Keywords: *Hyaluronidase enzyme, hyaluronic acid, banded tiger wasp*

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I. INTRODUCTION

The banded tiger wasps (*Vespa affinis*) are insects in order Hymenoptera, and are found in Southeast Asia including Northeastern of Thailand [1]. They are dangerous to people because of their nest is, widespread on trees or under eaves of houses. Their venom was used for defending themselves. Clinical manifestations when wasp bite is both local and systemic reactions such redness site bite area, pain, swelling, and headache [2]. The major proteins of venom gland include phospholipase, antigen 5, dipep-

*Correspondence concerning this article should be addressed to Sakda Daduang, Faculty of Pharmaceutical Sciences, Protein and Proteomics Research Center for Commercial and Industrial Purposes, Khon Kaen University, Khon Kaen, Thailand E-mail: sakdad@kku.ac.th

tidyl peptidase and hyaluronidase [3]. Phospholipase A1 (PLA_1) is one of the enzymes in phospholipase family that hydrolyzes phospholipid at the 2-acyl-lysophospholipid position to fatty acid and lysophospholipid [4]. Antigen-5 is an allergenic protein which causes hypersensitivity in human. This protein can find in plants and animals including wasp venom [5, 6]. Dipeptidyl-peptidases (DPPs) are exopeptidase that cleaves the polypeptide at N-terminal of peptide bond [7]. The hyaluronidase plays a role in degradation connective tissue of prey, and it facilitates spreading of another components into their body (spreading factor) [8]. In addition, that enzyme also is an allergenic factor [9, 10]. The hyaluronidases are a family of glycoside hydrolase (EC. 3.2.1.35) [11, 12]. This enzyme cleaves hyaluronic acid to tetrasaccharides at beta-1,4-glycosidic bond [13, 14]. In a previous study, hyaluronidase in venomous animals contains mixture biochemical and biophysical properties depend on species of animal. For example, the hyaluronidase venom of *V. tropica* has a molecular weight ranging from 46-47 kDa, pI 8.91 and optimal pH 3-4 [15, 16, 17]. The aims of this study are cloning and expression of recombinant hyaluronidase from banded tiger wasp (*V. affinis*) venoms and comparison different expression vector (pET-17b and pET-32a) in *E. coli* system.

II. MATERIAL AND METHODS

A. *Total RNA Extraction and cDNA Synthesis*

Total RNA was extracted from venom gland of *V. affinis* using Trizol® reagent (Ambion and Life technologies, USA). The quality of total RNA was investigated with absorbance at 260/280 nm by UV-Visible spectrophotometer and gel electrophoresis. After that, cDNA was synthesized from total RNA using ReverseAid first strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's protocol.

B. *Gene Cloning and Sequencing Analysis*

The specific primers were designed from hyaluronidase gene sequence of another wasp [15]. In addition, the specific PCR primers was obtained *Kpn*I and *Not*I restriction size position to ligate with cloning vector, pGEM-T easy vector (Invitrogen, USA) and expression vector, pET-17b and pET-32a vector (Invitrogen, USA) (Table 1). After that, the hyaluronidase gene (*VesA2* gene) was amplified with the cDNA as a template using polymerase chain reaction (PCR) followed by the conditions in Table 2. The PCR product was detected by agarose gel electrophoresis and purified with GenepHlow™ Gel/PCR kit (Geneaid, Taiwan) following manufacturer's protocol. The *VesA2* gene was ligated with pGEM-T easy vector using T4 DNA ligase at 4°C for 16-18 hrs and transformed into DH5α *E. coli* strain. The transformants (containing *pGEM-VesA2* plasmid) was verified by colony PCR method. The positive clones were cultured in LB broth containing 100 μg/ml ampicillin at 37°C overnight. The recombinant plasmids (*pGEM-VesA2*) were isolated using the Presto™ Mini Plasmid kit (Geneaid, Taiwan) according to manual's protocol. The isolated plasmids were sequenced and analyzed using the basic local alignment search tool (BLAST), https://blast.ncbi.nlm.nih.gov/.

TABLE 1
SPECIFIC PCR PRIMERS FOR VESA2 GENE

Primers	Product size (bp)	
Forward	5-GGTACCGCACCATCATCATCATCATTCCGAGAGACCGAAAAAAG-3	993
Reverse	5-GCGGCCGCCACTAGTTAACGGCTTCTGTCA-3	

TABLE 2
PCR CONDITION FOR VESA2 GENE

	Temperature	Time (min)	
Pre-denaturation	94°C	5:00	
Denaturation	94°C	0:30	25-35 cycles
Annealing	50°C	0:30	
Extension	72°C	2:00	
Final extension	72°C	7:00	
Storage	4°C	hold	

C. Gene Construction and Protein Expression

The *VesA2* gene was sub-cloned from the pGEM-T easy vector into both pET-17b and pET-32a expression vector. Briefly, the recombinant plasmids (*pGEM-VesA2*), pET-17b and pET-32a vector were digested with restriction enzymes *Kpn*I and *Not*I using double digestion method at 37°C for 3 hrs. After digestion, the reactions were analyzed by agarose gel and purified using GenepHlow^{TM} Gel/PCR kit as described above. The *VesA2* gene was ligated with both pET-17b and pET-32a expression vectors at 4°C for 16-18 hrs using T4 DNA ligase. The ligation products, *pET-17b-VesA2* and *pET-32a-VesA2* construction, were transformed into BL21(DE3) expression *E. coli* host and cultured on LB agar plate containing 100 μg/ml ampicillin at 37°C overnight. The colonies on agar plate were cultured in 5 ml LB broth containing 100 μg/ml ampicillin at 37∘C 14-16 hrs for the starter culture. After that, 100 μl of the starter culture was added in 10 ml of LB broth containing 100 μg/ml ampicillin and cultured at 37°C until the cell density reached to OD600 nm between 0.4-0.6. The cultured cells were incubated at 37°C for 4 hrs with 0.1mM IPTG. The cell pellet was kept by certification at 10,000×g for 3 minutes. The protein expression of hyaluronidase (recombinant *VesA2* protein) was analyzed using SDS-PAGE gels.

D. Hyaluronidase Activity Assay

Hyaluronidase activity was confirmed by gel zymography method that was modified from Mio and Sterm's reports [18]. The methods are similar to SDS-PAGE gels, but the hyaluronic acid substrate was obtained in separating gel. The 13% separating gel solution was prepared by adding 2,130 μl of acrylamide solution (30%; 37.5:1), 1,250 μl of 0.4 mg/ml of hyaluronic acid, 1,233 μl of 1.5 M Tris-HCl buffer pH 8.8, 333 μl of water, 50 μl of 10% SDS solution, 33 μl of 10% APS solution, and 2.1 μl of TEMED. After that, the 4% stacking gel was prepared following as: 265 μl acrylamide solution (30%; 37.5:1), 500 μl of 0.5 M Tris-HCl buffer pH 6.8, 1,215 μl of water, 20 μl of 10% SDS solution, 12.5 μl of 10% APS solution, and 6.25 μl of TEMED. This method was used electric potential at 150 volts. The substrate gel was incubated with 3% Triton-X 100 in 50 mM Hepes for 1 hr to remove SDS. Then, the gel was rinsed 2 times with water. After that, the assay buffer (0.1M acetate buffer pH 4.0 in 0.15M NaCl) was added and incubated at 37°C for 16 hrs. The gel was stained with Alcian blue solution for 1 hr and destained with 7% acetic acid until the observed lytic zone.

III. RESULT

A. Gene Cloning and Sequencing Analysis

Total RNA extraction from the venom gland of *V. affinis* was extracted from venom gland (Figure 1).

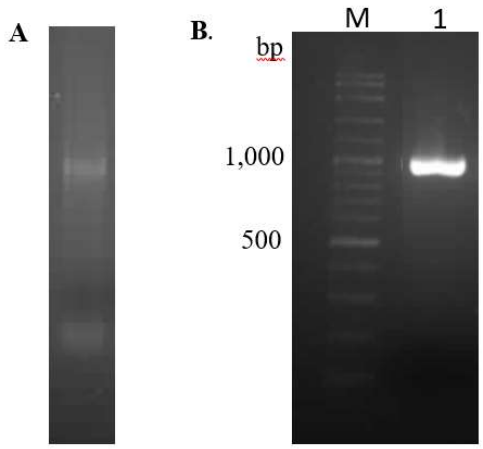

Fig. 1. Agarose gel analysis of (A) RNA extraction and (B) cDNA synthesis from venom gland of *Vespa affinis* were amplified using specific primer for hyaluronidase gene by RT-PCR technique. The product was about 1,000 bp in size. M: 100 bp DNA ladder, 1: hyaluronidase gene

The RT-PCR product, about 1,000 bp in size, using gene-specific primers was obtained (Figure 1). The PCR product band was purified and ligated with the pGEM-T easy vector. The recombinant plasmids (*pGEM-VesA2*) were transformed into DH5α *E. coli* strain and analyzed by colony PCR. The positive colonies clearly showed the products about 1000 bp in size (Figure 2, Lane 2-3 and 5-10). Then, the purified recombinant plasmids (*pGEM-VesA2*) were nucleotide sequenced to confirm the gene.

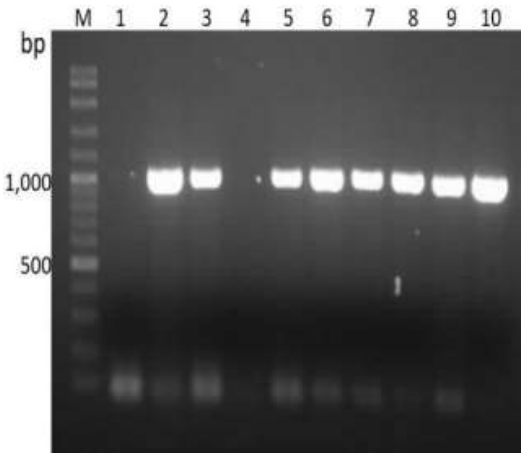

Fig. 2. Determination of recombinant gene (*pGEM-VesA2*) in *E. coli* DH5α by colony PCR technique. Lane 2-3 and 5-10 shown product size about 1,000 bp. M: 100 bp DNA ladder, 1-10: the number of *E. coli* DH5α colonies.

The obtained nucleotide sequences correspond to wasp venom hyaluronidase records in the GenBank database. Then, the nucleotide sequences were deduced to 331 amino acid residues using BLASTX online accessible tools. The amino acid sequences composed 96 residues with above 90% similarity to those of wasps, *Vespa magnifica* and *Vespula germanica* (Table 3).

TABLE 3
THE AMINO ACID SEQUENCE OF VESA2 WAS ANALYZED BY PROTEIN BLAST AFTER ALIGNMENT TO HYALURONIDASE FROM WASP VENOM

Description	% Identities
Hyaluronidase, Vespa magnifica	96
Hyaluronidase, Vespula germanica	92

B. Protein Expression and Hyaluronidase Activity Assay

The solubility of the recombinant products originally from both expression vectors had been investigated. The basic structures of pET32a and pET17b were shown in Figure 4. For the pET-17b plasmid, T7·tag and His·tag were at the N-terminus upstreamingly conjugated to the inserted *VesA2* (Figure 3), whereas for pET-32a plasmid, the fusion tags were thioredoxin tag, His·tag, and S·tag at the N-terminus (Figure 3).

The recombinant plasmids were transformed into BL21(DE3) expression host and induced by 0.1 mM IPTG. The recombinant VesA2 protein (39 kDa) from *pET17b-VesA2* and *pET32a-VesA2* constructs were about 41 (Figure 4A) and 59 kDa (Figure 4B), respectively, in size after analysis by SDS-PAGE. The hyaluronidase activity was demonstrated on substrate gel (Figure 4). Both recombinant VesA2 proteins showed clear bands in the size of 41 and 59 kDa, corresponding to those analyzed by SDS-PAGE.

Fig. 3. Schematic of rVesA2 protein with fusion protein from both expression vector. (A) Recombinant protein from pET17b vector was fusion protein of T7·Tag, His·Tag and rVesA2 with product size about 41 kDa. (B) Recombinant protein from pET32a vector was that of Trx·Tag™, His·Tag, S·Tag, rVesA2 and His·Tag with product size about 59 kDa

The induced cell pellets were determined their solubility. The pellet was freeze-thawed, sonicated and centrifuged at 10,000×g for 30 minutes. Then, the pellets were dissolved in 2 M and 4 M urea buffer. After centrifugation, the pellet and supernatant fractions of 2 M and 4 M urea were analyzed on SDS-PAGE (Figure 5). The insoluble rVesA2 proteins were found from both *pET17b-VesA2* (A) and *pET32a-VesA2* constructs (B). However, rVesA2 from *pET32a-VesA2* construct shown higher solubility in denaturing conditions (2 M and 4 M urea) than rVesA2 from *pET17b-VesA2* constructs.

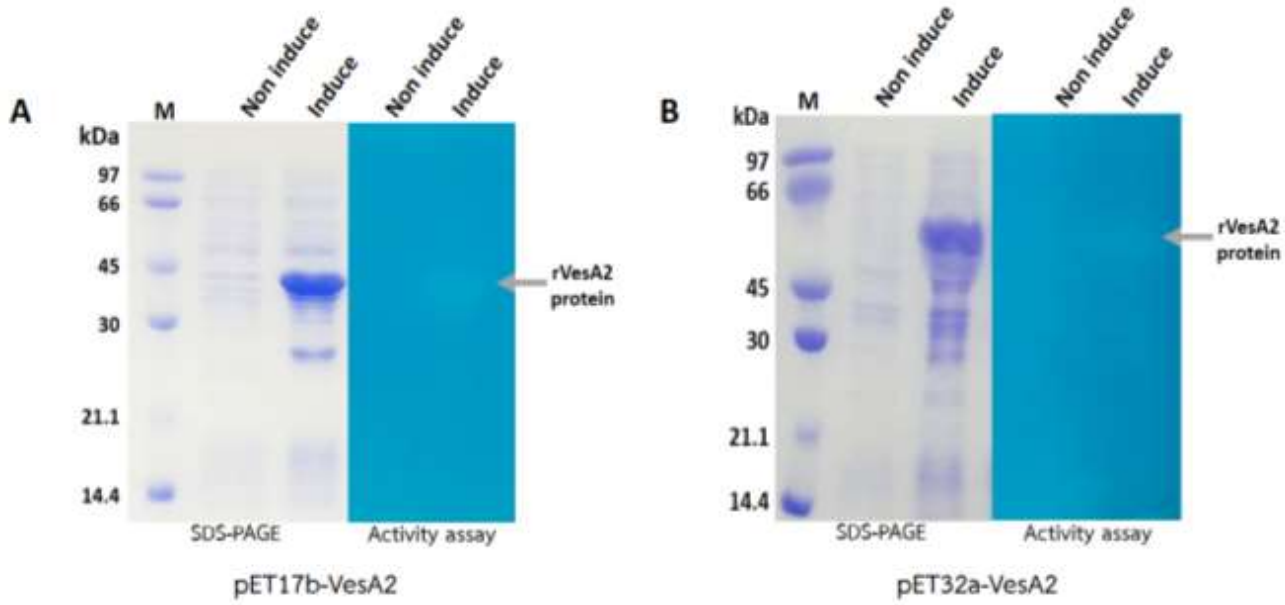

Fig. 4. SDS-PAGE analysis of recombinant VesA2 protein expression. (A) The expression of rVesA2 in *pET17b-VesA2* construction (41 kDa) and its hyaluronidase activity. (B) The expression of rVesA2 in pET32a-VesA2 construction (59.45 kDa) and its hyaluronidase activity. Products form both of vectors showed hyaluronidase activity in zymographic gel

The induced cell pellets were determined their solubility. The pellet was freeze-thawed, sonicated and centrifuged at 10,000×g for 30 minutes. Then, the pellets were dissolved in 2 M and 4 M urea buffer. After centrifugation, the pellet and supernatant fractions of 2 M and 4 M urea were analyzed on SDS-PAGE (Figure 5). The insoluble rVesA2 proteins were found from both *pET17b-VesA2* (A) and *pET32a-VesA2* constructs (B). However, rVesA2 from *pET32a-VesA2* construct shown higher solubility in denaturing conditions (2 M and 4 M urea) than rVesA2 from *pET17b-VesA2* constructs.

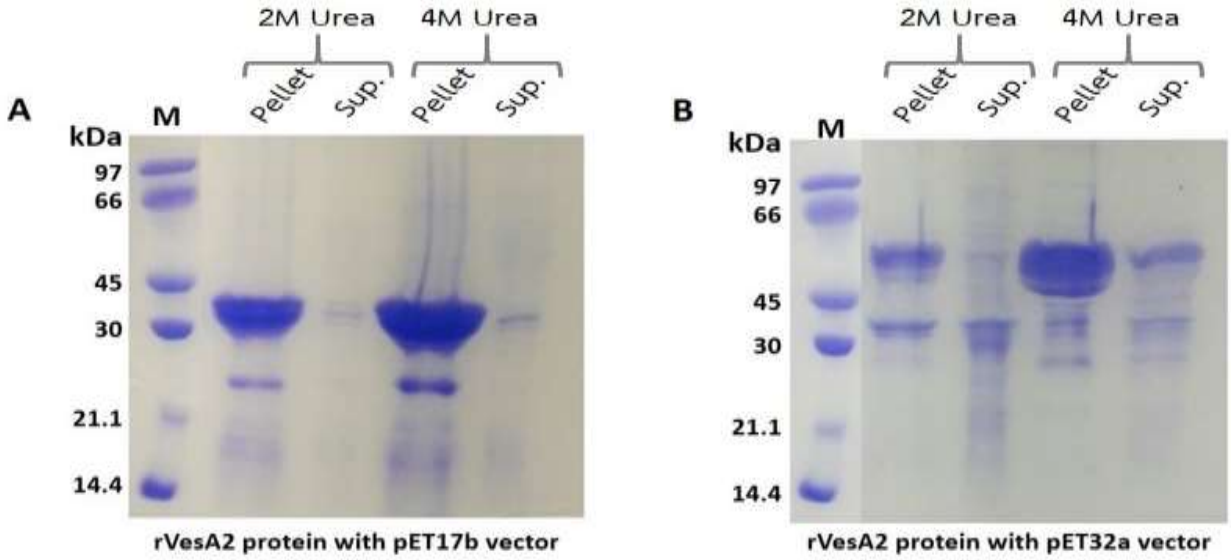

Fig. 5. the solubility of rVesA2 protein from pET17b (A) and pET32a vectors (B). The pellet (Pellet) and supernatant (Sup.) fractions were analyzed after rVesA2 protein was dissolved in denaturing conditions, 2M and 4M urea buffer

IV. DISCUSSION AND CONCLUSION

In this study, we described the cloning, expression, and characterization of rVesA2 derived from the tiger wasp, *V. affinis*, venom. The theoretical molecular weight and isoelectric points of mature VesA2 were about 39 kDa and 9.2, respectively, which corresponds to the experiments. Bacterial inclusion bodies are usually expressed at the high levels of protein expression [19]. The VesA2 was overexpressed mainly in insoluble form. However, the insoluble form has no effects on the HAase activity according to the zymographic gel assay [18]. The renaturing by 3% Triton-X detergent has a tendency to easily recover the activity. The refolding in gel, rVesA2 was able to degrade the substrate, hyaluronic acid, causing a lytic zone with electrophoretic mobility about 41 and 59 kDa in size after Alcian blue staining of the intact background polysaccharide, hyaluronic acid [18, 20].

However, the solubility was different. High concentration of urea (2 and 4 M urea) showed a tendency to synergize the solubility of fusion proteins expressed from the vectors with thioredoxin tag, a pET32a vector. Therefore, the addition of fusion Protein Tags, such as glutathione S-transferase (GST), thioredoxin (Trx), and Maltose binding protein (MBP) often improves the solubility of recombinant expression products [21, 22]. We clearly concluded that the tags just affected the solubility. The levels of fusion protein overexpression and HAase activity still showed similar results from both short-tag or long-tag vectors.

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