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Expression and characterization of a fibrinogenolytic enzyme from horsefly salivary gland

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ABSTRACT

Enzymes from various natural resources are valuable in management of thrombosis. Blood-sucking arthropods are one of these resources because they have a wide array of anti-hemostasis molecules in their salivary gland. However, it is difficult to purify enough protein samples from the salivary glands for pharmacological studies. In this work, a fibrinogenolytic enzyme (tablysin 2) identified from salivary glands of the horsefly *Tabanus yao* was expressed in *Escherichia coli* to further study its biological activities. The primary structure of tablysin 2 showed significant domain similarity to arthropod proteins from the antigen 5 family containing SCP domain, whose biological functions are poorly understood. Tablysin 2 cleaved the Aα and part of Bβ chains of fibrinogen and did not affect γ chain and fibrin. It inhibited platelet aggregation induced by ADP. It did not directly induce hemorrhage or activate plasminogen. The fibrinogenolytic activity of tablysin 2 provides a clue for the functions of antigen 5-related proteins in other haematophagous arthropods. This work demonstrate a method of expression of arthropod salivary proteins which are difficult to obtain from natural resources for further functional studies.

1. Introduction

Thrombosis is one of the main threatens in healthcare and often leads to death [1]. In the past decades, many anticoagulants have been developed for thrombus and these agents act on different targets in coagulations system, including platelets and various blood factors [2]. Among them, enzymes from human plasma, snake venoms, earthworm, etc. have been widely used in clinical [3].

Bloodsucking arthropods have evolved complex mechanisms to get blood meal. These arthropods reserve a wide array of anti-hemostasis molecules in their salivary gland [4–8]. One of them, horsefly, is notorious because it often disseminates filariasis among human and domestic animals when taking blood. On the other side, it is used as anti-thrombus materials for hundreds of years in China and some other Eastern countries [9]. Our previous work has revealed part of the anti-coagulant molecules in horsefly by protemomics coupling transcriptome with functional analysis [10,11]. Several families of proteins or peptides which exert mainly anti-

Abbreviations: IPTG, isopropyl β-D-1-thiogalactopyranoside; VPF, vascular permeability factor; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometer; CHCA, α-cyano-4-hydroxycinnamic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TBS, Tris buffered saline; PBS, phosphate buffered saline; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; PPP, platelet-poor plasma; PRP, human platelet-rich plasma.

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thrombosis functions were identified and characterized from 60000 pairs of salivary glands of the horse fly, including ten fibrinogenolytic enzymes hydrolyzing specially alpha chain of fibrin(-ogen), another fibrin(ogen)olytic enzyme hydrolyzing both alpha and beta chains of fibrin(ogen), ten Arg-Gly-Asp-motif containing proteins acting as platelet aggregation inhibitors, five thrombin inhibition peptides, three vasodilator peptides, one apyrase acting as platelet aggregation inhibitor and one peroxidase with both platelet aggregation inhibitory and vasodilator activities [10,11]. These studies not only facilitated the understanding of the molecular mechanisms of the ectoparasite-host relationship but also revealed the extreme diversity of horse fly anti-thrombosis components and the anti-thrombosis molecular mechanisms of the insect material in traditional Eastern medicine [9]. The diverse anti-thrombus molecules in horsefly also add a lot to our natural molecular reservoirs for the research and development of anti-thrombus drugs.

However, there are several hurdles for us to further study the anti-coagulant enzymes from horsefly. Firstly, it is very difficult to raise enough purified enzymes for pharmaceutical purpose due to the limited number of horsefly that we can get. Secondly, the horsefly salivary gland is tiny and it is arduous to dissect the salivary glands to obtain enough raw materials [12]. For example, it took several people and several weeks to collect thousands of glands to prepare the salivary gland extract. Thirdly, the purification procedures are complicated because the components in the salivary glands are extremely complex [10,11]. The enzymes we finally got from 60000 pairs of glands were just enough for molecular identification and characterization, not to mention detailed pharmacological and pharmacodynamics studies. In the proteomisc study, we have found the DNA sequences of some of these enzymes in the cDNA bank of *Tabanus yao* [10]. In this work, we expressed a fibrinogenolytic enzyme, tablysin 2, in *Escherichia coli* and purified it. At the same time, its anticoagulant activities were evaluated.

2. Materials and methods

2.1. Construction of recombinant vector

According to the previous reported sequence of tablysin 2 [10], the cDNA encoding tablysin 2 was synthesized. Rare codons for Asp and Pro were replaced by more frequently used ones based on *E. coli* codon usage. *E. coli* Rosetta-gami (DE3) and plasmid pET-32a (+) were used to express the recombinant tablysin 2. Two forward primers, NT10BDF-1 (5′-GACGACGACGACAAGAATGCTCAGTTATCGACGC-3′) and NT10BDF-2 (5′-GGGGTACCGACGACGACGACAAGAATGCTCAGTT-3′), and a reverse primer NT10BDR (5′-GGGAAGCTTCATAGTCCTCATATCGTTAA-3′) were designed. NT10BDF-1 contains the enterokinase cleavage site

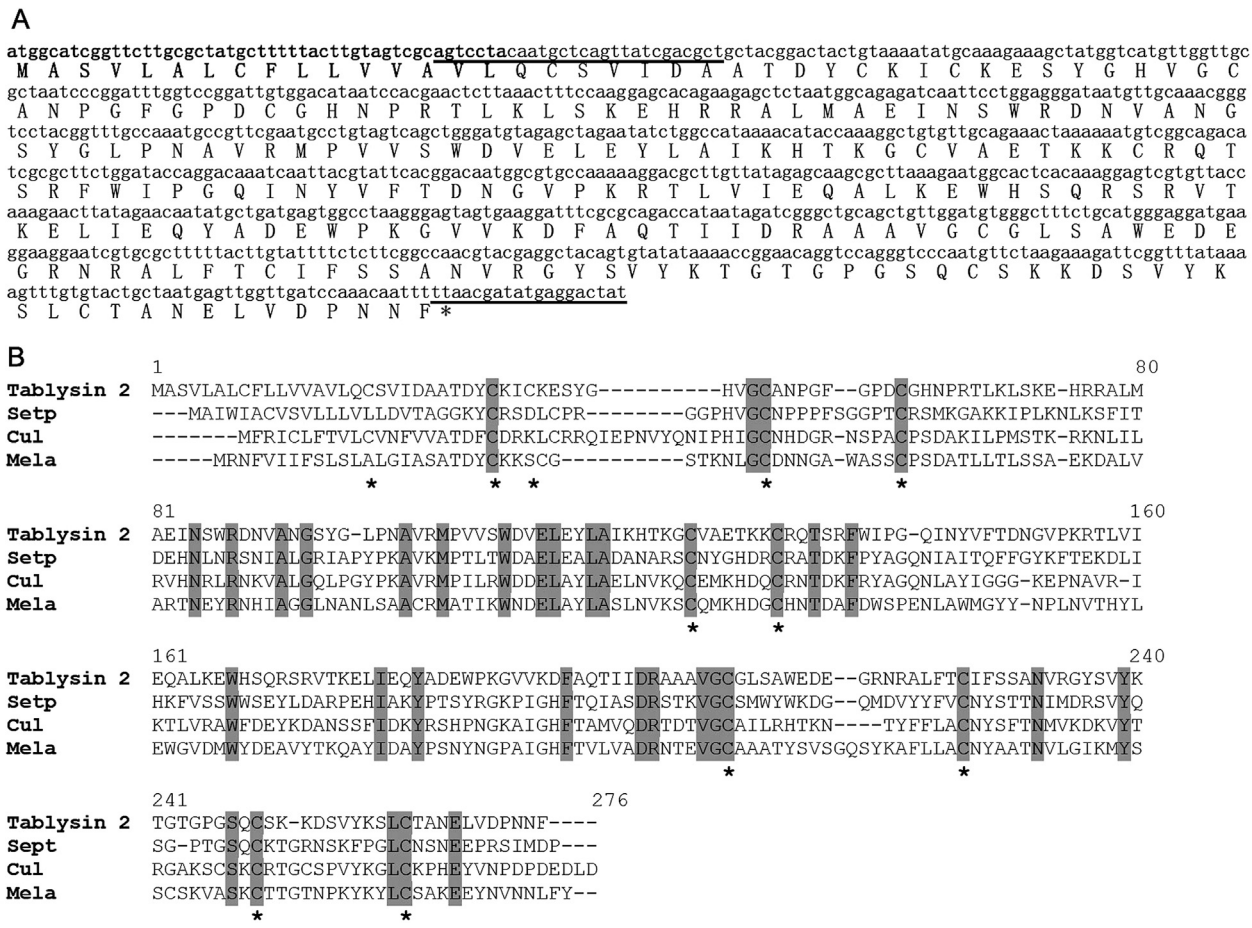

Fig. 1. The nucleotide sequence encoding tablysin 2 precursor (A) and the sequence alignment of tablysin 2 from *T. Yao* (ACS72298) with salivary antigen-5 related protein from *Anopheles stephensi* (AAO06821), Cul o 3 allergen from *Culicoides obsoletus* (AGI16776) and antigen 5-related protein from *Drosophila melanogaster* (AAB92563) (B). The signal peptide is in black. The asterisk (*) indicates the stop codon. The primer sequences used in cloning are underlined. The conserved amino acid residues in all the sequences are highlighted in grey. The bar (–) was introduced for optimal comparison. The asterisks indicate the cysteines in tablysin 2 that may form 5 disulfide bonds.