The University of South Bohemia in České Budějovice
Faculty of Science

Molecular cloning, expression, and purification of the tick salivary serpin IRS-7

Bachelor Thesis

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Annotation:

The aim of this thesis was to produce a recombinant IRS-7 using an *Escherichia coli* expression system along with SUMO fusion protein, purify it, and test this protein for activity in coagulation and protease inhibitor assays to be able to draw early conclusions about its functions in tick-host interactions.

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Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice …… ...…….

Pylypchuk Solomia

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List of abbreviations

AC − affinity chromatography

ADP – adenosine diphosphate

APC – activated protein C

APC's – antigen presenting cells

aPTT – activated partial thromboplastin time

bp – base pairs

BCA -bicinchoninic acid

BSA – bovine serum albumin

CatG – cathepsin G

CBB – Coomassie Brilliant Blue

CD – circular dichroism

cDNA – complementary DNA

DTT – dithiothreitol

E. coli **-** *Escherichia coli*

FT – flowthrough

FWD – forward

FII - prothrombin

FIIa – thrombin

FIXa:FVIIIa – intrinsic tenase complex

FXI, FX, FIX, FVIII, FVII, FV – an inactive form of coagulation factors

FXIa, FXa, FIXa, FVIIIa, FVIIa, FVa – activated form of coagulation factors

GP – glycoprotein

IB – inclusion body

IEC – ion exchange chromatography

IPTG – isopropyl β-d-1-thiogalactopyranoside

IRAC – *I. ricinus* anticomplement protein

***I. ricinus* –** *Ixodes ricinus*

Iris – *I. ricinus* immunosuppressor

IRS-2 – *I. ricinus* serpin-2

IRS-7 – *I. ricinus* serpin-7

LB – lysogeny broth

MHC – major histocompatibility complex

MM – master mix

mRNA – messenger RNA

MW – molecular weight

OD – optical density

PAI-1 – plasminogen activator inhibitor 1

PAR – protease-activated receptor

PCR – polymerase chain reaction

PGI2 – prostaglandin I2

POI – protein of interest

PT – prothrombin time

RCL – reactive center loop

REV – reverse

RT – room temperature

SAT – saliva-assisted transmission

SP – serine protease

SUMO – small ubiquitin-like modifier

TBE – tick-borne encephalitis

TCA – trichloroacetic acid

TF – tissue factor

TFPI – tissue factor pathway inhibitor

TF:VIIa – extrinsic tenase complex

TIL- – trypsin-inhibitor-like

tRNA – transfer RNA

TT – thrombin time

TXA2 – thromboxane A2

T7 RNAP – T7 RNA polymerase

UV – ultraviolet

VWF – von Willebrand Factor

Abstract

The saliva of ticks contains myriads of active substances that exhibit antihemostatic and immunomodulatory properties that are indispensable for tick's feeding success. Recently, a lot of attention in the scientific community has been brought to the study of tick salivary serpins because of the well-established importance of these proteins in regulating most of the vital functions of life within living organisms. Research on these pharmacologically active salivary components might result in the development of novel therapeutics or tick control methods. In this thesis, our research was narrowed down to the study of salivary serpin IRS-7 from the tick *Ixodes ricinus* − a European vector of tick-borne encephalitis and Lyme disease. Here, we produced a recombinant IRS-7 using an *Escherichia coli* expression system along with SUMO fusion protein, purified it, and tested this protein for activity in coagulation and protease inhibitor assays to be able to draw early conclusions about its functions in tick-host interactions.

1. Introduction

Ticks are hematophagous parasitic acarines that are divided into three major families: hard ticks (Ixodidae), soft ticks (Argasidae), and Nuttalliedae – the family containing only one species [1]. Ticks are ubiquitously distributed around the globe, preferring however warm and humid ecosystems. Since the beginning of the 20th century, as a result of anthropogenic land-use change, tick species *Ixodes ricinus* of the genus Ixodes residing prevalently in continental Europe has moved its habitat closer to urban and suburban areas [2]. Due to the climate-induced habitat shift and ticks being the carriers of multiple disease vectors including bacteria and viruses, pathogens causing various human diseases such as Lyme borreliosis, tick-borne encephalitis (TBE), anaplasmosis, etc. [3], the quest for understanding of their feeding strategies along with host-tick interaction grew in importance within the scientific community.

1.1 Feeding strategies and host-tick interaction

Argasidae and Ixodidae families employ different feeding strategies, when soft ticks feed repeatedly mainly for less than one hour and hard ticks get their blood meal only once from several days up to a few weeks [4]. Using their hypostome to puncture the skin of the host, ticks from within their saliva secrete bioactive substances at the feeding site that impede the host's defense mechanisms [5]. These mechanisms rely on hemostasis – the phenomenon of cessation of bleeding which includes platelet clot formation, triggering of coagulation cascade as well as the response from the innate immune system in the form of inflammation and complement activation [6].

1.2 Hemostasis

Despite being a very intricate mechanism that is immediately activated upon vessel injury, for simplicity hemostasis is conventionally divided into three major phases: primary, secondary, and tertiary. Primary hemostasis includes vasoconstriction and platelet plug formation, after which secondary hemostasis is launched culminating in activation of the coagulation cascade to form a stable fibrin clot; tertiary hemostasis aims at the dissolution of an aforementioned fibrin clot. [7]. As a matter of relevance to this thesis, only primary and secondary hemostasis will be uncovered in more detail.

1.2.1 Primary Hemostasis

Under normal physiological conditions, platelets float in the blood having a discoid shape and their adhesion to vessel walls is inhibited by the release of prostaglandin I2 (PGI2) and nitric oxide from endothelial cells [8]. Once the endothelial layer is damaged, its cells synthesize endothelin-1 that binds to embedded nervous receptors of the smooth muscle causing rapid vasoconstriction which is a short-lived narrowing of a blood vessel that impedes massive blood flow and thus its loss [9]. During primary hemostasis, much effort is aimed at encouraging the influx of platelets from the blood flow to the damaged area (Figure 1). To achieve that, nature has designed numerous strategies to cause platelet adhesion to the subendothelial layer. For instance, once vasoconstriction had occurred, von Willebrand Factor (VWF) – a glycoprotein (GP) that circulates freely in the blood, binds to fibrillar collagen exposed upon the injury [10]. Hence, since platelets contain receptors (GPIIb/IIIa and Ib-V-IX complex) with a strong affinity towards VWF [11] it massively encourages more platelets to adhere to the damaged surface. Additionally, platelets contain receptors (GPIa/IIa) that promote their direct adhesion to fibrillar collagen [12]. Functioning in an orchestrated manner, these processes induce the inflow of platelets from the blood and facilitate their adhesion at the site of injury. Followed by the adhesion, platelets get activated by their agonists such as ADP, thromboxane A2(TXA2) and thrombin (FIIa) released from dense granules of the platelets that render their shape pseudopodal. Lastly, the aggregation of platelets occurs forming an unstable but vital plug [7]. Important is to understand that the mechanisms mentioned above do not occur in such organized and coherent matter, they are all rather intertwined and happen simultaneously.

1.2.2 Secondary Hemostasis

According to a revisited theory of coagulation cascade which can be visualized in Figure 2, the line of coagulation amplification once the platelet plug had been formed is divided into three phases: the initiation, amplification, and propagation. During the initiation phase, tissue factor

(TF) exposed upon the tissue rupture binds to serine protease (SP) FVII. FVII, like many other major serine proteases, circulates in the blood in its inert zymogen form and is cleaved by proteolysis upon binding with TF forming an extrinsic tenase complex TF:VIIa [13]. Extrinsic tenase complex then activates minute amounts of SP's FX and FIX. Subsequently, produced FXa forms a prothrombinase complex with platelet-derived FVa in the presence of Ca^{2+} ions that cleaves prothrombin (FII) to thrombin (FIIa). During the initiation phase, only a small amount of thrombin is generated [14]. Nonetheless, these trace amounts of thrombin produced activate an even greater number of FV during the amplification phase. A positive feedback loop is thus being created since without cofactor FVa, FXa is unable to cleave a sufficient amount of thrombin and activate FVIII and FXI [15]. Finally, during the propagation phase, FXIa on the phospholipid surface of platelets activates even more FIX that associates with FVIIIa and forms intrinsic tenase complex FIXa:FVIIIa[16]. Intrinsic tenase in turn further catalyzes the conversion of FX to FXa. The amplification effect at each step is such that the intrinsic tenase ends up activating as much as a hundred-fold of FX compared to extrinsic tenase hence creating a sufficient amount of thrombin. Lastly, generated thrombin cleaves fibrinogen to form stable fibrin strands [8].

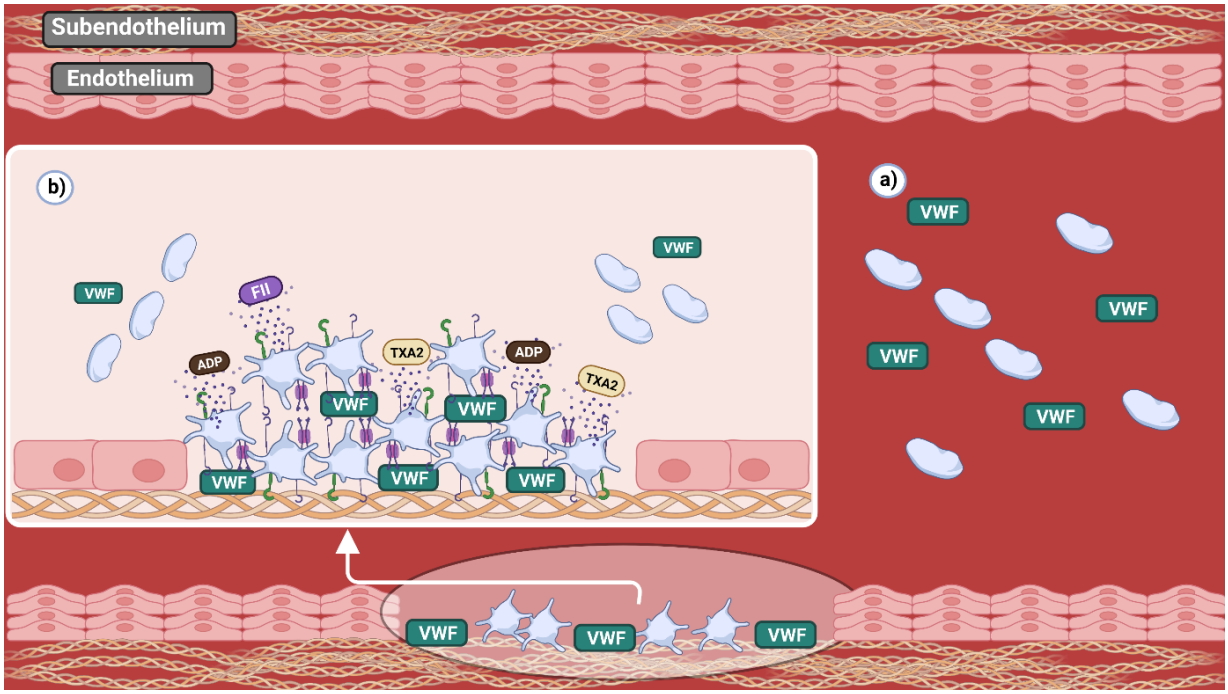

Figure 1: Shows primary hemostasis in simplistic way*: a) Inactivated platelets having discoid shape and von Willebrand factor (VWF) color-coded in green circulate freely in the blood vessels; b) Once the endothelial layer is damaged, platelets bind to the exposed collagen using GPIa/IIa receptors situated on their surface, their shape is rendered pseudopodal upon the instantaneous release of chemicals such as ADP, TXA2 and thrombin (FIIa) from the dense granules of the platelets. Simultaneously, VWF binds to the surface of the collagen. Since platelets are as well covered in receptors such as GPIIb/IIIa and GPIb-V-IX complex that show high affinity towards VWF, even more platelets get attracted from the blood flow. This cascade of events causes rapid aggregation of platelets at the site of the injury forming a temporary plug. The figure was created with BioRender.com.*

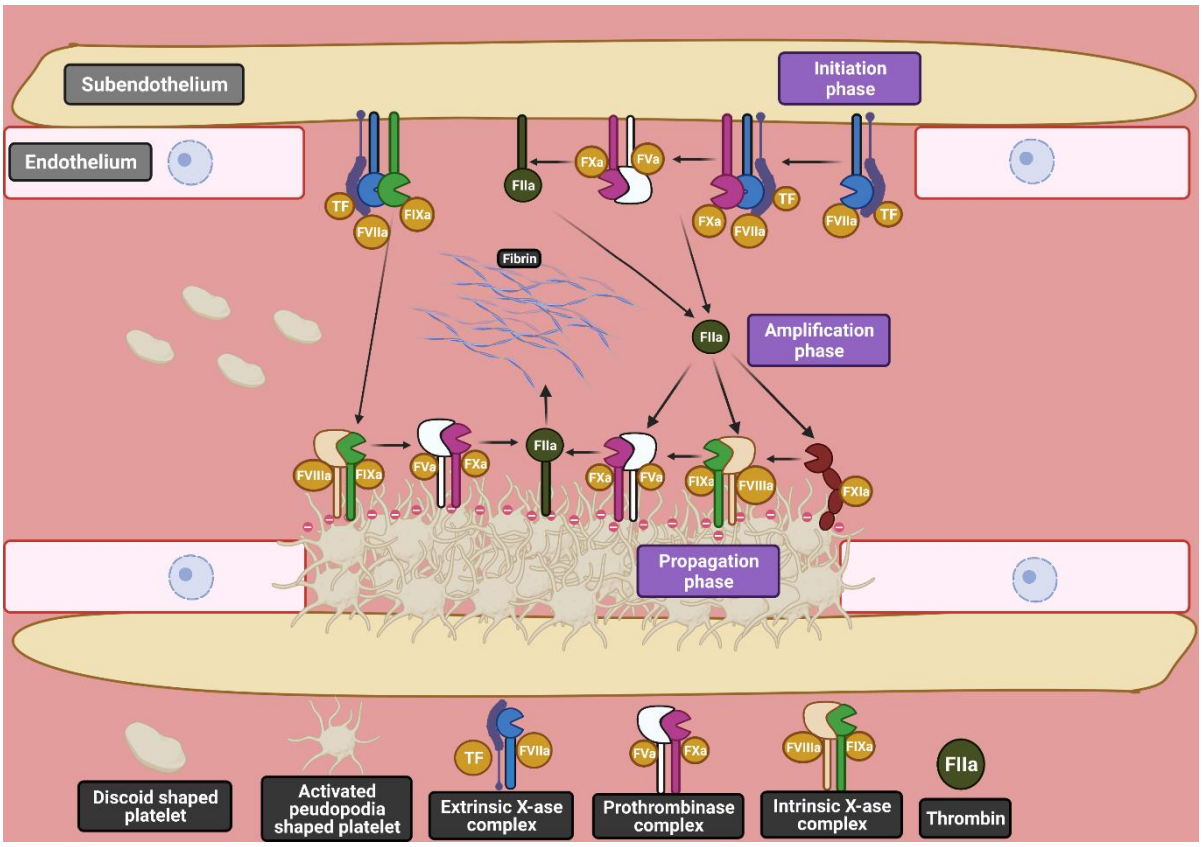

Figure 2: Illustrates the damaged blood vessel and the mechanisms of clot formation that follow instantaneously after the injury. *Three main phases of coagulation are shown in a simplistic manner to stress the most important processes: i) Initiation - during initiation tissue factor (TF) binds to FVII forming an extrinsic tenase complex TF:FVIIa. TF:FVIIa complex in turn activates minute amounts of FX and FIX. FXa then associates with platelet derived FVa in the presence of Ca^{2+} forming a prothrombinase complex FXa:FVa that cleaves the first traces of thrombin (FIIa). ii) Amplification – produced thrombin facilitates the activation of FV, FVIII and FXI thus creating an amplification positive feedback loop. iii) Propagation - propagation phase occurs at the phospholipid surface of platelets where FXIa activates more of FIX which in turn forms an intrinsic tenase complex FXIa:FVIIIa with FVIIIa. FXIa:FVIIIa further catalyzes the activation of FX. The figure was created with BioRender.com.*

1.3 Innate Immunity

The body does not only try to stitch the damaged areas, but it also had evolved strategies to protect itself from various types of infections. Another fundamental form of host defense thus involves simultaneous activation of innate immunity which is an ancient mechanism that works along complement proteins causing a rapid inflammatory response once the pathogen has passed the skin barrier [17]. Innate immunity, among other of its elements, is constituted of hematopoietic cells including mast cells and basophils, neutrophils, eosinophils, and monocytes that subsequently evolve into macrophages and dendritic cells [17][18]. Found in extracellular space, mast cells having detected a pathogen release chemical mediators such as histamines and proteoglycans causing edema at the site of the pathogen invasion and recruiting neutrophils from the bloodstream. Additionally, they release chymase and tryptase from their granules that

cleave proinflammatory mediators chemokines, and cytokines [19][20]. Basophils bear a rather similar immuno-regulatory function to that of mast cells likewise releasing histamines and proteoglycans inducing pain, fever, and vasodilation that helps fight the infection [21]. Next, neutrophils that get attracted to the site of the injury by chemotaxis battle microorganisms by phagocytosis; the contents of their azurophils granules – elastase, cathepsin G (CatG), and proteinase 3 – help to both digest ingested pathogens and to regulate the inflammatory processes by targeting cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-18 and moreover the protease-activated receptors (PAR's) [22]. Eosinophils that reside in tissues induce the degranulation of basophils and mast cells using their cytotoxic proteins [23]. The main *raison d'être* for dendritic cells is to engulf foreign particles, expose the cleaved antigens through the major histocompatibility complex (MHC) on their surface and then serve as antigen-presenting cells (APCs). By thus doing, they promote the stimulation of T-cells – the agents of adaptive immunity that only recognize the antigens that are bound to MHC [24]. Lastly, macrophages other derivatives of monocytes besides dendritic cells, are yet another type of phagocytes that are additionally involved in processes such as cleansing of cellular debris, tissue repair, cell regeneration, and regulation of inflammatory responses [25]. These elements of host defense are aimed to aggravate tick's feeding success [26].

1.4 How ticks overcome host-defense mechanisms

Being physiologically dependent on blood, ticks in accord with evolution developed strategies to overcome the host's intricate mechanisms of hemostasis and innate immunity mentioned in the previous chapters. Host defenses, vastly dependent on proteases, must therefore be disrupted by the tick to complete its blood meal [4]. The secret for such invasive artifice lies in the tick's saliva that is being injected into the host, and which contains a complex mixture of proteins that exhibit anti-hemostatic, anti-inflammatory, anti-complement, and immunomodulatory properties [27] [28]. Moreover, with growing research in the current field, grew as well the understanding that tick's salivary proteins display both redundancy and pluripotency. Redundancy implies the ability of multiple salivary proteins to target one component of the host's defense; pluripotency on the other hand means that one tick protein might target various players of hemostasis or innate immunity. It is therefore theorized that these proteins display somewhat orchestral functionality yielding an additive and potent effect [29] [30].

1.4.1 Tricking the host immune system

To be able to feed on the host undetected, ticks trick the host's immune response by locally inactivating it. As an example, recent research has shown that ticks secrete glycoproteins Evasins that inhibit signaling proteins chemokines responsible for leukocytes recruitment [31]. No leukocytes at the site of the bite mean no signs of inflammation; thus, no pain, itching, redness, or swelling can make the host aware of the presence of the parasite [32]. Some immunosuppressive proteins contained in the tick saliva such as *I. ricinus* anticomplement (IRAC) proteins I and II aim to block the host complement activation [33]; others impair the function of APCs [34]. Of importance is also to mention the ability of tick-borne pathogens to utilize salivary proteins of arthropods to facilitate their transmission to the host organism, a phenomenon known in the scientific community as saliva-assisted transmission (SAT) [28].

1.4.2 Suppression of host defense – protease inhibitors

Further weapons used in the tick's arsenal are protease inhibitors. Proteases themselves comprise a large multigenic family of digestive proteins that maintain various physiological functions in mammals [35]. These functions include protein metabolism and digestion, regulation of immunity, and coagulation cascade, where most of the clotting factors are serine proteases; their function also extends to regulating enzymes of the complement system, etc. [36] [37] [27]. There are four major groups of protease inhibitors to distinguish in tick saliva that counteract the cleaving and catalytic nature of proteases. The first group to be described is Kunitz-domain inhibitors: these are relatively small peptides of around 7kDa in weight having a disulfide-rich α/β structure [4]. Proteins with multiple Kunitz domains usually have anticoagulant properties. The next group to be looked at are trypsin-inhibitor-like (TIL-) protease inhibitors that also are small (6-9kDa) proteins with conserved 5 disulfide-bridge structure [38] [30]. TIL inhibitors show importance in immune response and carry anticoagulant and antimicrobial properties [39]. The third group is cystatins which regulate endogenous cysteine proteinases and carry antiviral, antimicrobial, and immunomodulatory functions [40]. Finally, serpins are probably the most well-studied superfamily of proteins whose role in hemostasis, immunity, and homeostasis is well established [41].

1.5 Serpins – Serine Protease Inhibitors

Serpins have succeeded in being ubiquitous serine protease inhibitors within most kingdoms of life [41]. A generic serpin is a large protein molecule consisting of around 400 amino acids (aa) [42] that fold to form three β-sheets (A, B and C), eight to nine α-helices (hA-hI) and an exposed

reactive center loop (RCL) [43]. The RCL that is approximately 17 aa long extends above the body of the serpin stretching between β-sheets A and C. RCL spans from residue P17 [44] of the strand (s) s5A of the N-terminal domain to the P3'(nomenclature after Schechter and Berger [45]) residue of the C-terminal s1C [46]. Serpins owe their biological success to the unique mechanism of inhibition that causes a conformational change of a serpin and interestingly renders its natural metastable conformation (known as "stressed") to a more stable cleaved (known as "relaxed") folding [42]. As shown in Figure 3, protease binds with its catalytic triad to the scissile region of RCL denoted as P1-P1' forming a typical Michaelis complex [47]. This attack of protease's catalytic triad on the scissile bond of the serpin leads to the formation of the covalent ester bond between serine of the protease and main-chain carbonyl carbon of the P1 residue of the serpin that eventually causes the cleavage of the peptide bond [48]. As a result, the strained RCL then pulls a protease downwards and inserts itself into the β-sheet A forming a new β-sheet [42]; by thus doing the protease folding is disrupted and therefore it ceases to the RCL region of the serpin facilitates the prediction of its inhibitory activity [50][51][52].

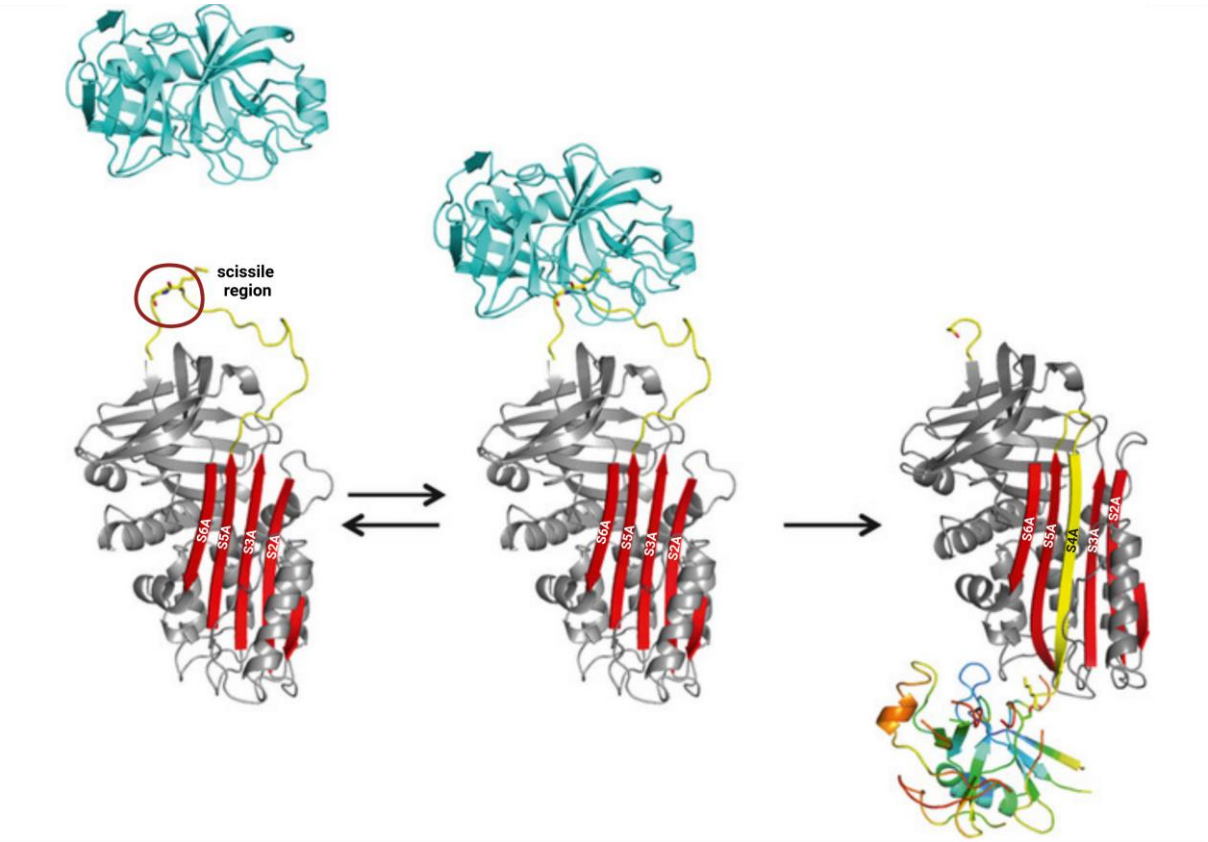

Figure 3: Serpin structure and mechanism of action. *On the left: i) The protease is shown in cyan and the serpin in its stressed conformation in gray; ii) β-sheet A of the serpin is color-coded in red numbered from right to left as s2A-s6A (skipping s4A); iii) RCL hangs above the serpin drawn in yellow, the scissile region of RCL is circled in red. In the middle: i) The protease-enzyme complex is shown; ii) Protease attacks the scissile region of RCL forming a covalent ester bond between amino acid serine of protease and carbonyl carbon of the P1 residue of the serpin. On the right: i) Serpin in its relaxed conformation is shown along with the colorful distorted protease; ii) P1-P1' bond of the RCL is cleaved and inserted into a newly formed s4A marked in yellow. Picture* adapted *from J.A. Huntington [48].*

1.5.1 Serpins in *I. ricinus*

Similarly, to mammals, serpins in ticks also maintain a range of physiological functions encompassing the regulation of internal processes such as initiation of the immune response, reproduction, and development [5][44]. Moreover, considerable amounts of anti-hemostatic and immunomodulatory proteins have been identified in the saliva of both hard and soft ticks [4]. Among various other functions, these proteins were found to inhibit thrombin [53], bear a tissue-factor-pathway-inhibitor-like nature [54], or interfere with signaling cytokines [55]. Despite the extensive knowledge of the existence of such molecular agents, only few of them remain structurally and functionally characterized. A peculiar example of characterized proteins originates from the salivary gland of *I. ricinus* tick named Iris (short for "*I. ricinus immunosuppressor*") [56]. Iris being a serpin that targets elastase-like proteases was shown to inhibit contact phase-activated coagulation pathway, hinder platelet adhesion and delay fibrinolysis [57]. Likewise, Iris also demonstrated the ability to modulate innate and adaptive immune responses by inhibiting the secretion of cytokines from T cells and macrophages [56]. Later, another serpin originating from *I. ricinus* termed IRS-2 was described [6]; its crystallographic data suggested a protein of 42.2kDa comprised of three β-sheets and nine α-helices. Further structural characterization of IRS-2, particularly its RCL sequence suggested the likelihood of IRS-2 inhibiting chymotrypsin-like enzymes [58]. As of its functional attributes, IRS-2 actively inhibited mast cell chymase and proinflammatory proteinase CatG, reducing edema formation at the site of the bite as well as decreasing the neutrophil influx [6].

1.6 Recombinant protein technology

Recombinant protein technology is a state-of-the-art tool enabling the production of a plentitude of heterogenous proteins useful for applications such as the development of therapeutics, cosmetic additives, or the cultivation of laboratory-assisted meat, etc. [59][60]. While being continuously refined, genetic and biochemical advancements thus far have allowed scientists to manipulate the genomes of both prokaryotic and eukaryotic hosts hence increasing the scale of manufacture of recombinant hormones, growth factors, antibodies, enzymes and vaccines. The prevalent host chosen for recombinant protein production, however, remains *E. coli* due to some of its salient features including well-described organism characteristics, short doubling time, and cost-efficient maintenance [61]. Moreover, in recent years new *E. coli* strains have been engineered accounting for the encountered production flaws such as poor protein yield, improper folding, or protein's poor solubility that leads to the formation of inclusion bodies [62].

1.6.1 Genetic Information Flow

Nowadays, it is widely accepted that DNA contains the information needed to produce a functional protein. The information encoded in DNA is passed by the means of messenger RNA (mRNA) in the process known as transcription; further down the line in the cytoplasm, mRNA in hand with transfer RNA (tRNA) and ribosomes perform the translation of the genetic code assembling an intended protein. As one can infer, the first step to producing the protein of interest (POI) requires coding DNA sequence. The discovery of viral reverse transcriptase in 1970 [63] allowed the conversion of single-stranded RNA into double-stranded complementary DNA (cDNA). This breakthrough in molecular biology accelerated the development of cloning techniques allowing the incorporation of the gene of interest into a suitable expression vector. In *E. coli* the expression vector is a plasmid which is a circular piece of DNA that carries genes responsible for evolutionary adaptations, acquisition of antibiotic resistance, and transfer of that genetic information to other bacterial cells [64]. Molecular biologists exploit these features of plasmids to engineer expression vectors with their consequent transformation into target bacterial host cells.

1.6.2 pET_SUMO expression vector

Various expression vectors have been designed so far, yet a pET expression system remains among the most successful ones when it comes to recombinant protein production [65]. pET series incorporate a T7 promoter system along with other crucial elements for an effective transcription and translation such as replicon, translation initiation regions, multiple cloning sites, and selectable markers [62]; in addition, affinity tags and fusion partners can as well be introduced into a plasmid [66][67]. In the T7 promoter system, the gene of interest is cloned downstream of the T7 promoter which is a nucleotide sequence very sensitive for recognition by T7 RNA polymerase (T7 RNAP) derived from bacteriophage [68]. This means that the transcription of heterogeneous genes situated downstream of the promoter region will be halted unless T7 RNAP is present. Using this knowledge, a special strain of *E. coli* called BL21(DE3) pLysS was designed to use along with the T7 promoter system because it carries a copy of a gene coding for a T7 RNAP in its chromosomal DNA [55]. The transcription of the T7 RNAP gene itself is regulated by the lac UV5 promoter [69] which in turn is silenced by the lacI repressor. The presence of a lacI repressor enables effective control of an unsupervised transcription of T7 RNAP and as a result the heterogenous gene as well. To release the lacI repressor and induce the transcription of the gene of interest, a dose-dependent addition of IPTG inducer which is a molecular mimic of allolactose can be introduced. Stochastic transcription of the T7 RNAP

gene also described as “leaky” expression is additionally regulated by T7 lysozyme - a natural inhibitor of T7 RNAP. This control of “leaky” expression by T7 lysozyme also accounts for the pLysS subscript in the name of the bacterial strain. Then, for plasmids to undergo replication the presence of a replicon is indispensable; pET vector series usually contain a pMB1 origin [70], but the origin of replication can be varied depending on the copy number of plasmids required. Another feature present in pET vectors is a selectable marker that confers resistance to various antibiotics; the markers ensure that the only bacteria which up took the plasmid survive. A more precise covering of the mechanism of action of the pET promoter system exceeds the scope of this thesis, nonetheless, it can be accessed in the cited literature.

1.6.3 His- and SUMO- tags

At this point, we have theoretically produced a sufficient amount of heterogeneous protein in the bacterial host, yet the challenges do not stop at this step. Next matters to think ahead are (i) how to purify and detect the POI from the mixture of other bacterial proteins after expression; (ii) how to protect the POI from protease digestion; (iii) how to augment the protein’s solubility and maximize its proper folding [66]. The fusion of the His-SUMO tag at the N-terminal of the POI addresses the issues stated above. Firstly, His stands for poly-His-tag which is the sequence of 6 histidine residues that show high affinity to metals such as Ni^{2+} or Co^{2+}; subsequent purification of the POI can thus be easily conducted by choosing the appropriate affinity column. Secondly, SUMO which stands for “small ubiquitin-like modifier” is a protein of around 100 amino acids whose 3-dimensional structure strongly resembles the regulatory protein found in most eukaryotes – ubiquitin [71]. Since heterogeneous proteins are seen as foreign by the bacterial cell, they usually become the main targets of digestive proteases. Here SUMO plays a crucial role: unlike ubiquitin which in eukaryotic cells targets the proteins for digestion, SUMO instead promotes the translocation of POI to different cell compartments thus saving it from digestion by bacterial proteases. Finally, some research papers show [72][73][74] that SUMO enhances the solubility of the target protein by acting as a nucleation site for folding. As result, proteins that are fused with SUMO have been observed to fold properly and be more soluble [71]. An increase in solubility of heterogeneous proteins, in turn, deters the formation of undesirable inclusion bodies. His-SUMO tag can be cleaved from POI enzymatically by SUMO C-terminal hydrolases-isopeptidase, specifically Ulp1. Cleavage by Ulp1 prevents the erroneous linear amino acid cleavage within the POI because Ulp1 recognizes the tertiary structure of SUMO, therefore, generating a native N-terminal of the target protein [66].

1.7 Ticks serpins as novel medicines

The high catalytic activity of proteases should be tightly regulated to prevent the pathologies of vital physiological functions that these enzymes maintain. However, the failure to regulate the activity of certain proteases can lead to their malfunction which marks the onset of numerous diseases. For instance, some studies indicate the correlation between the upregulation of cysteine proteases Cathepsin B [75] or Kallikreins [76] to tumor progression and malignancy. In Hemophilia - a severe bleeding disorder, the deficiency in coagulation factors FVIII and FIX disrupts the balance between pro- and anticoagulant activity of hemostatic proteins causing abnormal continuous bleeding in patients [77]. The pathogenesis of some autoimmune diseases is linked to the malfunction of CatG [78]. Considering that the list of the diseases caused by improper function of proteases could be perpetually extended, proteases, therefore, became major targets of drug research [79]. Since serine protease inhibitors control protease activity, the endeavor of medical research focused on hematophagous organisms might shed an auspicious light on the precise mechanism of how serpins influence the functions of molecules involved in immune response, coagulation, or signaling [27] [6] [80]. The ability of tick salivary proteins to disarm the players of the human defense mechanism is as well a point of very promising research to develop newer treatment possibilities for numerous human mal-conditions besides those mentioned above. For instance, the tick proteinaceous agent from the family of Kunitz domain inhibitors – Ixolaris has promising antithrombotic prospects because of its homology to tissue factor pathway inhibitor (TFPI) [81]; additionally, it also showed an anti-tumor property by blocking TF-dependent procoagulant activities in human melanoma cell lines [82]. Iristatin from the family of cysteine protease inhibitors identified in *I. ricinus* shows a range of immunosuppressive activities that might prove themselves useful in the treatment of immune diseases [83]. Even though the leap from scientific research of these proteins to their development into effective drugs and implementation in clinical practice might seem daunting, the list of potentially useful therapeutic derivatives of tick's salivary proteins keeps growing to the present day [84].

2. Aims

I. To clone salivary serpin IRS-7 derived from I. ricinus into pET-SUMO vector.

II. To overexpress IRS-7 in BL21(DE3) pLysS *E. coli* strain.

III. To isolate IRS-7 from bacterial cells and to purify the recombinant proteins with affinity- and ion-exchange chromatography.

IV. To test the functions of IRS-7 using coagulation and protease inhibitor assays.

3. Methods

3.1 General methods

3.1.1 Primers

Primers used to amplify the IRS-7 gene from tick's cDNA were designed and ordered from Generi Biotech. After the addition of 268.0µl and 315.0µl of H_2O to IRS-7 SUMO_FWD and IRS-7 SUMO_REV respectively, the stock concentration of both forward and reverse primers was 100pmol/µl. Before using them for PCR, primers were further 10x diluted. The sequence of both primers is shown below:

IRS-7_FWD.: 5'-GGA GAT GAG GAC AAA GTG AC-3'.

IRS-7_REV.: 5'-TTA GAG CTT GGT GAC CTG-3'.

3.1.2 High fidelity PCR

To amplify the gene of interest from the cDNA of adult *I. ricinus*, the polymerase chain reaction (PCR) was performed. Sample preparation included mixing on the ice of 12.4µl nuclease-free H_2O, 4µl 5X Phusion HF Buffer, 0.4µl dNTPs, 1µl of each of the two 0.01mM primers, 1µl of cDNA from transcripts in tick salivary glands, 0.2µl of Thermo Scientific™ Phusion Hot Start II (F-549S) polymerase to the total volume of 20µl. Cycling instructions are summarized in Table.1. After the last PCR cycle was finished, the addition of adenosine overhangs at the 3' end for the ligation step was performed by adding 1µl of Top-Bio™ Taq DNA polymerase to the reaction mixture and incubation for10 additional minutes at 72 °C.

Table 1: Cycling instructions for high fidelity PCR 3-step protocol.

	Temperature/ °C	**Time/ s**	**Cycles**
Initial denaturation	98	30	1
Denaturation	98	9	30
Annealing	50	25	
Extension	72	40	
Final Extension	72 12	7 min hold	1

3.1.3 Agarose gel electrophoresis

1.5% agarose gels were prepared by weighing 1.05g of agarose into a 250ml Erlenmeyer flask and dissolving it upon heating in 70ml of 1xTAE buffer. After the agarose had been completely dissolved, the solution was cooled down by exposing the Erlenmeyer flask to cold water. After the solution had been cooled, 7µl of SYBR® Safe DNA Gel Stain was added. The solution was then poured into the casting frame and let solidify for 20-25 minutes. 20ml of the sample with loading buffer and 10µl of Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder were loaded into the wells of the gel. Electrophoresis was carried out at a constant voltage of 100V for 45 minutes after which the DNA band was visualized under the UV light.

3.1.4 PCR clean-up and Gel extraction

DNA extraction from the agarose gel was performed according to the NucleoSpin® manual [87]. The DNA fragment was cut out with a surgical blade and transferred to a clean Eppendorf tube. According to the instructions in the manual, the dissolving of agarose was achieved by adding 230µl of NTI buffer to 115mg of agarose gel (< 2%). The sample was then incubated at 50 °C for 10 minutes allowing the gel to dissolve. NucleoSpin® Gel and PCR Clean-up Column was placed into a Collection Tube and loaded with the sample. To bind the DNA to the silica membrane of the column, centrifugation was executed at 11000xg for 30s, the flow-through was then discarded. 700µl of NT3 wash buffer (prior diluted with ethanol) was added to the column and the sample was centrifuged twice at 11000xg for 30s, the supernatant was then discarded. To ensure the proper removal of residual ethanol from the membrane, the column was centrifuged at 11000xg for 1 more minute. DNA elution was performed by placing the column into a new 1.5ml Eppendorf tube, ultra-pure H_2O was added, the sample was incubated at 70°C for 5 minutes and then centrifuged at 11000xg for 1 minute in an empty collection tube. The elution step was repeated by applying the eluted sample to the column again. The resulting DNA sample was quantified using Nanodrop.

3.1.5 DNA Ligation

Purified PCR-product was ligated into the pET-SUMO vector in the following manner: 1,1µl of the PCR-product(12,5ng/µl), 1µl of the 10xLigation buffer, 1,6µl of the pET-SUMO vector (25ng/µl) were mixed on ice along with 5,3µl of sterile H_2O, then 1µl of T4 DNA ligase was added and the sample was incubated at RT for 30 minutes. The ligation mixture was then transformed into One Shot TOP10 cells according to procedure 3.1.6 and grown according to 3.1.7.

3.1.6 Plasmid transformation

50µl of One Shot TOP10 chemically competent *E. coli* cells were used for ligation and transformation purposes. For subsequent protein expression with plasmid isolated from TOP10 cells and confirmed by sequencing – the same amount of BL21(DE3) pLysS cells were used. The bacteria were first thawed on ice, then 5µl of ligation product for cloning or 2.6µl isolated plasmid for protein expression were pipetted to the cells. For cloning, the TOP10 cells were incubated on ice for 30 minutes, and for protein expression, BL21(DE3) pLysS for 10 minutes. The cells were then subjected to the heat shock for 30 seconds at 42°C, subsequently ice-cooled for 2 minutes, and 500µl of pre-warmed S.O.C. medium was added to each vial. Afterward, the vials were shaken horizontally at 37°C for 1 hour at 225rpm.

3.1.7 Bacteria Cultivation – overnight and petri dish culture

Bacterial cultivations were performed in an LB medium containing kanamycin at the final concentration of 100µg/ml for all experiments. Bacteria grown in the S.O.C. medium according to section 3.1.6 were either spread on LB agar plates or directly added to the LB medium for overnight growth. The incubation of bacteria was carried out at 37°C and kept in the incubator overnight while liquid cultures were additionally constantly shaken at 200 rpm. The cultured bacterial cells were then used for subsequent experiments or stored at -80°C in the glycerol stock containing 770µl of the transformed cells and 230µl of the sterile glycerol.

3.1.8 Colony PCR

To screen the TOP10 bacterial colonies for the presence of the plasmids with the desired amplicon, single colonies were picked with a pipette tip from the agar plates and re-suspended in 10µl. Then around 1µl of those bacterial templates were mixed on ice with 12,5µl of PCR master mix (MM), followed by the addition of 0,5µl of T7 REV and IRS-7 FRW primers each and filled up to 25µl with nuclease-free water. The cycling conditions for the PCR reaction are shown in Table 2. After the plasmid with amplicon was detected by agarose gel, the positive bacterial cultures were grown overnight, the plasmid was then isolated.

Table 2: Cycling instructions for Colony PCR.

Step	Temperature/ °C	Time/ s	Cycles
Initial denaturation	95	180	1
Denaturation	95	30	34
Annealing	50	30	
Extension	72	120	

3.1.9 Plasmid DNA isolation

The plasmid DNA isolation from the transformed amplicon positive fully grown TOP10 *E. coli* colonies was performed according to the protocol of NucleoSpin Plasmid mini kit [87]. First, the saturated *E. coli* LB culture was centrifuged at 11000xg for 30 seconds, the supernatant was then carefully removed leaving the bacterial pellet for resuspension. To achieve cell lysis, 250µl of the resuspension buffer was added and then the pellet was resuspended by vortexing. Afterward, 250µl of the lysis buffer A2 was added and the tube was gently inverted 6-8 times for mixing, the solution was then let to incubate at room temperature (RT) for 5 minutes. After the lysate had become clear, 300µl of neutralization buffer A3 was added to the tube and mixed thoroughly by inverting until the blue sample turned completely colorless. Then the tube was centrifuged at 11000xg for 5 minutes to precipitate the cell debris. The binding of the plasmid DNA was done by decanting the supernatant from the previous step into the column in a collection tube with further centrifuging at 11000 x g for 1 minute. The washing of the silica membrane containing the plasmid DNA was achieved by adding 600µl of wash buffer A4 with subsequent centrifugation at 11000xg for 1 minute. To remove the residual ethanol from wash buffer, the solution was centrifuged once again at 11000xg for 2 minutes. Finally, the elution was performed by placing the column into a clean Eppendorf tube, adding 50µl of the elution buffer AE, incubating the tube for 1 minute at RT, and centrifuging the solution at 11000xg for 1 minute. The resulting isolated plasmid was then sent for Sanger sequencing. Having verified the presence of the insert by sequencing, the plasmid was transformed into BL21(DE3) pLysS *E. coli* cell strains for protein expression. For both small- and large-scale protein expression, transformed BL21(DE3) pLysS cells were grown overnight in an LB medium with the addition of 100 µg/ml kanamycin.

3.1.10 Small-scale pilot expression

To analyze the most optimal conditions of protein expression with the maximum yield, small-scale protein expression was performed. Four conditions were analyzed, namely induction of protein expression with IPTG at optical density (OD) 1.2 and 1.5 and bacterial growth at temperatures 30 and 37°C. First, the pipette tip with a scrape containing bacteria from glycerol stock was immersed into a 20ml LB medium followed by the addition of 10µl of 100µg/ml kanamycin and left to grow overnight. These colonies grown overnight were used to induce fresh medium, followed by incubation at 30- or 37°C, rotating at 200rpm to reach the OD between 1.2- or 1.5. Once the desired OD was reached, 20µl of 1M IPTG was added to induce the protein expression. Right after the inducer was added, 1ml of each of 4 samples was collected and centrifuged at 5000xg for 5 minutes. Afterward, the medium was discarded, and the bacterial pellet was frozen. This procedure was repeated at time points of 1,2,4,6,8, and 20 hours.

3.1.11 Bacterial cell lysis

To disrupt the bacterial cells after pilot expression, 500µl of 20mM Tris and 500mM NaCl(pH8) were added to each of the sample tubes. The tubes were vortexed to resuspend the pellet, then frozen in liquid nitrogen, followed by rapid warming up of the tubes to 56°C. The steps mentioned above were repeated 4 times. Afterward, the samples were centrifuged at 12000xg for 10 minutes. Finally, the supernatant was transferred to new tubes and both the supernatant and pellet samples were frozen to later be analyzed for the presence of proteins using SDS-PAGE gel.

3.1.12 SDS-PAGE

To prepare 12% polyacrylamide running gel: 4.5ml of 40% acryl/bisacryl amide was mixed in the beaker with 3,75ml of 4xTris-Cl/SDS (pH8.8), then the addition of 6.75ml of H_2O followed and finally the mixing was finished by adding 60µl of 10% APS along with 12µl of TEMED. The same procedure was repeated for the preparation of stacking gel but with the addition of 0,49; 1,25; 3,2ml of 40% acryl/bisacryl amide, 4xTris-Cl/SDS (pH8.8), and H_2O respectively followed by adding 30 and 6µl of 10% APS and TEMED. Having prepared gel mixtures, the running gel solution was first poured into the casting frame. The resulting SDS-PAGE gel was then let to solidify, followed by pipetting the stacking gel solution afterward. For the sample preparation, the master mix containing 7µl of NuPAGE^{TM} LDS sample Buffer(4x) was mixed with 3µl of reducing agent mercaptoethanol, to which 20µl of the supernatant protein sample was added. In the case of the pellet sample, it was first re-suspended in 20µl of H_2O followed by the addition of the master mix. Afterward, the samples were boiled at 95°C for 10 minutes.

Then, 15-25µl of each sample or 15µl of the marker were loaded into the wells. The gels were run at 200V, 120mA, and 60W for 1 hour in 1xELFO buffer. The gels were subsequently cooled, removed from the casting frames, and cleaned with deionized water 3-4 times to fully remove the running buffer. The gels were let soak in 0.1% Coomassie Brilliant Blue (CBB) staining solution for 1 hour. After the staining was completed, the gels were removed from the staining solution, rinsed with deionized H_2O, and then left in the destaining solution (10% acetic acid, 25% methanol in water) overnight.

3.1.13 Large-scale protein expression

Once the optimal conditions were assessed and the presence of the protein was verified on an SDS-PAGE gel, the method was scaled up to 3L of LB medium with the addition of 3ml of kanamycin at the final concentration of 100µg/ml. 3L of the LB medium were distributed to 6 Ultra Yield flasks, 500ml each. The flasks were incubated at 200rpm and 37°C to reach the preferable OD between 1,2. After reaching the OD=1,337, 0,5ml of 1M IPTG was added to each flask and incubated at 37°C overnight. Afterward, the flasks were centrifuged at 4200xg for 15 minutes at 4°C. Finally, the supernatant was discarded, and the pellet was collected with the help of PBS solution into 50ml falcon tubes. The pellets were then centrifuged once more at 4200xg for 10 minutes to remove the traces of medium and the pelleted bacteria were frozen.

3.1.14 Big-scale cell lysis

French Press cell lysis was employed to disrupt the bacterial cells after big-scale protein expression. First, the bacterial pellet was thawed and resuspended in 30ml of 20mM Tris and 500mM NaCl (pH8) solution by vortexing and shaking. Then, the cells were transferred into a clean beaker and filled up to 50ml with the Tris/NaCl solution with further addition of one protease inhibitor cocktail tablet. French Press system was washed 3 times with 25ml of H_2O, followed by one round of washing with the Tris/NaCl buffer. Afterward, the cells were one time pressed into a machine at 10000 psi, with the subsequent 2 rounds of pressing at 15000 psi. The cell lysate was collected in the clean beaker whereas the French Press pipes were washed again with appropriate solutions. After the disruption of the cells was performed, DNase I was added to the beaker with the lysate at the final concentration of 10 µg/ml, the solution was then stirred for 15 minutes. Next, the cell lysate was ultracentrifuged at 28000rpm and 4°C for 1h. Finally, once the ultracentrifugation was finished, both the supernatant and the pellet were frozen for future use.

3.2 Protein purification

3.2.1 Affinity Chromatography

Affinity chromatography was performed with HisTrap™ FF column with precharged Ni Sepharose® twice. First to purify the IRS-7 with the HisSUMO tag from the mixture of bacterial proteins after the cell lysis, and second to purify the IRS-7 after HisSUMO tag cleavage. In both cases the supernatant samples were filtered through the syringe filter before loading on the column; the sample after HisSUMO tag cleavage was additionally first centrifuged at 10000g for 10 minutes. Proteins bound to the column were eluted with a gradient of 20mM Tris+500mM NaCl+500mM Imidazole (pH8) solution with a flow rate of 4ml/min. The fractions (showing the UV absorbance at 280nm) were then run on an SDS-PAGE gel to confirm the presence of POI in corresponding fractions.

3.2.2 SUMO- and HIS-tag cleavage

To set up a cleavage reaction, the SUMO protease also known as Ulp from Invitrogen™ (12588018) was used. 30µl of SUMO protease was mixed with 8ml of the POI sample along with 17ml of 20mM Tris buffer (pH8) and DTT at the final concentration of 1mM. The solution was then incubated at RT overnight.

3.2.3 Ion exchange chromatography

Since the SDS-PAGE showed the presence of the POI, it was further purified using ion exchange chromatography with HiTrap® Q FF column. Gradient elution with subsequent increase of the ionic strength of the buffers was employed to purify the POI, with buffer A containing 20mM Tris(pH8) and buffer B – 20mM Tris+500mM NaCl (pH8). The flow rate of elution was 4ml/min and the eluted proteins were collected in 1ml fractions. The fractions (showing the UV absorbance at 280nm) were then run on the SDS-PAGE gel to confirm the presence of POI.

3.3 Protein Testing

3.3.1 Estimation of protein concentration – Bicinchoninic acid (BCA assay)

The microplate protocol of Pierce™ BCA Protein Assay Kit was employed to measure the concentration of IRS-7. Bovine Serum Albumin (BSA) standards of known concentrations namely 0, 25, 125, 250, 500,750, 1000, 1500, 2000 µg/ml were used for calibration. First, 25µl of each standard along with 3 aliquots of the protein sample were pipetted into 96-well microplates to which 200µl of working solution (50:1/reagent A: B) were added. The plate was then incubated at 37°C for 30 minutes. After the plate had been cooled to RT, the sample's absorbance at 562nm was measured with a Synergy H1 microplate reader (BioTek, Winooski, USA).

3.3.2 Coagulation assays

All the coagulation assays were performed at 37°C and coagulation time was measured using the Ceveron four coagulometer (Technoclone).

3.3.2.1 Prothrombin time (PT)

Prior to the PT-test, IRS-7(29,76µM) was diluted in 100µl to the final concentration of 6µM. 20µl of the IRS-7 or the control buffer containing 20mM Tris and 150mM NaCl (pH8) were distributed between 4 vials, to each of which 80µl of coagulation control N (normal plasma) was added. The samples were then incubated for 1 minute at 37°C. Then 200µl of Technoplastin stock was added to each vial and the time measurement began.

3.3.2.2 Activated partial thromboplastin time (aPTT)

20µl of IRS-7 at the final concentration of 6µM and the control buffer were distributed between 4 vials to all of which 80µl of coagulation control N was added. The solution was incubated at 37°C for 1 minute. After the incubation, 100µl of Dapttin reagent was added and again incubated at 37°C for 2 minutes. Afterward, 100µl of $CaCl_2$ that had been prewarmed to 37°C was finally added to the vials and the time measurement began.

3.3.2.3 Thrombin time (TT)

40µl of IRS-7 at the final concentration of 6µM along with control buffer were distributed between 4 vials to all of which 160µl of coagulation control N was added and the mixture was incubated at 37°C for 1 minute. After the incubation, 200µl of Thrombin was added and the time measurement began.

3.3.3 Formation of complexes between IRS-7 and proteases

Five serine proteases: Chymase, CatG, FXa, Chymotrypsin, and Trypsin were screened for the complex formation with IRS-7. First, the appropriate protease buffers were prepared and mixed with proteases at the final concentration of 1µM and 2µM. Then 1µM IRS-7 control along with 2µM IRS-7 to be mixed with proteases solutions were prepared. For the sample preparation, 10µl of proteases (C_{fin}=2µM) were mixed with 10µl of IRS-7 (C_{fin}=2µM); the controls were prepared by mixing 10µl of proteases or IRS-7 (C_{fin}=1µM) with 10µl of 20mM Tris+100mM NaCl(pH7.8). Then, the samples were incubated at RT for 1 hour. Subsequently, all samples were boiled at 95°C for 10 minutes before being analyzed by SDS-PAGE followed by silver staining. The concentrations and buffer constituents can be visualized in the table below.

Table 3: Reaction conditions for serine proteases.

Enzymes	Protease Buffers
Chymase	27mM Tris-HCl+ 150mM NaCl, pH8
Trypsin	50mM Tris+150mM NaCl+10mM $CaCl_2$, 0,05% Brij35, pH7.5
Chymotrypsin	20mM Tris+150mM NaCl+5mM$CaCl_{2+}$ 0,1%PEG6000+0,01% TritonX-100, pH7.5
FXa	20mM Tris+150mM NaCl+5mM $CaCl_2$, 0,1% PEG6000, pH7.5
CatG	50mM Tris-HCl+150mM+0,01%Triton, pH7.5

3.3.3.1 Silver Staining

After having run the polyacrylamide gel with desired proteins, the gel was incubated in a fixating solution consisting of 6,25ml of 20% trichloroacetic acid (TCA), 42µl of 37% HCHO and filled up to 100ml with 50% acetone. Afterward, the gel was rinsed 3 times for 5 seconds with UFH_2O followed by a longer wash in 100ml of UFH_2O for 20 minutes. Next, the gel was incubated in 100ml of 50% acetone in UFH_2O for another 20 minutes. Subsequently, the gel was immersed into a sensitizing solution consisting of 167µl of 10% $Na_2S_2O_3$ and filled up to 100ml with UFH_2O. The gel was briefly rinsed with UFH_2O again and then was incubated for 9 minutes in the silver dye solution which was comprised of 0.27g of $AgNO_3$ dissolved in 100ml of UFH_2O to which 250µl of 37%HCHO was added. Another rinsing with UFH_2O step was performed, after which the gel was finally immersed and incubated for 5 minutes in the developing solution consisting of 2.0g of Na_2CO_3 dissolved in 100ml of UFH_2O to which 42µl of 10% $Na_2S_2O_3$ along with 42µl of 37%HCHO were added. The staining termination step proceeded with immersing the gel into 1% acetic acid for 3 minutes. Lastly, the gel was rinsed with UFH_2O and visualized with ChemiDoc.

4. Results

4.1 Generation of IRS-7 gene amplicons

To amplify the gene of interest from tick's cDNA coding for IRS-7 protein, forward and reverse primers whose sequences are given in section 3.1.1 were designed and high fidelity PCR was performed (sec. 3.1.2). The resulting PCR samples were then visualized on an agarose gel. Theoretically, the amplicon of IRS-7 should have yielded the amplicon of approximately 1500 base pairs (bp). As can be seen in Figure 4, the amplification indeed yielded a single DNA band in that region (~1500 bp). Therefore, having concluded that the DNA band is IRS-7, we extracted it from the agarose gel and purified it (sec. 3.1.4). Then, this DNA sample was analyzed by NanoDrop; the concentration of the amplicon was found to be 12ng/µl.

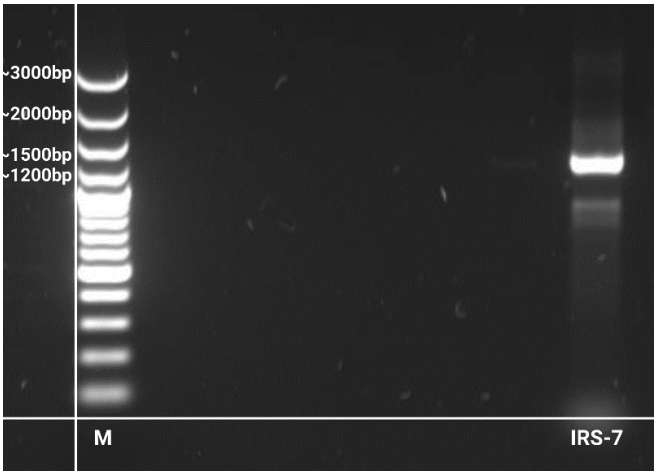

Figure 4: High Fidelity PCR results. *IRS-7 gene found in the range of 1500bp; M- molecular marker.*

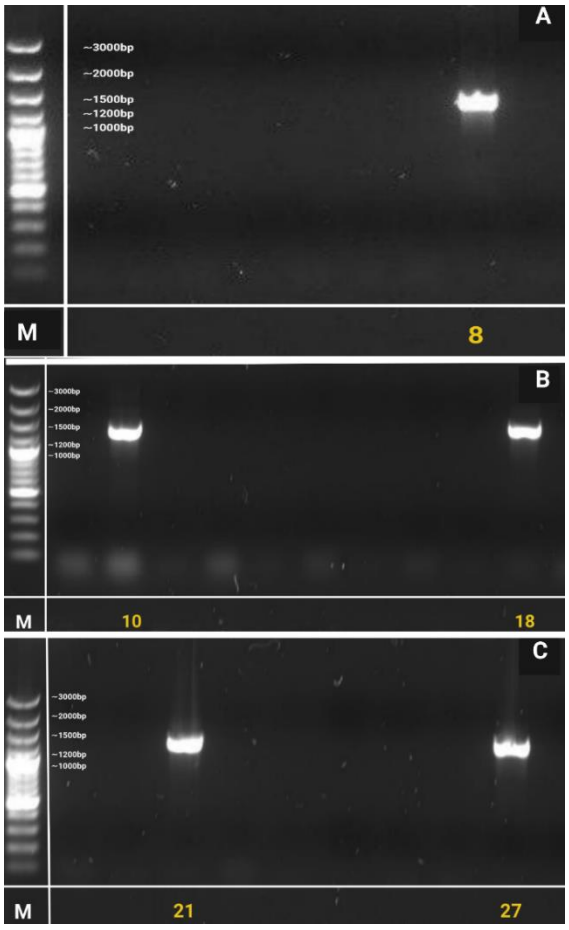

Figure 5: Colony PCR. *In positive colonies 8,10,18,21 and 27, the plasmid with incorporated amplicon was found in a region around 1500bp; M-molecular marker.*

4.2 Ligation and transformation

The next step was to ligate the product of the PCR reaction into the pET-SUMO expression vector with its subsequent transformation into the TOP10 Chemically Competent Cells. A colony PCR was done to verify the presence of the IRS-7 gene in the plasmid using 28 colonies that were chosen from the LB agar plate (sec 3.1.7 and 3.1.8). As a result, the agarose gel in Figure 5 had shown that colonies 8,10,18,21, and 27 yielded a positive clone with an incorporated plasmid bearing an insert found in a region around 1500 bp. Positive bacterial cultures were then inoculated into fresh LB media overnight and the plasmids were then isolated and sent for sequencing according to section 3.1.9.

4.3 Sequencing

The correct gene sequence in the plasmid was verified by Sanger sequencing at SeqMe company. After the sequence with the insert had been confirmed, the plasmid was transformed to BL21(DE3) pLysS cells for protein expression.

4.4 Small-scale Pilot expression

To screen for the optimal conditions for large-scale protein production, small-scale pilot expression with BL21(DE3) pLysS *E. coli* was performed according to section 3.1.10. The analysis was done with the soluble fraction since the primary goal was to produce the recombinant protein in its native conformation. Figure 6 shows the gel with bacterial proteins present in the supernatant after the bacterial cells had been disrupted with liquid nitrogen. In Figure 6B, one can see that the protein band is formed around the expected theoretical weight of IRS-7 of 42kDa (marked with the star) between 6 and 20h after the expression induction with IPTG when OD lays around 1.2, and the temperature is set to 37°C. Thus, the time point between 6 and 20h after induction was chosen to perform big-scale expression of IRS-7 described in the following section 4.5.

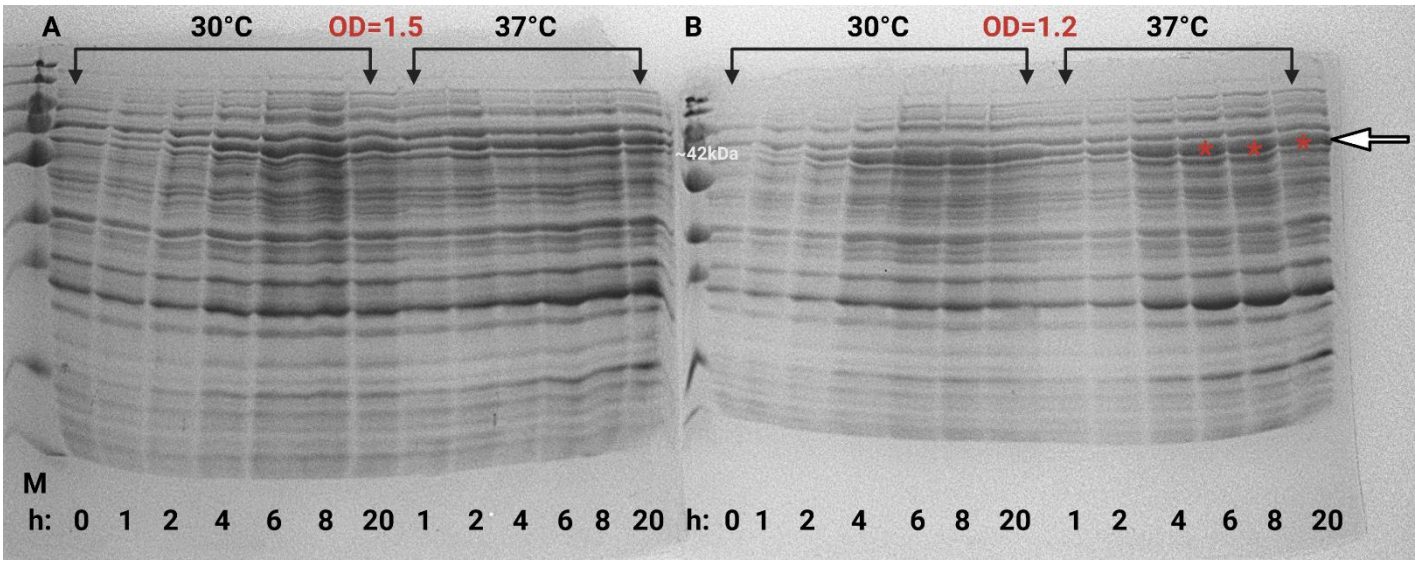

Figure 6: SDS-PAGE analysis of bacterial expression of IRS-7. *i) A – expression at OD=1.5 and temperatures 30- 37°C; ii) B- expression at OD=1.2 and temperatures 30-37°C; h – hours, corresponds to the time points after the IPTG addition; M – molecular marker.*

4.5 Large Scale Expression

Three liters of the medium were used to scale up bacterial protein expression. It took 4.5h for the bacterial cultures to grow up to an OD of 1.337 after which the IPTG inducer was added; the cells were then harvested 17 hours after induction. As described in section 3.1.14, resuspension buffer containing protease inhibitor tablet was added to the bacterial cells prior to cell lysis using French Press. Afterward, the cell lysate was ultracentrifuged and both pellet and supernatant samples were analyzed with SDS-PAGE. Since the SDS-PAGE analysis from this experiment showed only an undefinable smear, the data is not presented here. Nonetheless, supernatant samples were subjected to a series of purifications as described in the following section 4.6.

4.6 Purification

To first purify the serpin from the mixture of bacterial proteins and then from the cleaved His-SUMO tag, two subsequent runs of affinity chromatography were performed as described in sections 3.2.1 and 3.2.2. Fractions showing the UV absorbance at 280 nm were collected and analyzed with SDS-PAGE to confirm the presence of the purified protein. The fraction containing IRS-7 was then additionally purified with ion exchange chromatography according to section 3.2.3, and then run on the gel to confirm the presence of the protein.

4.6.1 Affinity Chromatography (AC)

During the first chromatographic run, IRS-7 was purified from bacterial cell lysate. After sample loading, the column was washed with 20mM Tris, 500mM NaCl, and 20mM Imidazole

(pH8) to remove bacterial proteins and any weakly-bound *E. coli* proteins. Any proteins which came out from the column before the wash step were collected and marked as flow-through (FT). The IRS-7 with His-SUMO tag was eluted with 20mM Tris, 500mM NaCl, and 500mM Imidazole (pH8). Figure 7 shows the chromatogram from the first purification, three fractions can be distinguished: A – containing FT with bacterial proteins; B – containing the proteins showing weak affinity to the column; C – elute with POI and His-SUMO tag. The presence of POI in fraction C was verified by SDS-PAGE shown in Figure 9. Then, the sample containing fraction C from the first chromatographic run was subjected to cleavage by Ulp SUMO protease and subsequently purified again by another run of affinity chromatography (Figure 8). In Figure 8, one can see the FT fraction D containing IRS-7 without the His-SUMO tag and fraction E containing uncleaved IRS-7 along with the His-SUMO tag itself. All the fractions mentioned above as well as the pellet left after the treatment with Ulp SUMO protease were analyzed by SDS-PAGE gel shown in Figure 9. The His-SUMO tag cleavage was confirmed by the gel since one can see a clear decrease in MW between fractions C and D. As a result, the presence of the pure POI in the FT fraction D was verified as it showed a single protein band with an expected molecular weight of ~42kDa (Fig. 9). This fraction was additionally purified by ion exchange chromatography according to section 3.2.3.

4.6.2 Ion Exchange Chromatography (IEC)

Ion exchange chromatography was performed to ensure that all the impurities are gone. Thus, IRS-7 was additionally purified using gradient elution with an increase in the ionic strength of the buffers (buffer A containing 20mM Tris(pH8) and B – 20mM Tris+500mM NaCl (pH8). The chromatogram in Figure 10 shows the results of purification where fraction G corresponds to the eluted pure POI whereas fraction F contains protein-free flowthrough. To verify the purity of IRS-7, fractions G, F, and H from IEC and additionally fraction D from AC (Sec. 4.6.1) run on the gel shown in Figure 11. Based on this gel, the presence of the pure IRS-7 in the fraction corresponding to the peak G was confirmed and found to be in the region around 42kDa.

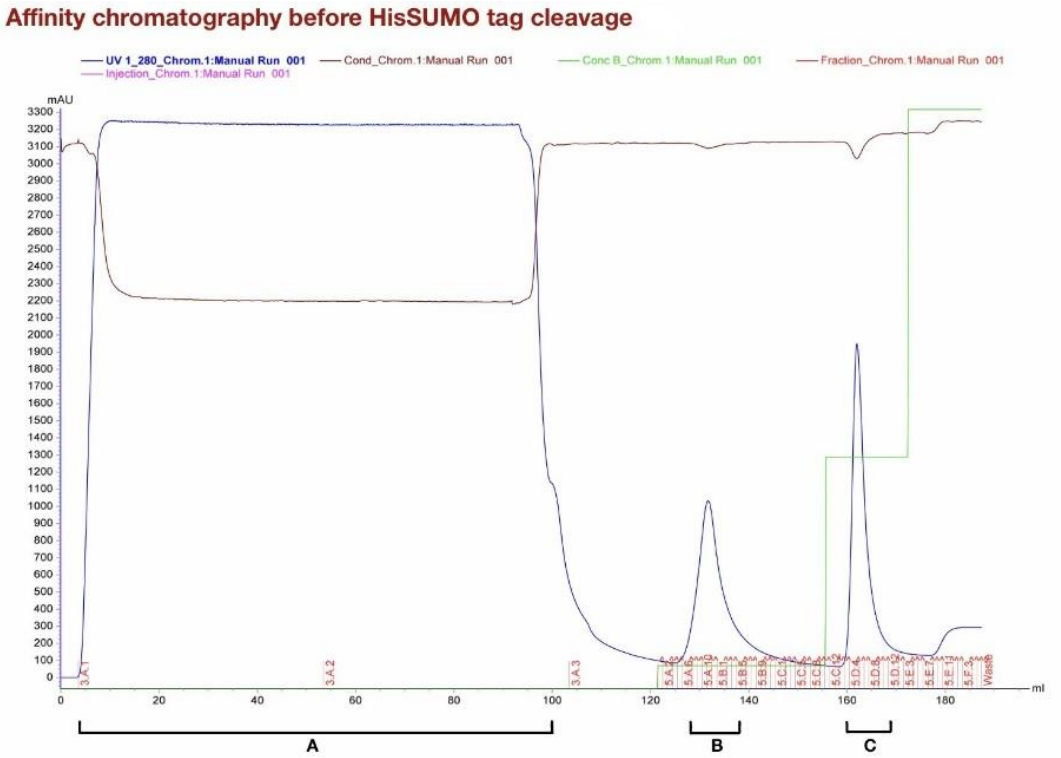

Figure 7: Affinity Chromatography of bacterial cell lysate. *A – contains flowthrough with bacterial proteins; B – shows proteins with weak affinity towards the column; C -represents elute with His-SUMO tag; blue line – UV absorbance in mAU; brown line – conductivity; green line – concentration of imidazole in buffer.*

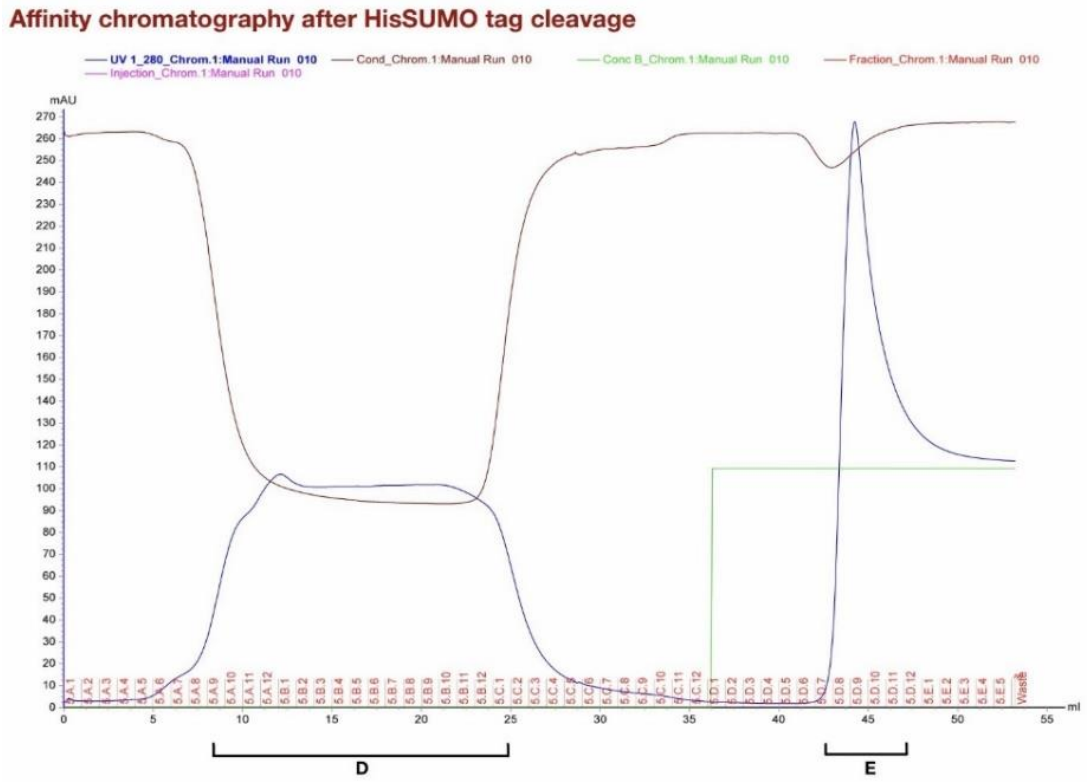

Figure 8: Affinity Chromatography after the His-SUMO tag cleavage. *D – shows FT containing the POI without His-SUMO tag; E – shows uncleaved IRS-7 with the His-SUMO tag itself; blue line – UV absorbance in mAU; brown line – conductivity; green line – imidazole buffer.*

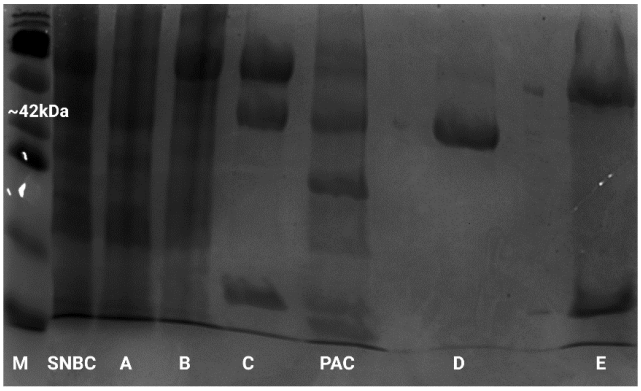

***Figure 9: SDS-PAGE after purification with affinity chromatography**; M – molecular marker; SNBC – shows supernatant before cleavage; PAC – pellet after cleavage; A, B, C, D, E – fractions of 2 subsequent AC's.*

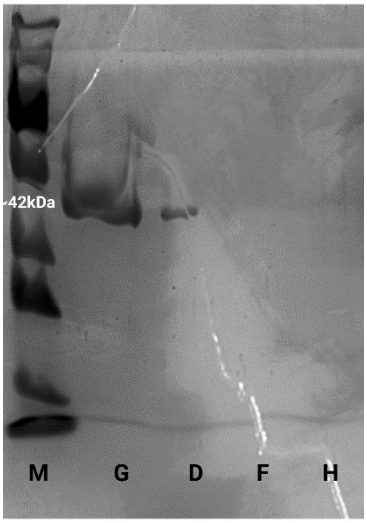

***Figure 11: SDS-PAGE after purification with IEC**; M – molecular weight marker; F, G, H – fractions of IEC; D – fraction of AC after His-SUMO tag cleavage.*

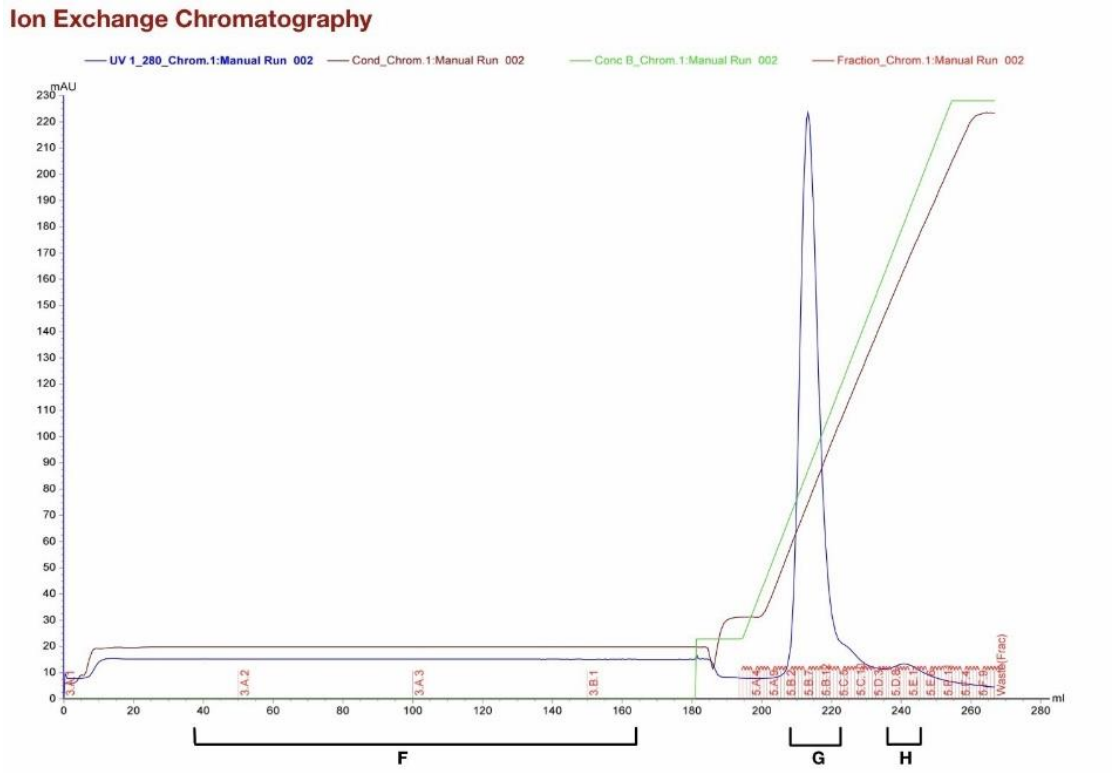

***Figure 10: Ion Exchange Chromatography**. G- shows purified POI; F – contains protein-free FT; blue line – UV absorbance in mAU; brown line – conductivity; green line – NaCl gradient.*

4.6 Estimation of protein concentration

Using the external calibration curve of the standards mentioned in section 3.3.1, the concentration of the IRS-7 was calculated and found to be 1,12mg/ml which corresponds to 29,76µM. Collected fractions from IEC (B4-C2 Fig.10) of purified POI were equal to 10 ml, therefore the amount of recombinant protein produced was 11,2 mg.

4.7 Coagulation assays

After having obtained a recombinant pure IRS-7, the next objective was to test this protein for its biological activity. Since tick's salivary proteins can be involved in hemostasis regulation, IRS-7 was tested for its ability to influence the clotting times of a plasma sample using various coagulation assays. PT is used to measure the clotting tendency of blood after thromboplastin (a mixture of tissue factor, $Ca^{2+,}$ and phospholipid) had been added and screens for the extrinsic and common pathways of coagulation; this test detects the abnormal functioning of factors II, V, VII and X. aPTT screens for deviations in functioning of factors VIII, IX, XI, X, II, and XII which are also the components of common pathways of coagulation [86]. TT test is done to measure the time it takes for a fibrin clot to form in plasma after the excess thrombin had been added; this test monitors the function of thrombin and fibrinogen [87]. Results of all three experiments, as can be visualized from Tables 4,5, and 6 (reported with the addition of standard error), fall into the normal physiological range being and are 12-15s for PT; 25-40s -aPTT; 17-25s – TT. Since the difference between the control and tested group was not significant, it was concluded that IRS-7 does not influence blood coagulation.

Table 4: Prothrombin Time Results.

Prothrombin Time	
Reagent	**Average Time/[s]**
IRS-7(6µM)	15.4 [±0.74]
Control	14.5 [±0.15]

Table 5: Activated Partial Thromboplastin Time Results.

Activated Partial Thromboplastin Time	
Reagent	**Average Time/[s]**
IRS-7(6µM)	31.8 [±0,15]
Control	34.2 [±0.25]

Table 6: Thrombin Time Results.

Thrombin Time	
Reagent	**Average Time/[s]**
IRS-7(6µM)	17.7 [±1,00]
Control	19.3 [± 0,40]

4.8 Formation of complexes with proteases

Since serpins are known to form covalent bonds with serine proteases, it was crucial to test the ability of IRS-7 to form covalent complexes with several serine proteases namely FXa, CatG, Chymotrypsin, Trypsin, and Chymase. The reactions with proteases were done *in vitro* and then the results were analyzed by SDS-PAGE and visualized with silver staining according to section 3.3.3.1 By looking at the gel in Figure 5, it is clearly seen that the reference pure IRS-7 band around 42kDa was found throughout all samples. Additionally, separate bands of lower molecular weights were identified corresponding to the pure proteases themselves. We did not detect any complex formation which would have been shown by a band at a molecular weight (MW) corresponding to MW of IRS-7+MW of protease. This result implies that IRS-7 did not form complexes with chosen proteases and therefore probably is not an inhibitor of these proteases. However, what can be observed is that in lanes corresponding to: CatG+, Chymotrypsin+, Trypsin+, Chymase+ IRS-7, a small decrease in molecular weight of serpin occurs (when compared to the 1st lane). The decrease in the molecular weight of the serpin and the presence of the separate bands corresponding to a particular protease (Fig. 12) might imply that these proteases cleave the IRS-7 in the RCL which results in the inactivation of the serpin and the release of the free protease [88].

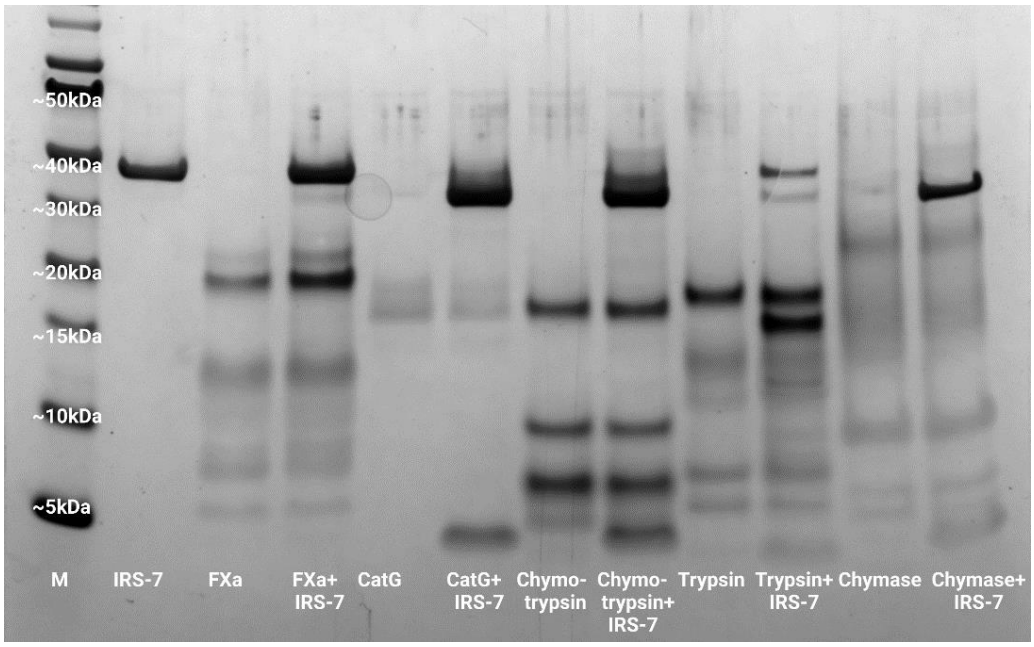

Figure 12: Testing of complex formation between IRS-7 and proteases. *Each lane corresponds to either the free protease (FXa, CatG, Chymotrypsin, Trypsin or Chymase) or protease-serpin mixture, along with pure IRS-7 for control. Since IRS-7 protein bands were found in MW region of ~42kDa in each well where IRS-7 was mixed with the corresponding protease, the conclusion could be drawn that IRS-7 did not form the complexes with chosen proteases. However, in lanes corresponding to CatG+, Chymotrypsin+, Trypsin+ and Chymase+IRS-7 a small decrease in MW of the serpins is apparent which implies the cleavage of the RCL of the serpin by protease. M – molecular weight marker.*

5. Discussion

As has been already introduced above, tick's saliva contains myriads of active substances that assist ticks in multiple intriguing ways such as: i) contribution to the tick's evasion of the host's defense mechanisms including immunity and hemostasis; ii) facilitation of the tick's feeding success; iii) or as a result of evolutionary co-existence of ticks with various pathogens – assistance in pathogen transmission. Serpins are among the active substances found in tick saliva, and the research on these proteins is particularly captivating because of the wide range of vital physiological functions that these molecules regulate. Therefore, knowledge of the functions of serpins contained within tick saliva might result in developing newer tick control methods and vaccines. Several serpins derived from the saliva of *I. ricinus* have been functionally and structurally described over the past decade [6][57]. Apart from two serpins Iris and IRS-2 derived from the saliva of *I. ricinus* which target elastase- and chymotrypsin-like proteases respectively, the functions of two new serpins namely Iripin-3 and Iripin-8 were partly decoded recently. Many serine proteases that are activated during hemostasis have been shown to favor arginine residue at the P1 site of the serpin for its proteolytic attack [93] [94]. Therefore, serpins Iripin-

3 and Iripin-8 appeared to be an interesting target of research since they both contain arginine in the scissile region of their RCLs. Indeed, the works of Chlastáková et al. [89] and Kotál et al. [90] have shown that serpins Iripin-3 and Iripin-8 both inhibited proteases involved in coagulation and immune response *in vitro*. It was found that Iripin-3 strongly inhibited kallikrein – protease involved in intrinsic coagulation pathway, fibrinolysis, and cleavage, and release of inflammatory mediator bradykinin [91]. Iripin-3 also inhibited type II transmembrane serine protease matriptase which is primarily responsible for the maintenance of the skin barrier function [92]. Likewise, Iripin-8 inhibited a wide range of proteases involved in the coagulation cascade (thrombin, FVIIa, FIXa, FXa, FXIa, plasmin, activated protein C (APC), kallikrein and trypsin) and was also found to inhibit intrinsic and common pathways of blood coagulation by prolonging clotting times in aPTT and TT coagulation assays. Since the experimental design for target proteases of serpins mentioned above was based on the amino acid sequence in the P1 position of the serpin, in our research we hypothesized that since IRS-7 has leucine in the P1 position, its main function could be that of inhibition of chymotrypsin-like proteases, similar to how P1 specificity is described in papers of M. S. Khan et al [48] and P. Zhao et all [51]. Despite our hypothesis, IRS-7 did not inhibit the chosen proteases and multiple reasons could have contributed to such an outcome. Firstly, it has been attested within the scientific community [97] that serpin's inhibitory activity is strongly dependent on being properly folded. The majority of serpins, with a notable exception of plasminogen-activator inhibitor 1(PAI-1) [96], spontaneously fold into a metastable highly-energetic active conformation in which the P1 residue of their RCL is solvent-exposed, and the difference in energy between the metastable conformation and potential low-energy serpin-protease complex is created. This difference in energy states implies that the serpin's active conformation is far from being the most stable one. On the contrary, a much more thermodynamically feasible conformation of serpin is the latent form in which the RCL inserts into β-sheet A as a fourth strand (s4A) accompanied by the extraction of strand 1 from β-sheet C. Another conformation where the serpin reaches its energetic minimum is termed cleaved and resembles the latent state only without the extraction of strand 1 from β-sheet C. Folding according to more energetically favorable pathways mentioned above, serpin acquires its energetic stability, sacrificing however its ability to inhibit the proteases because the loop with the recognition sequence is buried under the body of serpin and the energy difference no longer exists. The exact factors which influence the lingering of serpins in metastable conformation have not been elucidated yet. But some scientists argue [88,97] that mutations in the RCL of the serpins could at least explain their pre-mature transition to latency

or cleaved conformation and therefore might be the reason for serpin's dysfunction. For instance, such mutations could occur in the hinge region (residues P9-P15) – a highly conserved throughout the serpin family segment of RCL responsible for the loop insertion and the resulting metastable to stable (e.g., "stressed" to "relaxed") conformational change that is crucial for the inhibitory activity of serpin. Specifically, mutations in the P14 residue of the hinge region often render inhibitory serpins into substrates and allow the proteases to escape the serpin trap [52][95]. It is, therefore, possible that in our case such conformational features, the investigation of which would require more thorough research could have naturally occurred in the RCL of IRS-7. This might explain our results where hypothesized target proteases CatG, chymotrypsin, and chymase cleaved the serpin in its RCL and escaped the kinetic trap which resulted in the inactivated serpin and a free protease. Alternatively, it might also be that the features of the RCL of IRS-7 are such that its native conformation is in fact latent, resembling PAI-1. Therefore, our serpin could have naturally been in latent conformation, and only upon certain conditions (e.g., pH, salts concentrations, etc.) which are not known to us yet, could IRS-7 attain the active metastable fold. As a result, while existing in latent conformation, the serpin was rendered inactive and could not form complexes with target proteases. Yet another reason for the lack of inhibitory activity of IRS-7, could lay in the tendency of serpins to form heterodimers where the RCL of one serpin molecule inserts into β-sheet A of another thus surpassing the metastable form and becoming thermodynamically stable yet at the same time losing its functionality. It is worth mentioning that both latent and cleaved forms of serpin strongly resemble each other and therefore cannot be effectively distinguished only by using the reducing SDS-PAGE gel. Following the same line of thought, this technique also cannot detect serpin dimerization because of the loss of tertiary structure during denaturation. Finally, since the amino acid at the P1 position of the serpin was not recognized by any of the proteases tested and since serpin probably attained one of the three conformations described above, it also did not show a statistically significant delay in the coagulation assays (aPTT, TT, PT). Due to time constraints, we were not able to optimize our methods and further test IRS-7 for its function and the precise role of this serpin in the tick-host interaction or within the tick's body.

As a future perspective, it would seem reasonable to change some methodological steps in order to optimize the proper folding of IRS-7. As an example, before subjecting IRS-7 to the series of experiments, it might have been useful to perform the circular dichroism (CD) spectroscopy to screen for the secondary structure of the POI and be able to compare the resulting spectra to other known proteins similarly to how it was done in work of Kotál et al. and others [90, 99,

100]. Having the CD spectra, we could have better troubleshot the effects of temperature, mutations, or denaturants on conformational changes of the protein [98]. Continuing, to express IRS-7, we used BL21(DE3) *E. coli* strain optimized for the recombinant protein production transformed with pET_SUMO expression vector. The yield of 11,2mg of POI per three liters of the medium is relatively good yet our ambitions for the expression were set higher. Since we did not investigate the insoluble fraction after big-scale expression, it is very probable that some of the recombinant IRS-7 was trapped in inclusion bodies (IB) contributing to the lower amount of the produced protein. The SUMO fusion protein was used along the pET_SUMO vector and since it was described [66,70] to increase protein solubility and proper folding and to additionally guard the protein from protease degradation, we assumed that by using this expression system we would manage to produce correctly folded protein in the soluble fraction avoiding the need to refold IRS-7 from IB. In the light of the obtained results, alternatively, we could have tried using another expression vector namely pET_17b similarly to how J. Kotál did it in his master thesis [101], and obtain most of the POI aggregated in IB. To refold the protein from IB to its proper metastable conformation would therefore require further methodology optimization and additional experiments.

Concluding, given that IRS-7 most probably was in an inactive conformation, we cannot exclude the possibility that by attaining the proper native fold, IRS-7 could have inhibited the target proteases and showed statistically significant results in coagulation or immunological assays. Therefore, further *in vitro* and *in vivo* experiments and mechanistic studies are needed to elucidate the functions of IRS-7.

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