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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Biochemical approach to study the spider fang

Ana Cristina Julião Licuco

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Dissertation submitted to the University of Coimbra for compliance with the requirements to get the degree of Master in Biochemistry, conducted under the scientific supervision of Dr. Yael Politi (Max Planck Institute – Colloids and Interfaces) and Prof. Dr. Paula Veríssimo (University of Coimbra)

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Yael Politi (Max Planck Institute – Colloids and Interfaces) e da Professora Doutora Paula Veríssimo (Universidade de Coimbra)

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ABBREVIATIONS

abd – abdomen

APS – ammonium persulfate

BSA – bovine serum albumin

CE – Crude extract

CS – Chitin synthase

DDW – double distilled water

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

e.g. – (L. *exempli gratia*) for example

et al. – (L. *et alia*) and other

fan – fangs

G – guanidine hydrochloride buffer

i.e. – (L. *id est*) that is

IMAC – immobilized metal ion affinity chromatography

kDa – kilo Dalton

o.n. – overnight

r.t. – room temperature

RP-HPLC – Reverse-phase high-performance liquid chromatography

rpm – rotations per minute

tib – tibia

U – urea buffer

UDP-GlcNAc – UDP-*N*-acetylglucosamine

SUMMARY

The environmental context where some animal are found may often explain some of their characteristics. This is not different for arthropods whose body is covered by an exoskeleton, the cuticle, a structure that, among other functions, confers protection, shape and defense against parasite invasion. This is possible thanks to the organization of the exoskeleton in different layers with very specific characteristics.

The cuticle of arthropods, composed of chitin and protein, exhibits notable variations in both organization and local microstructure. This situation occurs for all body anatomical structures containing cuticle and with different patterns of microstructural and chemical gradients between them.

This work aims to give information about the organization of the cuticle of arthropods, using the spider *Cupiennius salei* as a model, and focusing the attention on the protein content. The project was developed assuming the existence of so far unidentified metal-dependent proteins, where the metal coordination is possible due to the presence of histidines, situation that also contributes to the mechanical properties of the cuticle. The project comprises the establishment of a principle for extraction, purification based on zinc affinity and characterization of proteins from cuticles of abdomen, tibia and fangs of *Cupiennius salei*. The optimization of the protein extraction and purification will allow the characterization of these proteins and the possible establishment of homology with proteins, of other organisms, with similar amino acid compositions and sequences and/or common functions.

Key words: arthropods, *Cupiennius salei*, cuticle, proteins, histidine, zinc affinity

RESUMO

O contexto ambiental em que alguns animais são encontrados pode muitas vezes explicar algumas das suas características. Tal não é diferente para os artrópodes cujo corpo é coberto por um exosqueleto, a cutícula, uma estrutura que, para além de outras funções, confere protecção, forma e defesa contra invasão de parasitas. isto é possível graças à organização do exosqueleto em diferentes camadas com características bastante específicas.

A cutícula dos artrópodes, composta de quitina e proteínas, exhibe oscilações notáveis quer a nível de organização quer a nível de micro-estrutura local. Esta situação ocorre em todas as estruturas anatómicas do corpo que contêm cutícula e com diferentes padrões micro-estruturais e gradientes químicos entre elas.

Este trabalho tem por objectivo facultar mais informação acerca da cutícula de artrópodes, usando como modelo a aranha Cupiennius salei, e focando atenções no conteúdo proteico. O projecto foi desenvolvido assumindo a existência de proteínas metalo-dependentes, até agora não identificadas, onde a coordenação metálica é possível devido à existência de histidinas, situação que contribui igualmente para as propriedades mecânicas da cutícula. O projecto compreende o estabelecimento de uma metodologia para a extracção, purificação tendo por base a afinidade por zinco e caracterização de proteínas da cutícula de abdomen, tibia e presas de Cupiennius salei. A optimização do processo de extracção e purificação permitirão a caracterização dessas proteínas e o possível estabelecimento de homologias com proteínas, existentes noutros organismos, com composições de aminoácidos e sequências semelhantes e/ou funções comuns.

Palavras-passe: *artrópodes, Cupiennius salei, cutícula, proteínas, histidina, afinidade por zinco*

Chapter 1

General Introduction

1. GENERAL INTRODUCTION

1.1. Arthropods and its exoskeleton

1.1.1. Chitin and cuticle: formation and composition

The study of arthropods can be done from several points of view and according to different objectives.

Before starting an extensive discussion on the structure and composition of the arthropod exoskeleton, two concepts must be clarified, i.e. the concept of chitin and of cuticle.

Chitin (Figure 1.1) is a large homopolymer of β -(1-4) linked N-acetyl-D-glucosamine. One of its

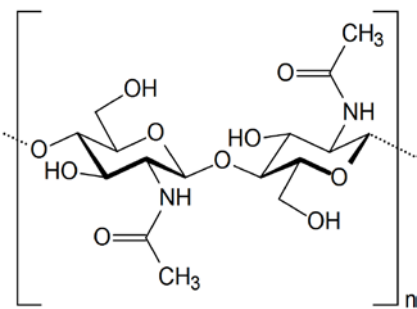


Fig. 111. Structure of the chitin molecule, showing two of the N-acetylglucosamine units that repeat to form long chains in β -1,4 linkage.

common forms is the α -chitin, (typically arranged into crystalline fibrils of approximately 3 nm wide and 300 nm long (Neville *et al.*, 1976, Vincent and Wegst, 2004).

The polymerization is catalyzed by chitin synthase, a membrane-bound glycosyltransferase (Cohen, 2001) abundantly produced by invertebrates. In arthropods, its formation and deposition is a highly complex of biochemical and biophysical events which starts intracellularly and ends extracellularly (Merzendorfer and Zimoch, 2003). Outside the cells, the chitin organizes into supra-macromolecular structures, which then form the cuticle and the semi-permeable membranes, with non-cellular structure, of the animal.

The chitin formation and regulation process is not entirely known yet but the general cascade of events is summarized by:

- 1) Synthesis of the monomers: Sequential biotransformation of sugars such as trehalose or glucose (Figure 1.2) that comprises phosphorylation, amination and formation of the substrate UDP-N-acetylglucosamine (UDP-GlcNAc), which take place in the cytoplasm;
- 2) Chitin synthase assembly: Synthesis and assembly of chitin synthase (CS) units,

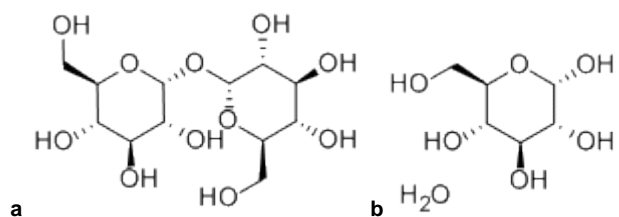


Fig. 1.2. Scheme representing the structure of (a) trehalose and (b) glucose.

followed by their translocation, proper insertion into plasma membranes and activation. A CS unit is one enzyme molecule which is part of a cluster of closely topologically packed molecules. This arrangement eventually ensures coalescence of nascent chitin polymers into a crystalline fibril;

- 3) Polymerization and assembly: The catalytic step is performed by CS, followed by orientation of the long-chain chitin molecules and translocation of polymers across the plasma membrane to the extracellular space, where they crystallize to form microfibril by inter-chain hydrogen bonding;
- 4) Association with cuticular proteins and fibers formation.

The cuticle is a non-cellular material secreted by the epidermis. It constitutes the exoskeleton, including the tracheal ducts, i.e. the internal respiratory tubes of arthropods. This structure gives to arthropods support, shape, means of locomotion, water-proofing and diffusion control. The cuticle material shows a range of localized mechanical specializations such as high or low compliance, wear resistance, etc.. The cuticle also provides place for muscle attachment, function as a temporary food store and it is a major barrier to parasitism and disease (Vincent and Wegst, 2004). The components of the cuticle can be varied in orientation and volume fraction resulting in a fibrous structure with highly anisotropic properties. The wide range of mechanical properties is governed by different parameters: chitin nanofibres volume fraction and orientation, type of protein, water content and degree of cross-linking of the protein, lipid, metal (Zn, Mn, Fe) ions, and calcium carbonate. Therefore, and as stated also by Vincent and Wegst (Vincent and Wegst, 2004), it can be concluded that the cuticle is a multifunctional material.

The cuticle is subdivided into epicuticle and procuticle (Santhanam, 1955). The epicuticle consists of a thin outer waxy coat, that moisture-proofs the other layers, and of an inner lipo-protein layer. The procuticle consists of two chitin-containing layers, the exocuticle and the endocuticle, which are chemically hardened and unhardened proteins, respectively. The chitin fibrils are coated by a tightly bound protein layer and embedded in a protein matrix (Neville *et al.*, 1976; Politi *et al.*, 2012) composed of several proteins, many of which contain post-translational modifications (Willis, 1999). Many of these proteins contain conserved sequences responsible for forming specific chitin-protein interactions (Neville *et al.*, 1976; Hamodrakas *et al.*, 2002).

Each body segment and limb section of the animal is encased in hardened cuticle. The joints between body segments and between limb sections are covered by flexible cuticle. Cuticular lipids,

the proportion of chitin, the nature and quantity of sclerotizing agents, and the sequence of the constituent proteins, determine the physical properties of the cuticle (Willis, 1999). Depending on the functional regions considered, there is a variation in the chitin to protein ratio, as well as a difference on the amount and type of cross-linking (Andersen et al., 1996; Andersen *et al.*, 1995; Andersen, 2010) and the content of adsorbed water.

1.1.2. Proteins involved in the cuticle, their characteristics and importance

As already mention, the chitin occurs together with large quantities of protein, in a ratio that differs between animal, structures and also depending on the cuticle hardness (Fraenkel and Rudall, 1947). In arthropod cuticle, the protein component is usually subdivided into proteins that are covalently bound and proteins that bind non-covalently to chitin or another component. Non-covalently bound proteins are those extracted by various solvents and the differences in solubility presented by them reflects differences in association with other cuticular components.

To address the site of synthesis of cuticular proteins, different methods have been applied. A detailed explanation is provided by (Willis *et al.*, 2005). Briefly the methods are summarized below.

- Northern blot analysis: gives information on where (what tissue) and in which stage mRNA is present for a particular cuticular protein;
- Radioactive labeling: incubation of epidermis or integument *in vitro* with radioactive amino acids, separate the proteins and compare the eletrophoretic mobility of the labeled proteins to proteins isolated from clean cuticle;
- In vitro protein translation: isolation of mRNAs from tissues followed by translation *in vitro* with commercially available wheat germ extracts or rabbit reticulocytes and compare the translation products to known cuticular proteins;
- *In situ* hybridization: localization of specific DNA or RNA sequence in a section of tissue, using a probe which is a labeled complementary DNA or RNA strand;
- Immunolocalization: visualizing labeled proteins within the endoplasmatic reticulum and Golgi apparatus, by immune-labeling.

From these kinds of researches it is concluded that the cuticular proteins with known sequences or for which specific probes are available, are synthesized by the integumental sections analyzed. Not less important is to note that different proteins are synthesized at different times

during a molt cycle and in distinct anatomical regions. There are also some cuticular proteins which production is dependent and specific for the life stage of the organisms. The techniques mentioned above also revealed that cuticular proteins synthesis is temporal and spatially controlled at the level of transcription.

After investigation suggesting that most exoskeletal proteins seem to resemble each other (Hackman and Goldberg, 1976), the research on cuticular proteins had a decreased interest and these proteins were, for a long time, neglected (Willis, 1987). Nevertheless, nowadays a fairly completed protein database for insects' cuticle is found, but the same is not true for crustacean and arachnid cuticle.

Recently, it has been shown that exoskeletal proteins are structural components of the chitin-protein microfibers and are involved in the interactions between microfibers, the interfibrillar matrix and also the mineral in calcified exoskeletons (Andersen, 1999; Willis, 1999; Andersen, 2000).

The proteins are often considered the most important component for determining the mechanical properties of a cuticle (Hojrup *et al.*, 1986a; Willis, 1987), especially as both chitin and sclerotizing agents interact with them.

Among the proteins found in the cuticle it is possible to identify the following groups:

- Non-structural proteins such as pigments (e.g. insecticyanines, two different yellow proteins and putatively β -carotene binding), enzymes (e.g. the ones involved in sclerotization, in molting and in digesting the old cuticle), defense proteins (e.g. antibacterial peptides, prophenoloxidase, zymogen form of a serine proteases and also proteins related to the defense protein scolexin) and arylphorins (proteins with high content of aromatic amino acids and some lipid);
- Structural proteins which have as feature either a low or inexistent level of cysteine and methionine residues, as most of structural cuticular proteins, and the occurrence of post-translational modifications (which is an extremely important condition to the properties of the exoskeleton's matrix).

Some examples of structural proteins comprise the (a) resilins, which are characterized by high concentration of glycine and proline and involve cross-linking of di- and tri-tyrosine residues, and (b) proteins containing motifs such as:

- R&R Consensus which original motif is a part of a longer conserved sequence (the pfam00379 region) and has three distinct forms: RR-1: proteins associated with soft

cuticles; RR-2: proteins associated with hard cuticles, and RR-3: proteins derived from post-ecdysial cuticle, soft or hard (Andersen, 2000; Willis *et al.*, 2005);

- Repeats of A-A-P-(A/V) that it is thought that may or may not be associated with R&R Consensus;
- Stretches of glycine, leucine and tyrosine (beginning G-Y-G-L or G-L-L-G).

The specific distribution of cuticular proteins suggests that the properties of individual protein contribute to the physical properties of the cuticle, i.e. its rigidity or flexibility (Shawky and Vincent, 1978; Andersen *et al.*, 1995). The flexibility of the cuticle can be related essentially to the features of the cuticular proteins present. Once considered the classification of the cuticle as flexible (or soft) and rigid (or stiff), some conclusions regarding the characteristics of the proteins in each one of the ends can be established namely by the occurrence of amino acid residues such as glycine, alanine, tyrosine and histidines. One of the main examples is the higher glycine content and the relative lower alanine content in soft cuticle, in comparison to in stiff cuticles. Regarding tyrosine and histidine, soft cuticles are rich in the first and extremely poor in the second, while the inverse occurs in the hard cuticles (Bordereau and Andersen, 1978; Willis, 1987).

Most of what is known about exoskeleton composition and cuticular proteins in arthropods come from studies performed during many years in insects and crustaceans (Fraenkel and Rudall, 1947). Until 1982 there was no cuticular protein sequence reported in literature. This situation changed when partial sequences of cuticular protein from *Drosophila* started being published at this year and after. Partial sequences from cuticular proteins of *Sarcophaga* and *Locusta* followed later as result of studies developed by different research groups (Henzel *et al.*, 1985; Hojrup *et al.*, 1986a; Hojrup *et al.*, 1986b). Scorpions, lobsters, crabs and others comprise some of the other models that have been extensively studied. Although this list is already extensive, it is not enough for a full understanding of the cuticle (Willis *et al.*, 2005).

1.2. *Cupiennius Salei*: Species among a phylum – features and behaviors

1.2.1. Description of the model used and comparison with species of the same genus

The origin of arthropods may have happened during Cambrian – the first geological period of the Paleozoic Era –, idea that comes from how the arthropod-like trace fossils from this Era resemble to centipede (Budd and Telford, 2009).

Following the traditional classification, the phylum Arthropoda is described as including myriapods (millipedes and centipedes), chelicerates (spiders, scorpions, mites and ticks), hexapods (insects) and crustaceans (crabs, shrimps, barnacles and woodlice) – collectively known as the Euarthropoda (Budd and Telford, 2009) -, and other genus. Therefore, this phylum comprises a large number of species and individuals, also corresponding to the most numerous phylum of all living organisms, both in number of individual and species (Minelli *et al.*, 2013; Giribet and Edgecombe, 2013), and in which the sizes range between microscopic until some centimeters. One of the important characteristics of this phylum is the very well adaptation of its individuals to dry environments (Cloudsley-Thompson, 1975), mainly thanks to the evolution of respiratory and excretory systems over the years. Their internal anatomy, as the external, is built of repeated segments, throw hemolymph flows in an open circulatory system.

The need of molting for arthropods is related with the fact that the exoskeleton doesn't grow with them. Thus, the epidermal cells of immature insects become detached from their cuticle, grow by cell division or cell enlargement (or both) and secrete a new and extensible cuticle with folds – a process that occurs periodically (Schneiderman and Gilbert, 1964).

The genus *Cupiennius*, was described in 1891 by Eugène Simon, a French arachnologist. Although it is still under debate, to which family it belongs. Nowadays, with improvements in technology and with the wider variety of tools and techniques available, a large number of *Cupiennius* have been studied and characterized. These studies allowed the identification of nine species of *Cupiennius* genus: *C. getazi*, *C. coccineus* and *C. salei* (the large-bodied species), *C. granadensis*, *C. foliatus*, *C. cubae* and *C. panamensis* (the smaller species), *C. remedius* (recently discovered – 1992 – and named by (Barth and Cordes, 1998)) and *C. celerrimus*¹.

There is a set of features that easily identify *Cupiennius* genus: the size and relative position of their eight eyes, the arrangement of spines on the legs and the third claw on the tarsus together

¹ Although *C. celerrimus* had been described by Simon in 1891, as for a long period of time it was not seen again, arachnologists decided to eliminate it from the genus, but since 1995, when it was seen again and described, it was, once again, included on the list

with claw tufts in which the hairs point apically to diagonally downward, but never diagonally upward as in other genera (Figure 1.3).

1.2.2. The *Cupiennius salei*

As suggested by Barth (Barth, 2002), *Cupiennius salei* can be described as a “sit and wait hunter that patiently waits for its victim in its hideout”. This description is due to an observation of the behavior of this spider that only reacts (and acts) when it is close enough from the prey, throwing itself and grabbing it with fast and precise, from the first reaction until the prey is bitten.

The safety of *C. salei* during the hunting process is because of a dragline, produced by it, that attaches it to its waiting site.

The spider touches the prey using the tip of its first pair legs and, after the bitten, it only starts eating the prey when it has stopped moving.

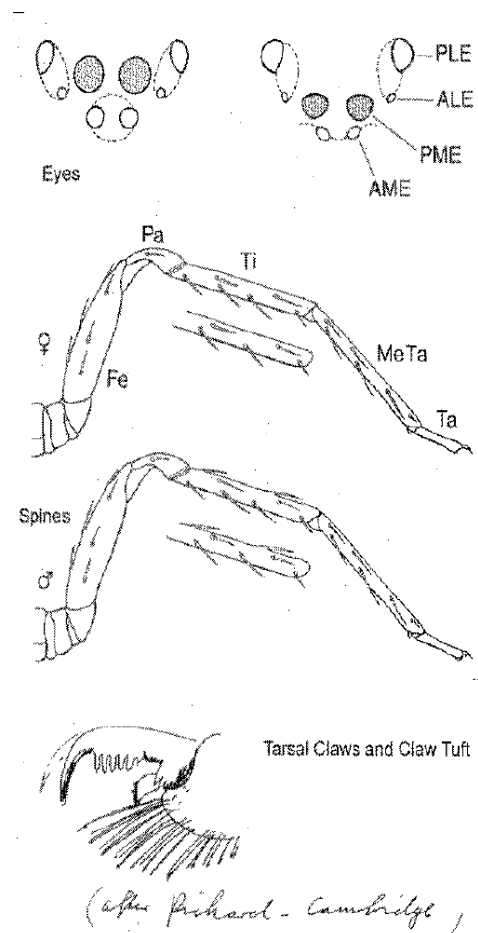


Fig. 1.3. Characteristics of the genus *Cupiennius*. **Top** The position of the eyes in frontal (left) and dorsal view (right). PME Posterior median eyes; PLE posterior lateral eyes; AME anterior median (principal) eyes; ALE anterior lateral eyes. **Middle** Bristles on the walking leg; female: legs I and II, tibia of legs III and IV; male: legs I and II, tibia legs III and IV. Fe Femur; Pa patella; Ti tibia; MeTa metatarsus; Ta tarsus. **Bottom** Third claw and claw tufts (Drawing M. Melchers). Adaptation from (Barth, 2002)

1.3. Abdomen, tibia and fangs in *C. salei*: structure, function and chemical composition

Like in all arthropods, the exoskeleton of *C. salei* derives its macroscopic properties from several factors. The protein/fiber volume fraction, the fibers orientation, the cross-linking, the presence of metals as well as the hydration level of the material.

The organization of the layers-containing chitin and matrix proteins is due to fibrils arranged within curves and in-between two laminae. The planes of fibrils overlapped show a rotation of 180°

from one lamina to other, which is the result of the small rotation angle of each successive layer (Bouligan, 1972).

These aspects are undoubtedly significant for the structure and characteristics of the exoskeleton, however, in this set of properties the matrix-protein content is many times undervalued with a tendency to generalizations that are not always correct.

1.3.1. The abdomen in *C. salei*. Comparison with analogue structures in other models

The abdomen (Figure 1.4), also called opisthosoma, is the hindmost section of the spider's body and includes the heart, reproductive and respiratory organs, digestive tract, and silk spinning glands. Unlike the others segments of the spider's body, this one has a soft and expandable exoskeleton.

So far there are no studies reporting the abdomen's composition, of *C. salei*, in terms of cuticular proteins. Micro- and ultrastructural analysis, directed to the localization and identification of cuticular proteins, have not been applied to this anatomical structure as well, so the information available is limited.

Some studies on abdomen cuticle of arthropods have been done along the last years, intended to understand the chitin structure and the composition in soft cuticles (Fraenkel and Rudall, 1947; Neville *et al.*, 1976; Iconomidou *et al.*, 2001). In relation to cuticular proteins content, some information is available regarding studies in insects (Andersen *et al.*, 1995; Willis, 1999). From these studies it was established that the abdomen is an anatomical structure in arthropods where cuticle has soft characteristics such as a higher level of chitin and low level of proteins, which are usually interacting by weak bonds and with little occurrence of cross-linking.

1.3.2. The tibia in *C. salei*. Comparison with analogue structures in other models

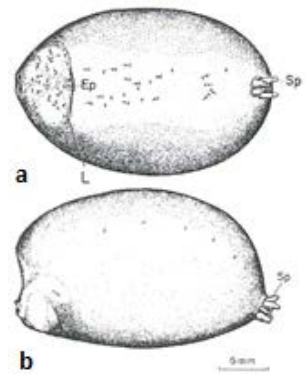


Fig. 1.4. Scheme showing the anterior (a) and lateral (b) sides of the abdomen of *C. salei*. Ep epigynum; L lung slit; Sp spinnerets. Adaptation from (Barth, 2002)

Hunting spiders are well adapted to fast locomotion. For these, factors like the number of legs, their localization and anatomical disposition have an important contribution. As in all spiders, *C. salei* has eight legs. From studies performed either on this species or others from the same genus, it is known that the spiders' hemolymph powered "hydraulic" system allows them to move and walk (Kropf, 2013).

Each leg is filled with nerves that respond to stimuli from outside, recognized by chemical and mechanical sensors.

Every leg has seven segments which, beginning closest to the body, comprise (Figure 1.5): coxa and then trochanter (both are short), then a long femur and a knee-like patella, then a slender tibia and metatarsus, and last, at the very tip, is the tarsus which usually has two or three claws.

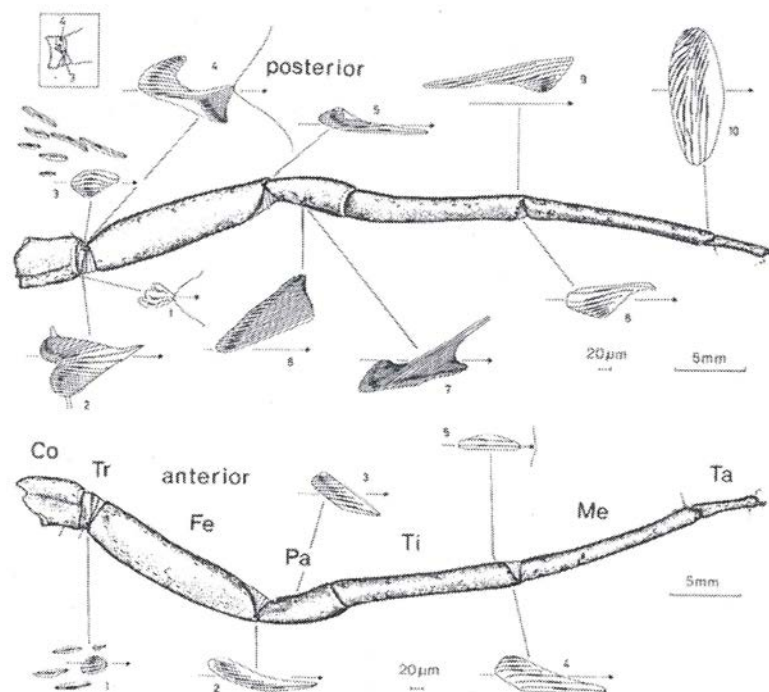


Fig. 1.5. Scheme showing the segments of the spiders' leg. *Co* coxa; *Tr* trochanter; *Fe* femur; *Pa* patella; *Ti* tibia; *Me* metatarsus; *Ta* tarsus. Adaptation from (Barth, 2002)

The tibia, the 5th segment of the leg is mainly a target of studies related with tensile forces and vibration mechanisms during movement. As can be expected, the layer arrangement of the cuticle follows the traditional order (from outer to the inner layer): epicuticle, exocuticle, mesocuticle and endocuticle (Figure 1.6). It is known that the exoskeleton of this part of the body is essentially hard due to a packing of the chitin fibrils and the reason for this stiffness is attributed to the presence of a high content of protein, in relation to chitin, mainly arranged and cross-linked

(protein-protein and protein-chitin) (Iconomidou *et al.*, 2005) and perhaps maintaining covalent interactions between them and chitin.

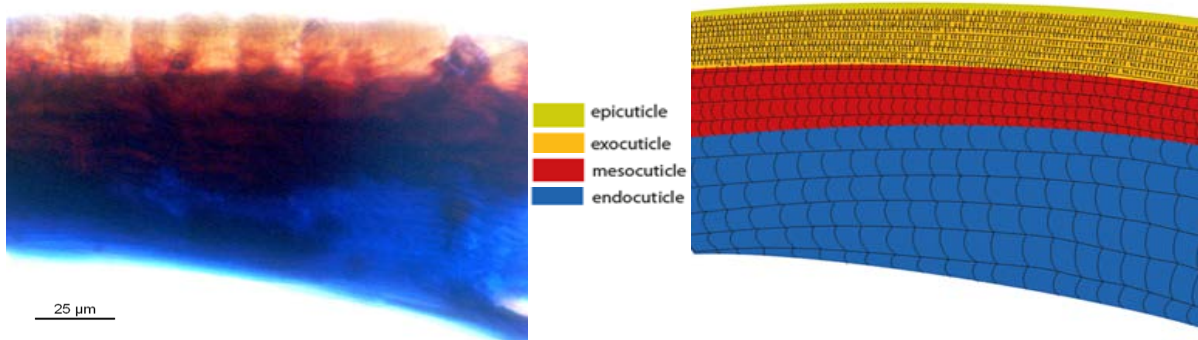


Fig. 1.6. Mallory staining of the leg and scheme representing the cuticle layers of this anatomical structure of *C. salei*. (Figure provided by Dr. Clara Valverde)

1.3.3. The fang in *C. salei*

1.3.3.1. The relevance of the fangs and of their study

Cupiennius salei, as a hunter animal, has its fangs as the primary structure getting in contact with the internal structure of the prey. The prey, perhaps an insect, and the fang cuticle are made of a similar chitin-protein composite. The fang is located on the spider's chelicerae and might be comparable with a small injection needles (~1.5-3 mm long) with a single opening of the venom canal on the dorsal side (Figure 1.7) and that *C. salei* uses to inject the paralyzing venom into the prey.

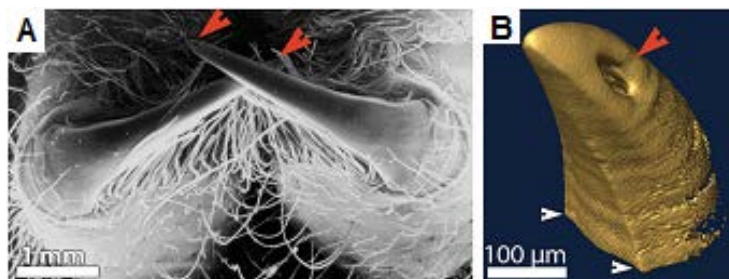


Fig. 1.7. A) SEM image showing the spider's chelicerae with the fangs. Orange arrows point to the opening of the venom canal. B) The tip of the fang as reconstructed by μ -computer tomography. Orange arrow points to opening of venom canal. White arrow-heads mark the two reinforcement ridges that run down up to one half of the fang's length. (Politi *et al.*, 2012)

1.3.3.2. Cuticular arrangement and nature of different substances over the fang

As the structure made of cuticle with a typical layers arrangement, it is possible to distinguish in fangs the epi-, exo-, meso- and endocuticles (Figure 1.8A). These layers exhibit gradients in both morphology and thickness that vary from the fang tip to its base. The edge of the fang tip has a thickness about 10 μm and is made only of epicuticular material, composed of globules 10-50 nm in diameter. Comparably, the epicuticle thickness in other parts of the fang, is thinner (<10 μm) (Figure 1.8 B and C). Focusing on the surface or the fang tip, it is possible to see small pores that are continuous with the rich pore-canal system that runs parallel to the long axis of the fang. However, it is known that this canal system is structurally unrelated to the venom canal (Politi, unpublished data), and the individual pore-canals bend towards the surface only proximal to the opening of the venom canal.

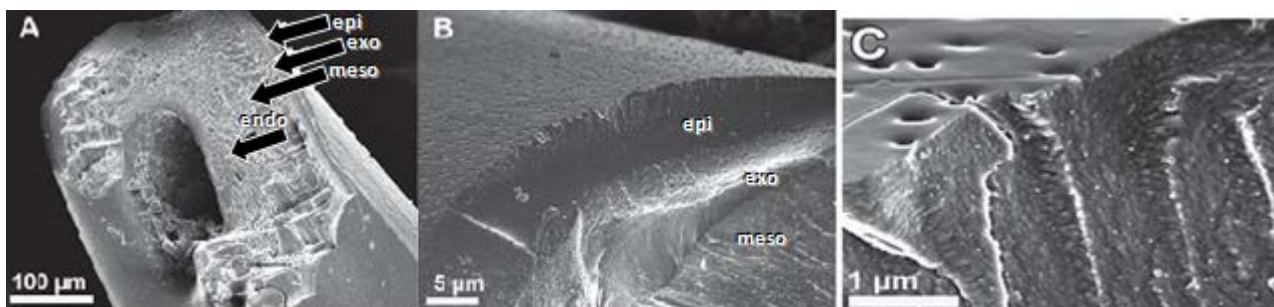


Figure 1.8. SEM images of a fracture surface of the fang. A) A fracture through the venom canal opening and B) on the dorsal side, to the right of the canal opening. Note many small pore-canals in (B). The epi-, exo- and mesocuticle can clearly be seen. C) High magnification image of the epicuticle, showing the texture of the protein globules. The surface shows multiple small openings of the pore canals. (adaptation from Politi *et al.*, 2012)

The exocuticle in the fang is structural as plywood with thin lamellae about 300 nm width. The fibers are coated by a thick globular protein matrix. The mesocuticle is organized by dense and oriented fiber columns that run parallel to the fang long axis. The exocuticle encases the entire fang below the epicuticle, and extends all the way down to the base of the fang, while the mesocuticle with columnar structure is found more centrally (Politi *et al.*, 2012).

Inwards and adjacent to the mesocuticle, the endocuticle occurs as a plywood structure composed of lamellae 1-1.3 μm thick.

The hardness and stiffness of the cuticle of the fang decrease steeply when evaluated from its tip towards its base and this change is related with decreased amount of chitin and an increase cross-linking of the protein matrix close to the very tip.

According to one study performed by Politi and her co-workers (Politi *et al.*, 2012), various transition metals and among them, zinc are present at the fang tip, with a highest concentration at the distal part of the tip, together with small amounts of iron (Fe) and copper (Cu) (Figures 1.9 and 1.10). The presence of the metal ions was suspected to be a main factor in hardening of the fang cuticle.

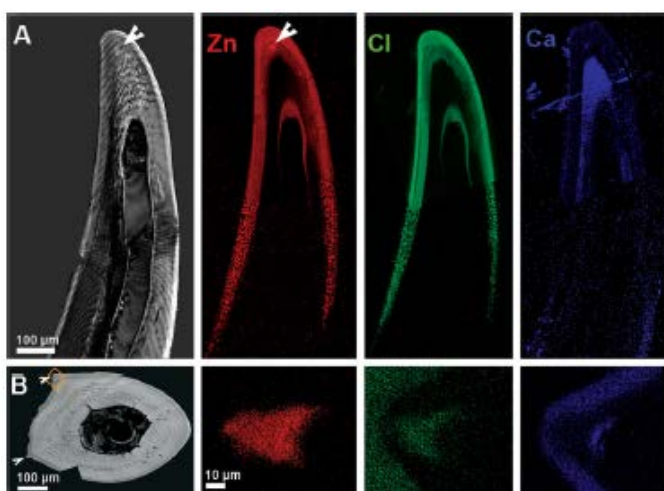


Fig. 1.9. **A)** Scanning acoustic microscopy (SAM) image of a longitudinal section of the fang and the Zn (red), Cl (green), and Ca (Blue) distribution maps from energy dispersive X-ray spectroscopy (EDX) measurements. The different grey levels in the SAM images correspond to acoustic reflectivity. The arrowhead points to a spot ca. 40µm wide of higher reflectivity, where also a higher concentration of Zn is observed without concomitant increase in Cl. In the EDX maps, long acquisition maps of the tip region are superimposed on short acquisition maps of the lower half of the fang. The striped pattern in the SAM map results from surface waves of the sample occurring during the measurement. **B)** SAM map of a cross section of the fang in the mid-range of its length. White arrowheads point to the two reinforcement ridges. Zn (red), Cl (green) and Ca (blue) EDX maps of a highly magnified picture of the upper ridge in the SAM image are shown. The epicuticle at this height of the fang is rich in Ca, while the reinforcement ridges are rich in Zn and Cl. (from Politi *et al.*, 2012))

Amino Acid Analysis

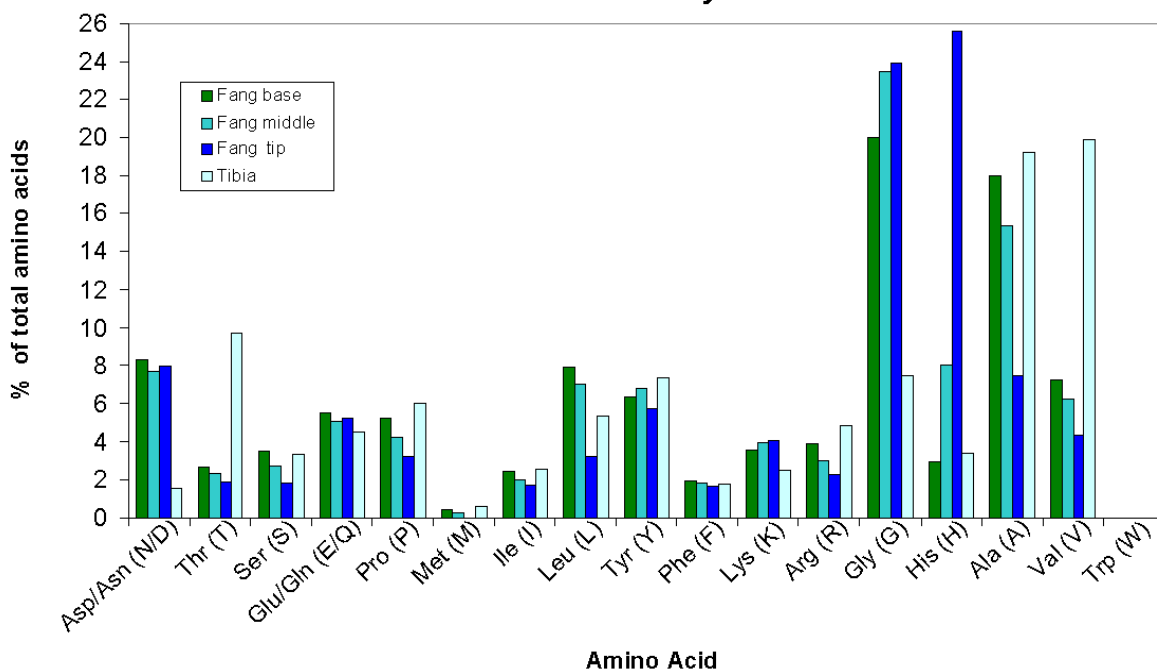


Fig. 1.10. Results of the amino-acid analysis. Amino-acids are labeled by their conventional 3 and one letter abbreviation. Asp and Asn represent one column together since they are not distinguished in the analysis due to the oxidation of Asn during acid treatment. Major differences are observed in the concentrations of Asx, Gly, Ala, Val and His. (from supplement of Politi *et al.* 2012)

Another parameter to take into account when studying the fangs is its amino acid composition, stressing the increase in histidine, from 3% at the base to 26% at the tip of the fang (Politi *et al.*, 2012). In general histidines are involved in intermolecular hydrogen bonding interactions with neighboring protein side chains, covalent interactions with sclerotizing cross-linking agents and, in coordination with divalent metal ions such as Mg^{2+} , Ca^{2+} , Ni^{2+} , Cu^{2+} or Zn^{2+} (Remko *et al.*, 2010). Taking into account the correlation between increased concentrations of both Zn and His at the spider fang tip, the idea that Zn is involved in intermolecular cross-linking via histidine residues might be a reality. The difference in the Zn/Cl ratios found in the spider fang suggests either that Zn coordination is not related to Cl, or that more than a single coordination environment exists.

1.3.3.3. Comparison with analogue structures in other models

The structure, function and chemical organization of the invertebrate biting and piercing anatomical organs give reason for their potential for the development of synthetic materials. Thus, it is necessary to understand the relationship between composition, architecture and mechanical properties of the biological structures. To define the organic composition and, in particular, the structural proteins that make up the scaffolding framework in anatomical structures like jaws and mandibles, some research on the topic has been developed.

In 2008, Broomell and his co-workers purified from the fang-like jaws of the marine polychaete *Nereis virens* a protein which is rich in glycine and histidines (Broomell *et al.*, 2008a). Their results give reason to the idea of structures mostly composed of proteins containing a metallic center with the main features observed in cuticular proteins from hard cuticles: high abundance of histidine and glycine residues.

The use of transition metals by the protein of fang-like jaws was also explored by Broomell and his co-workers (Broomell *et al.*, 2008b) where the emphasis is made on zinc, as major contributor to the hardness and stiffness. Earlier, a study from Lichtenegger and colleagues (Lichtenegger *et al.*, 2003) stated how distribution of zinc correlates with the mechanical properties of the jaw material in worm species *Nereis* and *Glycera*. Thus, this study showed a new process to make materials harder and stiff, in contrast to the process that requires calcification that was mainly considered until then as a common process occurring in the cuticle of lobsters, crab and

mollusks. In 2006, Broomell and colleagues proved that zinc plays a critical role in mechanical properties of histidine-rich *Nereis* jaws (Broomell *et al.*, 2006). Researches following the same direction were held earlier by other authors (Bryan and Gibbs, 1979; Bryan and Gibbs, 1980; Schofield and Lefevre, 1989).

The study of the role of zinc as hardness enhancer for materials is not exclusive for the anatomical structures described above, but is also exploited synthetically to increase the hardness of silk produced by spiders (Lee *et al.*, 2009).

1.4. Dissertation outline

The presence of cuticular proteins in the exoskeleton is an essential feature in a structural context. They contribute for shape, thickness and stability of the exoskeleton. Considering the technological advances and the growing interest in material following the biological fundamentals, the study of cuticular protein provides information that may be useful to the development of materials with high stability and resistance.

The main objective for this work is to extract, purify and characterize cuticular proteins. To achieve this, the following objectives are pursuit:

- Evaluating the efficiency of cuticular protein extraction from different anatomical structures of *C. salei*.
- Establishing an efficient extraction protocol.
- Purification of specific proteins using the zinc affinity as a selective tool for His-rich proteins.
- Determine the amino acid content and sequence profiles mostly found in this kind of proteins.
- Establish preliminary homology with other cuticular proteins already found and characterized in other arthropods;
- Understand the physico-chemical arrangement of cuticles in *C. salei*.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1. Biological material

The biological material used in this work was obtained from adult spiders scarified after their second molt, the spiders, are stored at -20°C in 70% ethanol until the moment of use. The spiders were kindly provided by Prof. Dr. Friedrich G. Barth, University of Vienna.

2.1.1. Obtaining material

Biological material for experiments was collected from three different anatomical structures in of the body of *C. salei*: abdomen (abd), tibia (tib) and fangs (fan).

In abdomen, slices of approximately 1 cm² were cut out from the cuticle dorsal center side. The external part of the slice was cleaned from hairs and the interior from possible attached internal tissues.

Tibias were collected from legs making a cut on each tibia's extremity, approximately 1 mm from the junction with the patella and the tarsus. The conjunctive tissue and muscle were carefully removed from the interior.

Fangs were uncovered from the chelicerae, cut out from them and the conjunctive tissue still attached was removed.

In all cases, after collection of the material from the animal, it was carefully washed first with double distilled water (DDW) then with acetone. After drying, the material was made in powder using liquid nitrogen and the mortar and pastel and the resultant powder, was carefully weighted.

2.1.2. Preparation of aqueous extract from cuticle of *C. salei* and separation of the protein content from chitin

The powder cuticular material was exposed to one of three solutions described below, either individually, or in a sequential manner.

Urea buffer (U): 8 M urea in 5% (v/v) acetic acid containing 1 mM of protease inhibitors (1 mg/ml of stocks of 10 mM leupeptine and 1 mM pepstatin)). Urea is a powerful denaturant agent, disrupting the non-covalent bonds in the proteins. After mixture homogenization, the soluble extract was left overnight (o.n.) at 4°C or at room temperature (r.t.) with shaking.

Double distilled water (DDW): In order to ascertain the presence of water-soluble proteins in the cuticles, the process of extraction was also done using DDW containing 1 mM of protease inhibitors (the same conditions as described before). The homogenized mixture was left, as before, o.n. at 4°C or at r.t. with shaking.

Guanidine hydrochloride buffer: The last condition of extraction applied was guanidine buffer (G) at pH 8.9 (8 M guanidine hydrochloride, 1% (v/v) MSH buffer – 210 mM mannitol, 70 mM sucrose and 5 mM HEPES, pH 7.4 –, 100 mM tris-base and 1% (v/v) of 100 mM PMSF). Guanidine is one of the strongest protein denaturants, unfolding the well-ordered structures in proteins most of the times in an irreversible way. The homogenized mixture was incubated at 60°C for 2 hours with vigorous shaking.

The three buffers mentioned were used either in separate extraction processes or in sequenced extraction process, i.e.:

- 1) A known amount of powder material was extracted in DDW, another in urea and another in guanidine. After period of extraction, the soluble extract was centrifuged at 13200 rotations per minute (rpm) during 10 minutes – using Centrifuge 5415R, or at 10000 rpm during 15 minutes – using BECKMAN Allegra™ 64R Centrifuge –, both at 4°C.
- 2) A known amount of powder material was extracted first in DDW, then after collection of the supernatant by centrifugation (at 13200 rpm during 10 minutes – using Centrifuge 5415R – or at 10000 rpm during 15 minutes – using BECKMAN Allegra™ 64R Centrifuge –, both at 4°C), the pellet was used for extraction in urea and finally after collection of the supernatant by centrifugation, the pellet was used for extraction in guanidine.

In all cases, the average volume of extraction buffer used per powder material was 2-4 ml for 4-10 mg powder sample.

At the end of each extraction, the pellet-containing chitin was dried and characterized by FTIR (see section 2.4.3.). The supernatant, named crude extract, was collected for further analysis.

2.1.3. Dialysis of the crude extract

Urea and guanidine were removed from the crude extract using dialysis (SERVAPOR® dialysis tubing MWCO 12000-14000 RC from PALL – Life Sciences) against 50 mM acetic acid buffer in different conditions:

- starting with dialysis buffer at pH 5 and then at pH 7;
- starting with dialysis buffer containing 2 M urea at pH 5 and then only the dialysis buffer at pH 7.

2.2. Protein purification

2.2.1. Fast protein liquid chromatography (FPLC)

This kind of chromatography is used to purify specific proteins out of a mixture of proteins. Separation is possible due to the different affinities that mixture of proteins has either for the moving fluid (mobile phase that will pass through the column) and for the stationary phase of the column (through which the mobile phase passes).

2.2.1.1. HiTrap™ IMAC HP column

The column used in this study is HiTrap™ IMAC HP (1 ml), from GE Healthcare, which is pre-packed with IMAC Sepharose™ High Performance, and is ideal for immobilized metal ion affinity chromatography (IMAC). This column was chosen as the aim of the project is to identify the zinc binding protein from the spider fang. Therefore, the stationary phase was immobilized with 0.1 M ZnCl₂ (10x volume of the column). Pre-equilibration was performed using 8 M urea in 5% (v/v) acetic acid, pH 7.5 (10x volume of the column). The crude extracts were loaded after adding to them 20 mM of phosphate buffer, pH 7.4, and adjusting the pH to 7.5. The column loading process was performed using peristaltic pump, with a flow rate of 1 ml.min⁻¹.

2.2.1.2. Purification step using BioLogic DuoFlow™ Chromatography System

The purification was carried out using FPLC BioLogic DuoFlow™ Chromatography System, from Bio-Rad. The column was connected to the system and prewashed with a linear gradient between 100% to 95% of buffer A (20 mM phosphate buffer, 50 mM sodium chloride and 8 M urea, at pH 7.5) and 0% to 5% of buffer B (20 mM phosphate buffer, 50 mM sodium chloride, 8 M urea and 250 mM imidazole, at pH 7.5). The column was then eluted using a linear gradient between 95% to 0% of buffer A and 5% to 100% of buffer B. The gradient flow rate was 1 ml.min⁻¹ and 0.5 ml fractions were collected when absorbance at 280 nm was above 0.100 arbitrary units (AU).

2.3. Analysis of the protein content

2.3.1. Protein quantification

The amount of protein from extraction and from eluted purified fractions was quantified by Bradford protein assay (Bio-Rad), using bovine serum albumin (BSA) as standard. Briefly, 950 µl of Bradford reagent was added to 50 µl of sample test at adequate dilution factor and the absorbance was read at 595 nm. The protein quantification was performed based on calibration curve prepared for this purpose.

2.3.2. Gel electrophoresis

Gel electrophoresis gives information on the molecular weight, charge and the presence of subunits in the proteins. This technique also provides information about the purity of a particular protein preparation, allowing monitoring of the extraction and purification processes.

In this work, the protein samples were separated using different gel electrophoresis methodologies as described below.

2.3.2.1. Electrophoresis in continuous systems

2.3.2.1.1. Acetic acid-urea gel electrophoresis

A continuous 15% acetic acid-urea polyacrylamide gel electrophoresis, which allows protein separation based on both the molecular size and charge, was used. In this system, urea is added to the gel increasing the proteins' coefficient of friction and as a result there is a change on their electrophoretic mobility.

The samples were prepared in sample loading buffer (9 M urea, 5% (v/v) acetic acid and methyl green acting as a tracking dye). Running was performed in a Mini PROTEAN® Tetra Cell, from Bio-Rad, during 1.5 hour at constant voltage (150V).

2.3.2.2. Electrophoresis in discontinuous systems

2.3.2.2.1. SDS gel electrophoresis (SDS-PAGE)

Discontinuous 10%, 12% and 15% polyacrylamide gel electrophoresis were used according the procedure of Laemmli (Laemmli, 1970). This process guaranties separation based on molecular mass of the proteins in the presence of sodium dodecyl sulphate (SDS), i.e., in denaturing conditions.

The samples were first denatured at 90°C for 3 min in presence of sample loading buffer containing Tris-HCl, pH 6.8, SDS, glycerol, bromo phenol blue and β -mercaptoethanol (Thermo Scientific). To estimate the molecular mass of the separated proteins, each gel was also loaded with colored protein standards: PageRuler™ Prestained Protein Ladder (from 10 to 170 kilo Dalton (kDa)) and/or Spectra Multicolor Low Range Protein Ladder (from 1.7 to 40 kDa), (Thermo Scientific). The running was carried out using running buffer 100 mM Tris/Bicine, 0.1% (w/v) SDS in a Mini PROTEAN® Tetra Cell, during 1 hour and 20 minutes at constant voltage (100V).

2.3.2.2.2. Tricine gel electrophoresis (Tricine-SDS-PAGE)

A discontinuous 12% tris-tricine acrylamide gel electrophoresis was used for the analysis of low molecular mass proteins. Similar to the process described before, this method allows separation based on the molecular mass of the proteins, but with increased separation resolution for proteins of low molecular weight due to the functional group of Tricine that defines the electrophoretic mobility of the trailing ion (Tricine) relative to the electrophoretic mobilities of proteins.

The samples were loaded on the gel in the presence of sample loading buffer containing Tris-HCl at pH 6.8, SDS, glycerol, Coomassie Blue G-250 and dithiothreitol (DTT) (commercially produced – Thermo Scientific – or homemade). Protein standards were run in the gels in order to estimate the molecular mass of the proteins separated: PageRuler™ Prestained Protein Ladder and/or Spectra Multicolor Low Range Protein Ladder. Running was carried out during 1 hour and 10 minutes at constant voltage (100V), in a Mini PROTEAN® Tetra Cell, with the inner chamber containing cathode buffer (0.1 M Tris, 0,1 M Tricine and 0.1% (w/v) SDS at pH 8.3) and the outer chamber containing anode buffer (0.2 M Tris-HCl at pH 8.9).

2.3.3. Protein staining

The gel staining, in order to reveal the proteins separated by electrophoresis, was performed during 20 min in 0.1% (w/v) Coomassie Brilliant Blue G250, 10% (v/v) acetic acid and 50% (v/v) methanol. De-staining was done by several washings in de-staining solution containing 30% (v/v) methanol and 10% (v/v) acetic acid.

2.3.4. Transferring into a PVDF membrane

Protein transference from gels to PVDF membranes was performed in aim to examine them further by amino acid analysis and sequencing.

The protein separated by electrophoresis was transferred to BioTrace™ PVDF membrane 0.45µm, from PALL. The process was performed in transferring buffer at pH 10 containing 10 mM CAPS, 10% (v/v) methanol, within Mini Trans-Blot Cell (Bio-Rad) during 1 hour at constant voltage (100V).

The protein staining on the membrane was done using Ponceu S Stain at r.t. incubation for 20 minutes. The membrane was destained by washing in DDW.

2.3.5. Determining the presence of glycosylations

The detection of glycoproteins was done in products of electrophoresis, using Glycoprotein Detection Kit (GLYCOPRO from Sigma).

2.3.6. Amino acid analysis

2.3.6.1. Sample preparation

The samples were prepared by 24 hours protein hydrolysis in 6N HCl at 110°C. After hydrolysis, the solution containing hydrolyzed protein was clean up from HCl by successive evaporations and washings with DDW. The remaining residue containing hydrolyzed proteins was resuspended in appropriate volume of sample dilution buffer 0.12 N at pH 2.20 (Sykam).

2.3.6.2. Amino acid identification

This process was carried on SYKAM Automatic Amino Acid Analyzer S 433, according to the Ninhydrin post-column derivatization method, using the software ChromStar7. This technique combines the advantages of the ion exchange separation method with the technique of high performance liquid chromatography.

To obtain the amino acid content, a pH and salt gradient is used between 100% to 0% of buffer A (0.12 N sodium citrate buffer at pH 3.45) and 0% to 100% of buffer B (0.20 N sodium citrate buffer at pH 10.85). The gradient flow is $0.45\text{ml}\cdot\text{min}^{-1}$, with increasing temperature from 52°C to 74°C. The identification of the amino acids is done at absorbance of 570 nm except for proline/hydroxyproline for which the absorbance used is 440 nm.

2.3.7. Sequencing using Edman degradation

Products of protein purification were sequenced by N-terminal analysis using the Edman degradation method. The analysis was done in Proteome Factory AG.

2.4. Analysis of the non-protein content

2.4.1. Determination of chitin content

Cuticles from abdomen, tibia and fangs, collected as described on section 2.1.1., were subjected to protein removal by 0.3% sodium hypochlorite (NaClO) in acetate buffer (1 ml per mg of material). The mixture reacted for 3 to 24 hours at 70°C and after all protein removal the remaining material was extensively washed with DDW and dried for 48h at 50 °C.

2.4.2. Attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR)

FTIR measures how well a sample absorbs or transmits light at each wavelength in the IR spectral region. ATR-FTIR is a technique for direct analysis of samples in solid or liquid state without the need of prior preparation. In this technique, a beam of infrared light is passed through an ATR crystal in contact with the sample and then it reflects at the internal surface. The spectrum at the IR region probes the vibration of the molecules, providing information about present chemical groups in the sample. For example, the N-C bond in the amide group absorbs at 1000-1350 cm^{-1} while C=O absorbs at 1640-1490 cm^{-1} . Macromolecules like chitin and proteins have a characteristic fingerprint spectrum which can be used to identify their presence and state.

ATR-IR was performed using the HYPERION 3000 FT-IR Microscope, from Bruker, controlled by OPUS 7.0 software. Cuticles made in powder, collected from abdomen, tibia and fangs, were placed on the surface of glass blades and analyzed in reflection mode before and after protein extraction and after subjection to protein removal by NaClO. Infrared spectra were obtained at a resolution of 4 cm^{-1} and a zero-filling factor of 2, using 32 scans to get spectra between 4000 cm^{-1} and 600 cm^{-1} .

Chapter 3

Results

3. RESULTS

3.1. Extraction of protein from cuticle of different anatomical structures of the wandering spider, *Cupiennius salei* – A proof of principle

Working plan was defined based on need to establish an efficient protocol for extraction of cuticular proteins from exoskeleton of *C. salei*. Tasks for this propose comprised:

- testing the possibility to extract cuticular proteins;
- once proved the possibility of obtaining protein, the efficiency of protein extraction using different extraction buffers in non-sequenced manner was studied (due to the need of identify proteins showing different solubility and to attempt to increase the amount of extracted protein improving conditions of extraction);
- perform to comparison between protein extraction profiles for soft and hard cuticles, applying the established conditions of extraction in sequenced manner;
- evaluation of the efficiency of dialysis process in crude extracts containing urea and guabidine.

To illustrate results of protein extraction, electrophoresis was performed, which modality was chosen according to characteristics of each crude extract, and amount of protein was quantified using Bradford Protein Assay.

3.1.1. Protein extraction, quantification and respective profiles

The first way to test the extraction of cuticular proteins from *C. salei* was performed using abdominal cuticle in 8 M urea buffer. Table and Figure 3.1 show the result obtained by several protein extractions from cuticle of abdomen reveling that extraction process gives a large number of proteins with different molecular weight.

Table 3.1. Dry weights and percentage of protein extracted from cuticle of opisthosome (abdomen) of *C. salei* in 8 M urea buffer

Crude extracts of abdomen (n=4)	dry weight of abdominal cuticle (mg)		% protein
1		3,50	22,93 (± 2,58)
2		3,94	26,77 (± 1,36)
3		6,59	18,13 (± 1,02)
4		5,26	15,89 (± 4,19)

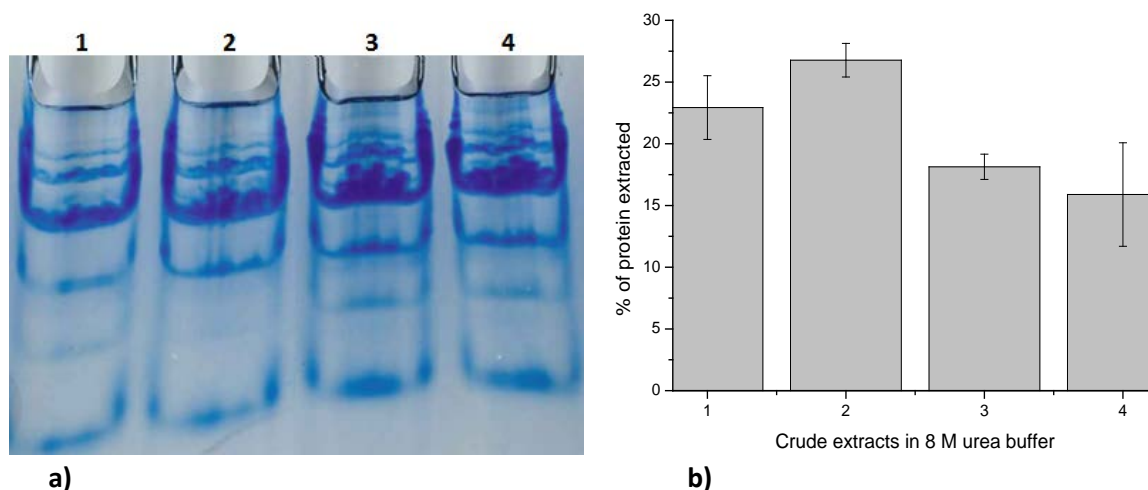


Fig. 3.1. Protein profile of crude extract of abdominal cuticle of *C. salei* (n=4) extracted in urea buffer. a) 15% acetic acid urea gel stained by Coomassie Brilliant Blue showing the protein profile of crude extracts 1 (3.50 mg), 2 (3.94 mg), 3 (6.59 mg) and 4 (5.26 mg). **b)** Percentage of protein extracted from crude extracts of abdomen. Values are expressed in relation to initial dry weight of the material analyzed.

Distinct conditions of extraction were applied then and results (Table and Figure 3.2) indicate that the amount of extracted protein from abdominal cuticle is different depending on the buffer in which it is extracted. Another observation to these results is the low molecular weight observed for a large amount of the extracted proteins. During dialysis in 50 mM acetic acid buffer, the extracted proteins from the crude extract tend to precipitate (Figure 3.2a), as can be noticed by the abrupt decrease in protein concentration after dialysis.

Table 3.2. Percentage of protein extracted from cuticle of opisthosome (abdomen) of *C. salei* in different buffers.

		dry weight of abdominal cuticle (mg)	% protein
Crude extract of abdomen (n=1)	in DDW	3.63	1.14
	in U	4.85	18.45
	in G	4.57	28.35
Crude extract of abdomen after dialysis (n=1)	pellet from U	4.85	1.26
	pellet from G	4.57	0.98
	supernatant from U	4.85	0.00
	supernatant from G	4.57	0.00

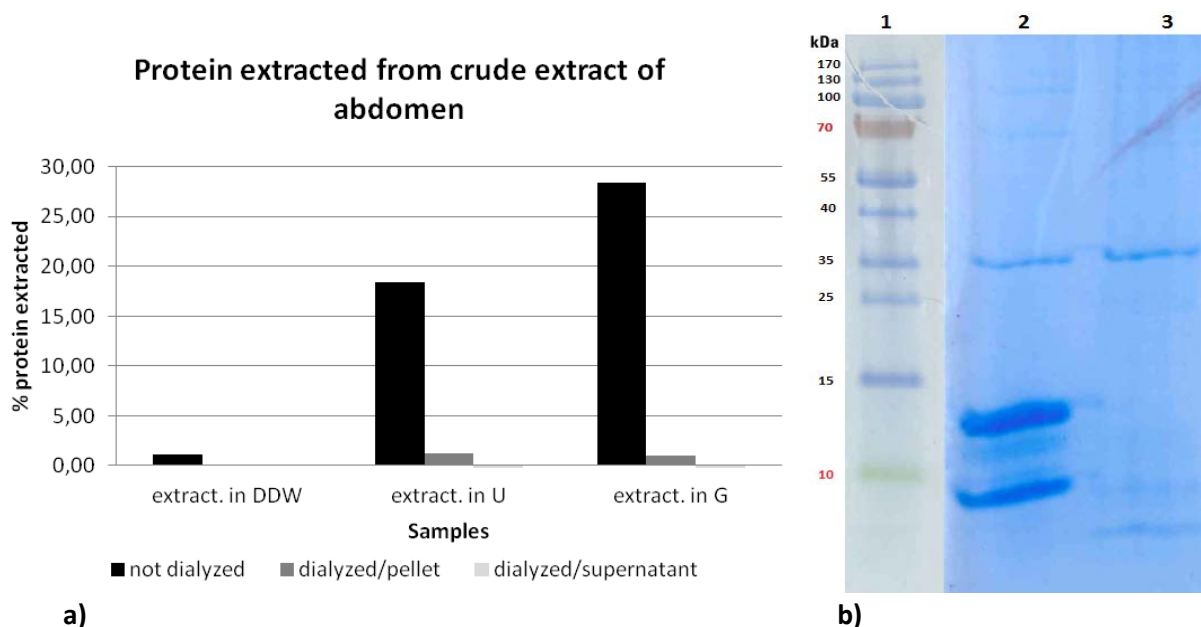


Fig. 3.2. Protein profile from crude extract of abdominal cuticle of *C. salei* (n=3) extracted in double distilled water (DDW), in 8 M urea buffer (U) and in 8 M guanidine buffer (G). a) Crude extracts of cuticle in DDW, U and in G, before and after dialysis against 50 mM acetic acid buffer. The percentage of protein is presented in relation to initial dry weight of the material. **b)** 12% tricine-SDS gel stained by Coomassie Brilliant Blue showing the protein profile from crude extract of abdominal cuticle of *C. salei* extracted in U and G and subjected to dialysis. 1) marker; 2) pellet from dialysis of crude extract in U; 3) pellet from dialysis of crude extract in G dialyzed.

Finally, comparison between protein contents of hard and soft cuticles, using abdominal and leg cuticle (tibia), was obtained. At this level, proteins were sequentially extracted by homogenization of cuticular powder materials in different buffers, i.e., first in DDW, then the remaining pellet, containing chitin and proteins, was homogenized in 8 M urea buffer and the

remaining pellet from this step was homogenized in 8 M guanidine (Table and Figure 3.3). Here it is seen that most of the protein is already efficiently extracted in urea buffer, however some of the protein is only soluble in guanidine buffer. Major differences observed between both cuticles are, first, the amount of extracted protein from abdomen is much larger and, second, a larger abundance of low molecular weight proteins is present in cuticle of tibia than in cuticle of abdomen.

Table 3.3. Percentage of protein extracted from cuticle of opisthosome (abdomen) and tibia of *C. salei* sequentially in different buffers.

		dry weight of abdominal cuticle (mg)	% protein
Crude extract of abdomen (n=1)	in DDW	10.58	1.84
	in U		27.03
	in U dialyzed / supernatant		8.17
	in G		1.57
Crude extract of tibia (n=1)	in DDW	11.98	1.86
	in U		16.65
	in U dialyzed / supernatant		2.34
	in G		4.03

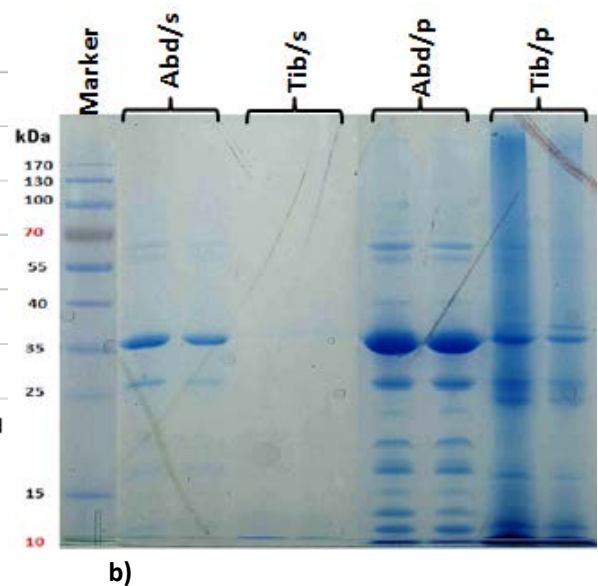
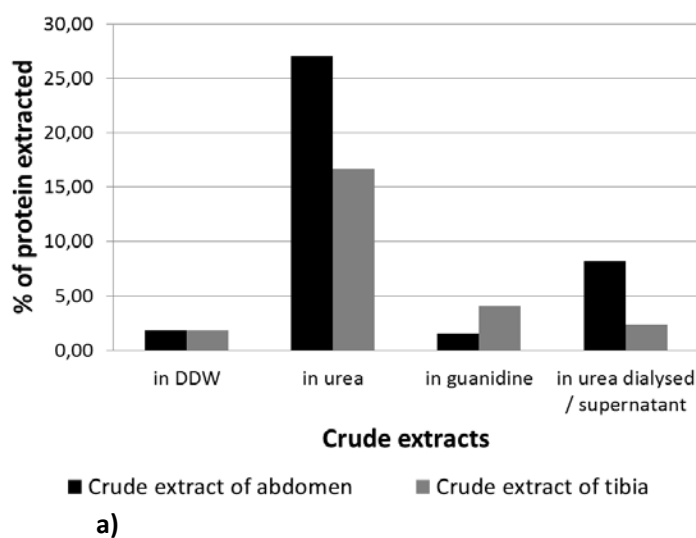


Fig. 3.3. Protein profile from crude extracts of cuticle of abdomen and tibia of *C. salei* sequentially extracted in double distilled water (DDW), in 8 M urea buffer (U) and in 8 M guanidine buffer (G) and then dialyzed. a) Crude extracts of cuticles from abdomen (10.58 mg) and tibia (11.98 mg) in DDW, in U and in G, before and after dialysis. The percentage of protein is expressed in relation to the dry weight of the initial material. **b)** 12% SDS gel of crude extracts protein profile of cuticle of abdomen and tibia of *C. salei* sequentially extracted in urea buffers before and after dialysis against 5 mM acetic acid buffer. Staining by Coomassie Brilliant Blue. (Abd/s) abdomen supernatants; (Tib/s) tibia supernatants; (Abd/p) abdomen pellets; (Tib/p) tibia pellets.

This group of results allowed the establishment of protocol principle to be used on further steps of the project in order to extract proteins. Thus, this principle follows the steps listed below:

- 1) Collect, clean, grind and weight, by this order, cuticular material
- 2) Homogenize the cuticular powder material in DDW for extraction o.n. at 4°C
- 3) Centrifuge the crude extract, keep the supernatant and dry the pellet containing chitin and proteins
- 4) Homogenize the previous pellet in 8 M urea buffer for extraction o.n. at r.t.
- 5) Centrifuge the crude extract, keep the supernatant and go further with the pellet containing chitin and proteins
- 6) Homogenize the previous pellet in 8 M guanidine hydrochloride buffer for extraction during 2h at 60°C
- 7) Quantify protein extracted in each step and subject it to electrophoresis.

3.2. Extraction, purification and characterization of protein from different cuticular anatomical structures of *C. salei*

After defined the protocol for protein extraction, there were conditions to repeat this process in order to obtain material to be chromatographically purified. The process was applied either in abdomen and tibia, which study and protocol optimization was performed during first stage of the project, and also in fangs. Crude extracts selected for this propose were those with proteins soluble in 8 M urea buffer. As the last and principal objective of this work is the development of an approach for studying of cuticular composition in fangs of *C. salei*, purification parameters were established following this idea. Thus, as it is known that fang cuticle has in its constitution histidine-rich proteins with zinc ions associated, it was selected a chromatographic system that would allow purification of protein with such features: the HiTrap™ IMAC HP loaded with 0.1 M ZnCl₂.

Because amino acids present in proteins are one of the ways to proceed to their characterization, proteins purified from cuticles of each anatomical structure were subjected to amino acid analysis. Proteins which amino acid content was considered relevant for the context of this work, i.e., high amount of amino acid residues, generally characteristic of cuticular proteins in

arthropods (alanine, glycine, cysteine and specially histidine), were analyzed in order to obtain their N-terminal sequence.

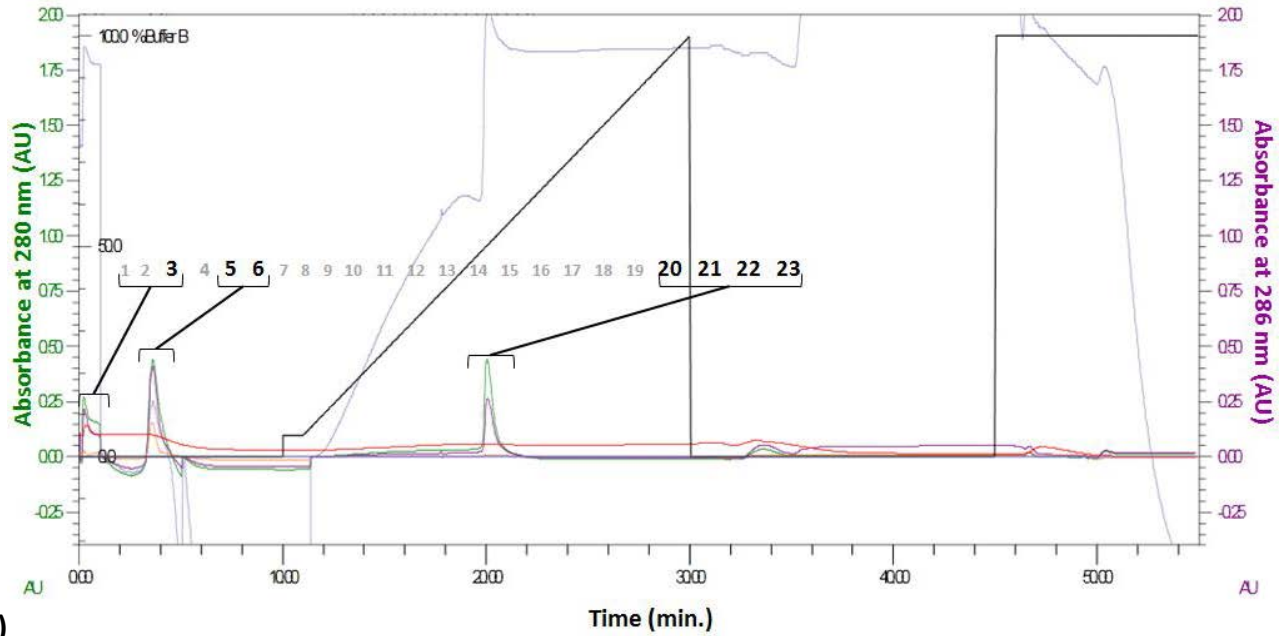
It is known that glycosylations are common and a feature of cuticular proteins. They can be related to function of such proteins, being responsible for signalization of where and/or when these proteins should occur and play their role. So, in this work, glycoproteins detection was envisaged as an advantage for the process of proteins characterization, approach that was then performed for crude extracts in DDW and urea buffer and, for second situation, also for products of purification.

3.2.1. Affinity chromatography

Proteins extracted from cuticles of abdomen, tibia and fangs in 8 M urea buffer were purified using HiTrap™ IMAC HP loaded with 0.1 M ZnCl₂, in FPLC BioLogic DuoFlow™ Chromatography System, and following a protocol explain in section 2.2.1. The prewashing of the column, with a linear gradient between 100% to 95% of buffer A and 0% to 5% of buffer B allowed the exclusion of proteins not specifically attached to zinc of stationary phase of the column. The elution by linear gradient between 95% to 0% of buffer A and 5% to 100% of buffer B released proteins that were establishing with zinc strong interactions.

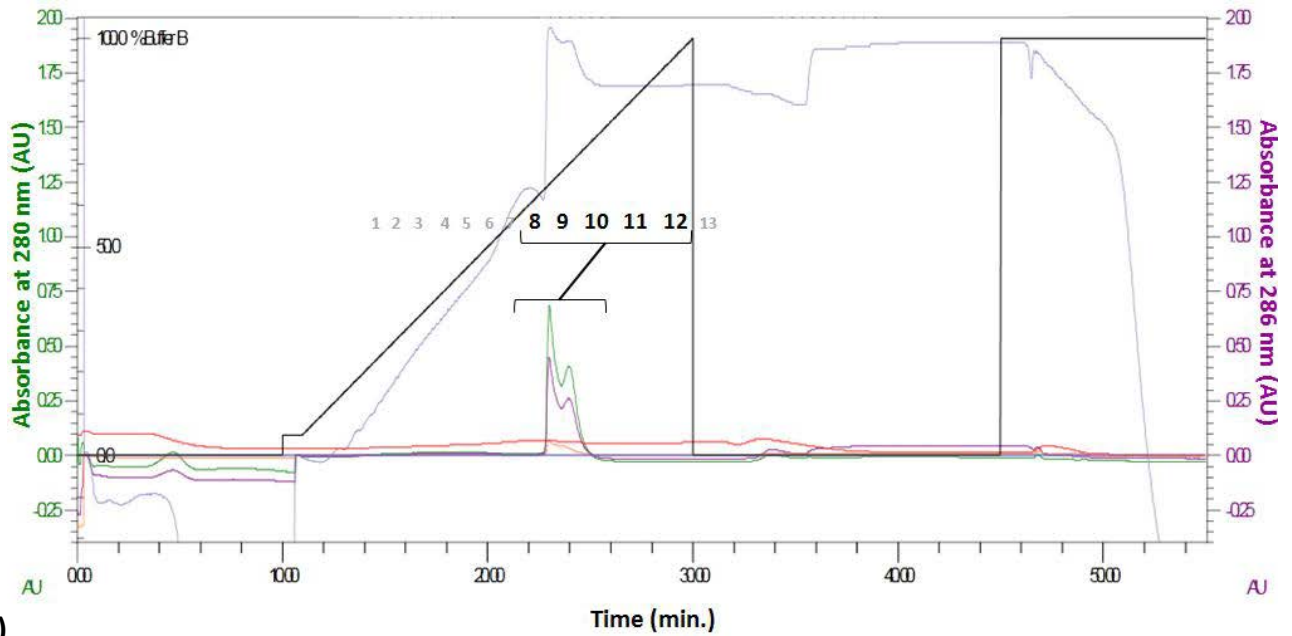
Chromatograms for one single experiment on each anatomical structures studied are shown in Figure 3.4. Total chromatograms for all experiments carried on at this stage of the work are presented in the appendix Figure I. 0.5 ml fractions were collected when absorbance at 280 nm reached 0.100 AU and are indicated by numbers along the chromatogram.

Chromatogram from crude extract of abdominal cuticle in 8 M urea buffer



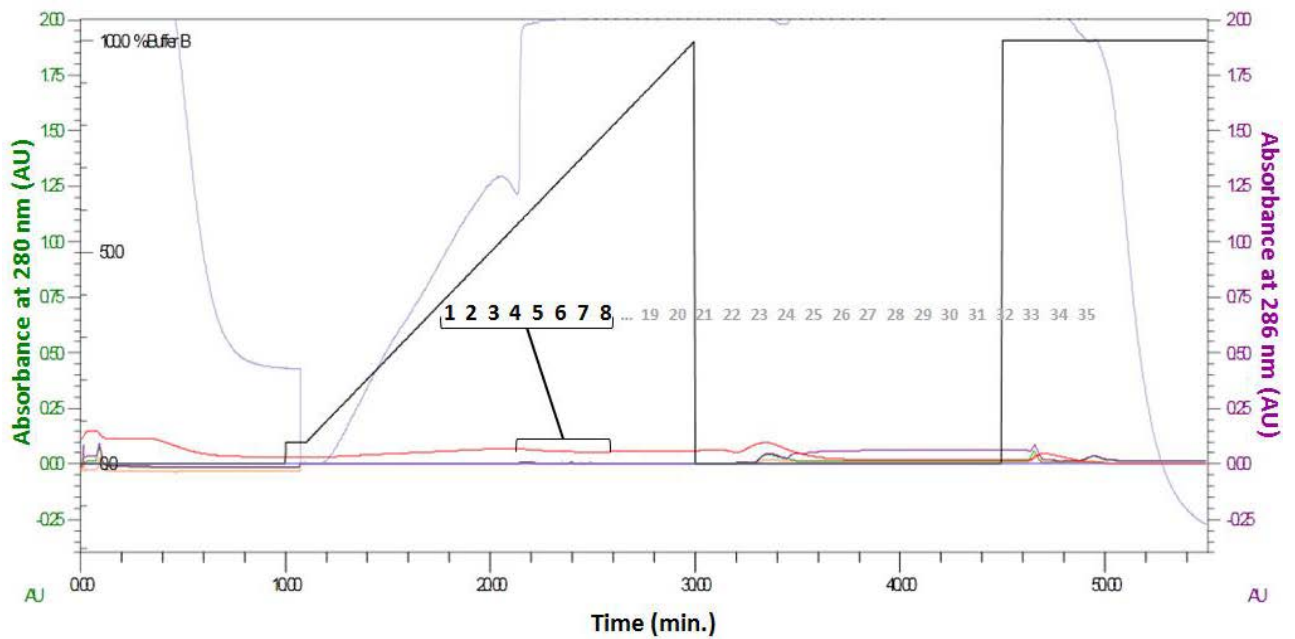
a)

Chromatogram from crude extract of tibia cuticle in 8 M urea buffer



b)

Chromatogram from crude extract of fang cuticle in 8 M urea buffer



c)

Fig. 3.4. Affinity chromatography of crude extracts of abdomen in 8 M urea purified in HiTrap™ IMAC HP loaded with 0.1 M ZnCl₂. a) Purification of cuticular protein from abdomen. b) Purification of cuticular protein from tibia. c) Purification of cuticular protein from fangs. Numbers indicated in each chromatogram correspond to elution fractions collected (**black** – fractions considered relevant; **grey** – fractions considered irrelevant).

It was quantified the concentration of fractions collected during purification, when absorbance at 280 nm was above 0.100 AU or high enough in comparison to surrounding values. Results are summarized in Table 3.4 and Figure 3.5 and they show not only the amount of protein present in each fraction collected but also stress the idea of dependence of initial protein concentration to load in the column in order to have an efficient chromatographic process – note the case of fangs.

Table 3.4. Percentage of protein extracted and purified from cuticle of opisthosome (abdomen), tibia and fangs of *C. salei*

		Dry weight of cuticle (mg)	% Protein extracted / purified ²
Extraction / Abdomen (n=2)	Crude extract in DDW		0.87 (± 0.75)
	Crude extract in U	8.610 (± 1.25)	46.36 (± 7.77)
	Crude extract in G		1.69 (± 1.70)
Flow-throw	4.93 (± 1.53)		
Purification / Abdomen	Fractions eluted in pre-washing		1.65 (± 2.41)
	Fractions eluted in linear gradient		2.60 (± 0.59)
Extraction / Tibia (n=2)	Crude extract in DDW	21.588 (± 1.66)	0.56 (± 0.35)
	Crude extract in U		21.64 (± 0.60)
	Crude extract in G		1.07 (± 1.26)
Purification / Tibia²	Flow-throw		3.94 (± 4.21)
	Fractions eluted in linear gradient		10.82 (± 2.39)
Extraction / Fangs (n=2)	Crude extract in DDW	5.640 (± 2.22)	0.64 (± 1.01)
	Crude extract in U		12.55 (± 5.47)
	Crude extract in G		4.13 (± 1.03)
Purification / Fangs²	Flow-throw		13.25 (± 3.76)
	Fractions eluted in pre-washing		8.93 (± 5.28)
	Fractions eluted in linear gradient		12.86 (± 4.33)

² The percentage of protein extracted is expressed in relation to the dry weight of the initial material. The percentage of protein purified is expressed in relation to the total amount of protein loaded into the column (the extracted in urea).

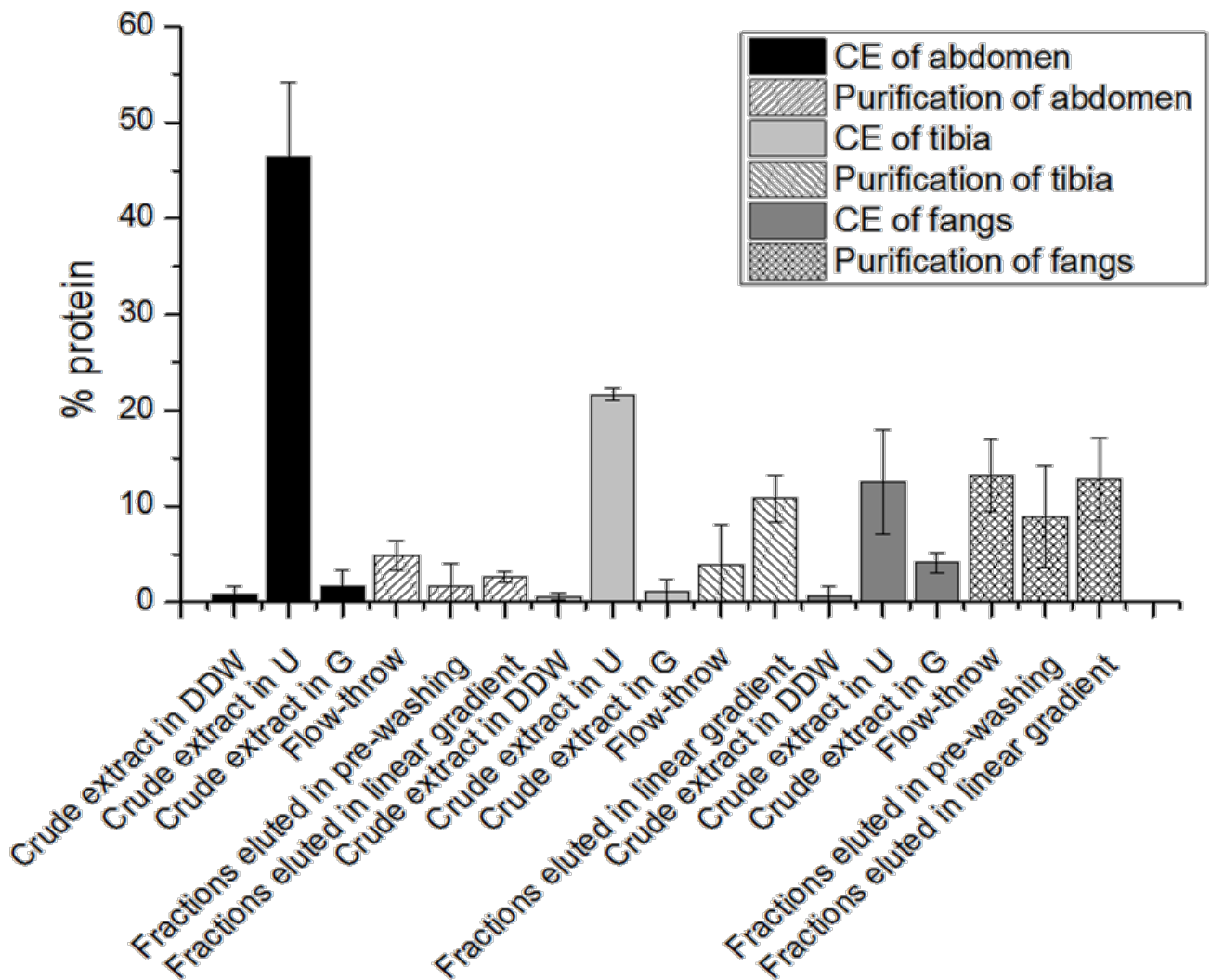


Fig. 3.5. Percentage of protein obtained from crude extracts of cuticle of abdomen, tibia and fangs of *C. saiei* sequentially extracted in double distilled water (DDW), in 8 M urea buffer (U) and in 8 M guanidine buffer (G) and then purified in IMAC column loaded with $ZnCl_2$. Crude extracts of cuticles from abdomen, tibia and fang in DDW, in U and in G, and then purified. The percentage of protein extracted is expressed in relation to the dry weight of the initial material. The percentage of protein purified is expressed in relation to the total amount of protein loaded into the column (the extracted in urea).

This group of results reveals a more pronounced amount of protein purified from crude extracts of tibia, then from crude extracts of abdomen and, lastly, from crude extracts of fangs.

3.2.2. Amino acid content

Cuticular proteins, purified from crude extracts of abdominal and tibia cuticles (see chromatograms a and c in appendix Figure I), separated by electrophoresis were again transferred

to PVDF membranes. Protein revelation in each membrane showed bands consistent with those presented in Figure 3.6 indicated below.

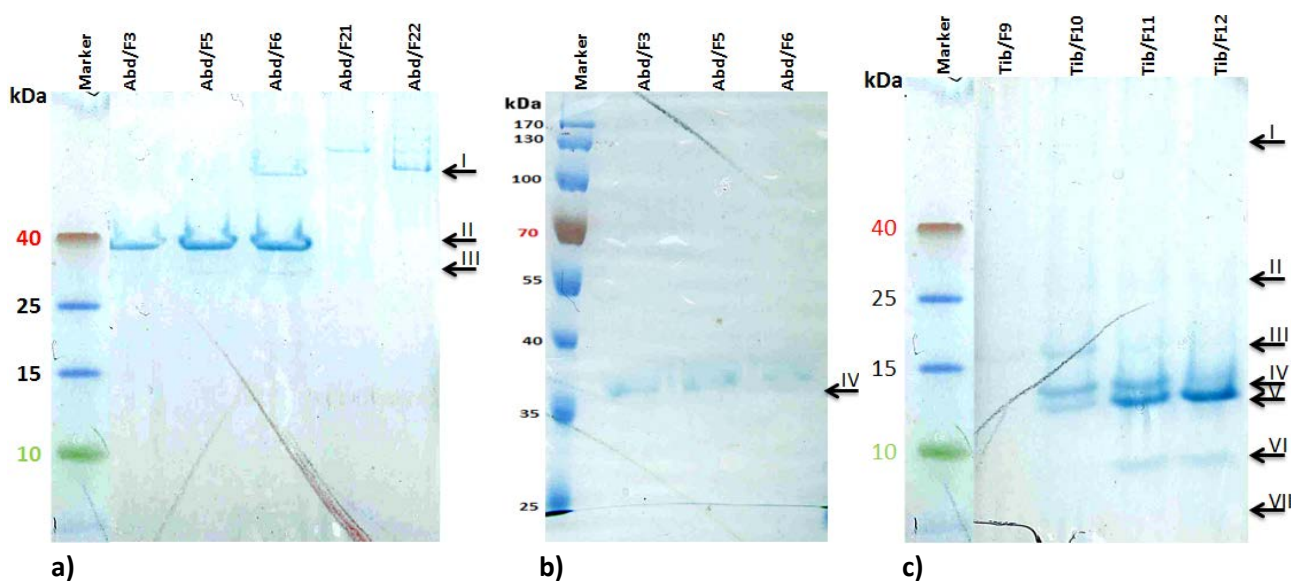


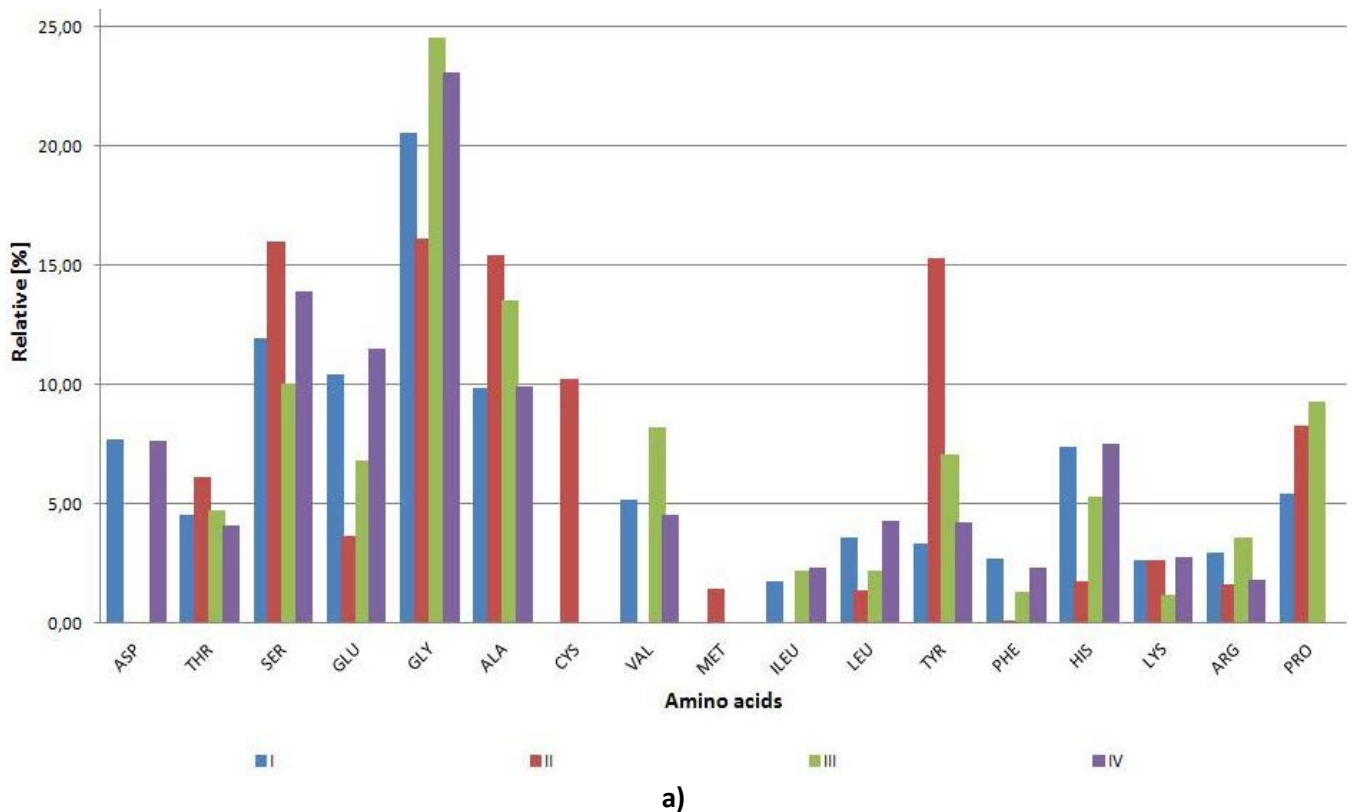
Fig. 3.6. Protein profile from crude extracts of cuticle of abdomen and tibia of *C. salei* sequentially extracted in double distilled water (DDW), in 8 M urea buffer (U) and in 8 M guanidine buffer (G) and which products of extraction in U was then purified in IMAC column loaded with $ZnCl_2$. a) 12% tricine-SDS gel showing the product of purification of abdomen. b) 10% SDS gel showing the product of purification of abdomen. c) 12% tricine-SDS gel showing the product of purification of tibia. (Abd/F) fractions referring to purification in abdomen; (Tib/F) fractions referring to purification in tibia. All gels were stained with Coomassie Brilliant Blue. Arrows point to band corresponding to stained protein considered relevant and the numbers indicate the identification attributed to each one of these bands for the further procedures.

Bands corresponding to stained protein (those marked with black arrows in previous figure), were carefully cut out from the membranes and subjected to amino acid content determination. Amino acid content of respective remaining materials obtained after protein extraction process was also evaluated. The results are summarized in Tables 3.5 and 3.6 and also in Figures 3.7 and 3.8.

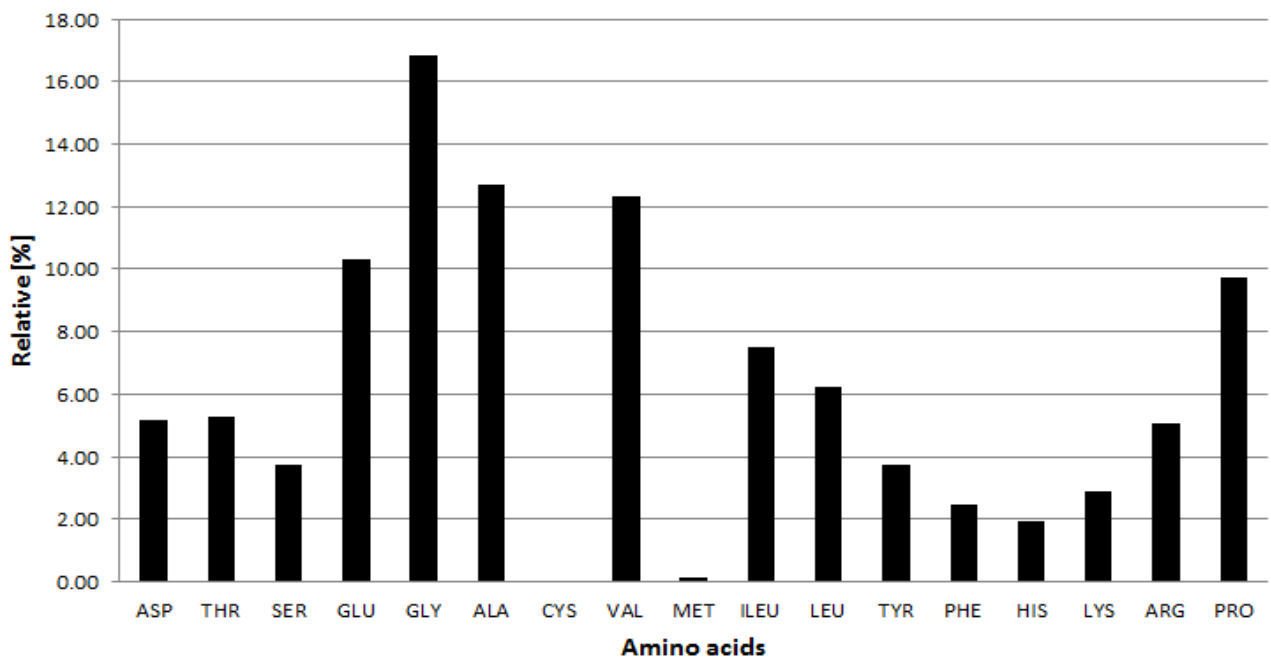
Table 3.5. Relative percentage of amino acids in cuticular proteins purified from opisthosome (abdomen) of *C. salei* and still attached to chitin after extraction.

Amino acids	Purified protein (%)				Protein remaining in the cuticle (%)
	I	II	III	IV	
ASP	7,67	0,00	0,00	7,65	5.18
THR	4,56	6,10	4,76	4,12	5.26
SER	11,91	15,98	10,06	13,92	3.75
GLU	10,45	3,64	6,80	11,52	10.29
GLY	20,51	16,10	24,53	23,05	16.84
ALA	9,85	15,41	13,53	9,92	12.68
CYS	0,00	10,21	0,00	0,00	0.00
VAL	5,20	0,00	8,21	4,54	12.33
MET	0,00	1,42	0,00	0,00	0.14
ILEU	1,79	0,00	2,18	2,34	7.49
LEU	3,58	1,41	2,20	4,26	6.22
TYR	3,34	15,26	7,07	4,21	3.73
PHE	2,69	0,15	1,30	2,33	2.47
HIS	7,40	1,75	5,29	7,53	1.94
LYS	2,67	2,64	1,17	2,79	2.86
ARG	2,94	1,65	3,59	1,82	5.06
PRO	5,46	8,27	9,31	0,00	9.75
Allo-Ile ³					0.34

³ Allo-Ile was measured only for remaining protein attached to chitin in the end of extraction process.



a)



b)

Fig. 3.7. Amino acid content of cuticular proteins from abdomen. a) Cuticular proteins purified from crude extracts of abdomen in 8 M urea buffer. The relative concentration of amino acids is expressed in relation to total amount of protein present on band cut out from the membranes. Each sample analyzed corresponds to bands marked on Figure 3.5a and b with arrows. **b)** Cuticular proteins attached to chitin after protein extraction process. Relative concentration of amino acids is expressed in relation to total amount of protein present on mixture of protein and chitin.

Amino acid analysis from bands containing purified elution fractions (from abdomen) revealed presence of high levels of serine, glycine, alanine, and tyrosine residues for the three bands marked with black arrows on Figure 3.6a. However, sequencing of these proteins was not possible. Abdominal cuticular proteins still attached to chitin after extraction process show high level of glutamate, glycine, alanine, valine and proline residues.

Table 3.6. Relative percentage of amino acids in cuticular proteins purified from tibia of *C. salei* and still attached to chitin after extraction.

Amino acids	Protein purified (%)							Protein remaining in the cuticle (%)
	I	II	III	IV	V	VI	VII	
ASP	0,00	0,00	7,57	7,66	7,27	5,47	7,55	9.18
THR	5,09	3,19	4,14	4,16	4,66	2,50	4,32	2.53
SER	10,58	5,36	6,51	4,99	4,10	6,73	12,74	2.87
GLU	11,59	5,58	6,44	5,35	0,06	14,62	8,17	4.61
GLY	20,61	13,01	16,20	16,45	4,21	16,36	25,02	19.19
ALA	15,44	15,26	21,76	24,22	15,33	7,80	9,19	19.30
CYS	0,00	0,00	0,02	0,00	27,70	0,00	0,00	0.00
VAL	5,46	4,66	5,90	7,27	7,15	10,55	4,93	8.31
MET	0,00	0,00	0,30	0,67	1,07	0,00	0,00	0.39
ILEU	1,84	1,04	2,23	1,22	0,90	1,27	2,01	2.70
LEU	3,91	2,13	3,93	2,31	1,64	3,93	5,09	7.11
TYR	4,15	4,69	5,12	6,98	8,02	5,09	5,18	5.60
PHE	3,20	1,62	2,78	2,83	2,21	2,84	2,01	1.46
HIS	8,53	34,28	4,22	3,97	3,94	7,73	10,52	4.53
LYS	2,89	1,56	2,42	1,75	1,20	4,10	2,35	1.82
ARG	1,78	1,81	1,67	2,16	1,86	0,77	0,93	3.78
PRO	4,92	5,80	8,80	8,03	8,69	10,24	0,00	6.62
Allo-Ile ⁴								0.06

⁴ Allo-Ile was measured only for remaining protein attached to chitin in the end of extraction process.

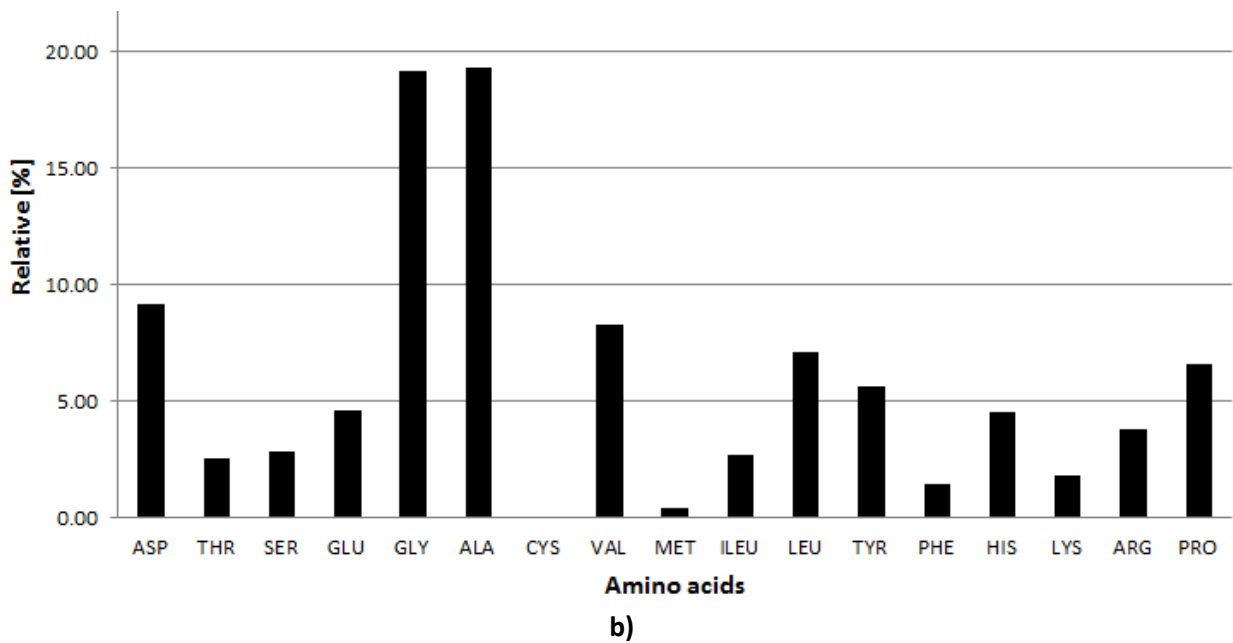
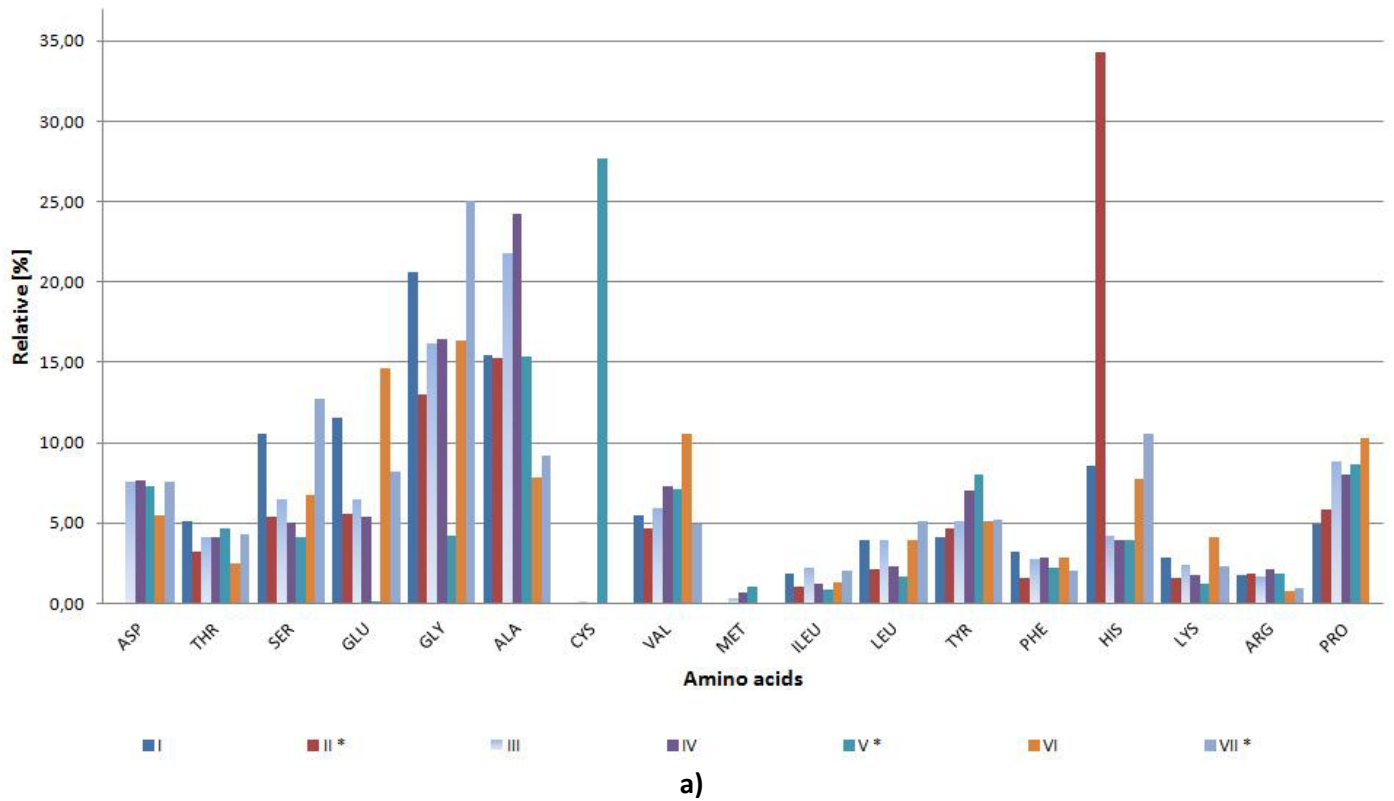


Fig. 3.8. Amino acid content of cuticular proteins from tibia. **a)** Cuticular proteins purified from crude extracts of tibia in 8 M urea buffer. The relative concentration of amino acids is expressed in relation to total amount of protein present on band cut out from the membranes. Each sample analyzed corresponds to bands marked on Figure 3.5c with arrows. Samples marked with * were considered relevant for further analysis. **b)** Cuticular proteins attached to the chitin of tibia after protein extraction process. The relative concentration of amino acids is expressed in relation to total amount of protein present on mixture of protein and chitin.

For the total of seven bands marked with black arrows on Figure 3.6c, for three of these bands representing proteins, amino acid analysis from bands containing purified elution fractions (from tibia) revealed presence of high levels of alanine, cysteine and histidine residues. Consequently, these proteins, marked on Figure 3.8a with * (bands II, V and VII), were considered relevant for determining their sequences (see next section: 3.2.3). In the other hand, protein still attached to chitin after extraction process reveals high level of glycine and alanine.

As observed in Table 3.4 and Figure 3.5, the amount of cuticular proteins extracted from fang cuticle was no too high. For this reason respective chromatography resulted in low amount of protein purified. In these conditions, proteins separated by electrophoresis were not detected in gel and their transference to PVDF membrane, in order to proceed for amino acid analysis, was not possible. Nonetheless, amino acid content of remaining material obtained after protein extraction process was evaluated and results are illustrated by Table 3.7 and Figure 3.9. They suggest a high amount of glycine and alanine, mainly.

Table 3.7. Relative percentage of amino acids of cuticular proteins from fangs of *C. salei* still attached to chitin after extraction.

Protein remaining in the cuticle		Protein remaining in the cuticle	
Amino acids	(%)	Amino acids	(%)
ASP	7.71	ILEU	2.00
THR	1.99	LEU	7.93
SER	2.44	Allo-Ile	0.00
GLU	4.12	TYR	6.46
GLY	23.03	PHE	1.46
ALA	19.60	HIS	5.23
CYS	0.00	LYS	1.93
VAL	7.58	ARG	3.26
MET	0.19	PRO	5.05

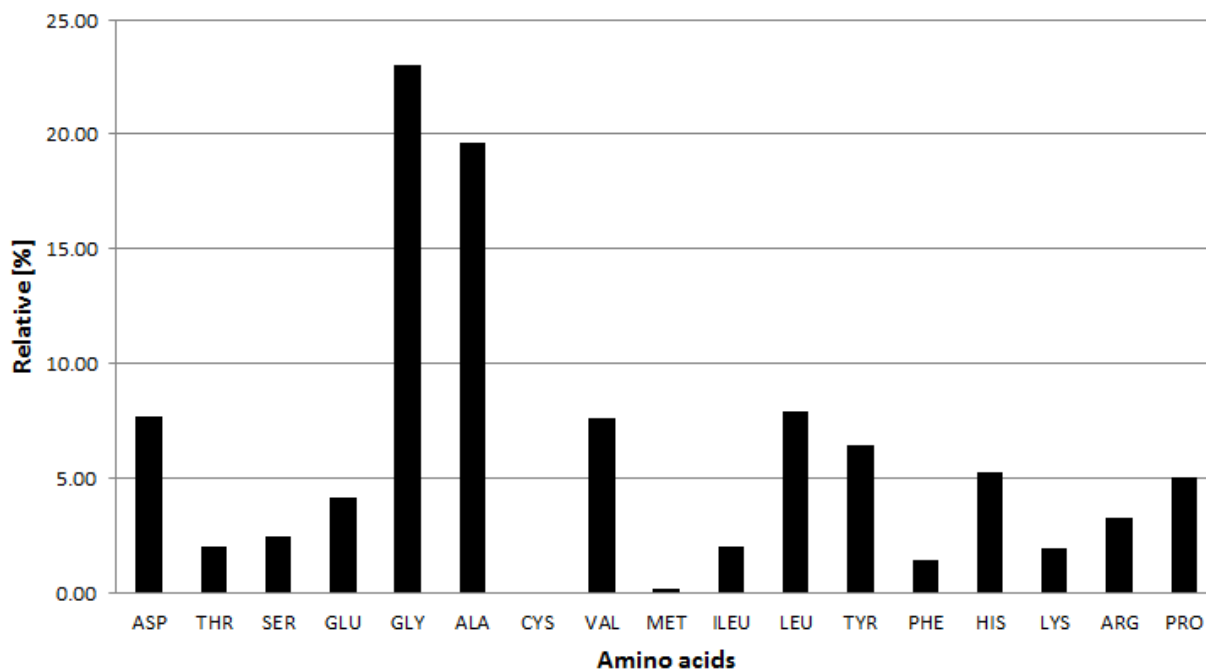


Fig. 3.9. Amino acid content of cuticular proteins attached to chitin of fangs after protein extraction process. The relative concentration of amino acids is expressed in relation to total amount of protein present on mixture of protein and chitin.

3.2.3. Protein sequences and respective homologues

As already mentioned on previous section, three proteins purified from crude extract of tibia and which amino acid content was determined were considered relevant. These proteins are those identified with Roman numbers II, V and VII on Figure 3.6 and which amino acid content result is marked with * on Figure 3.8. Proteins were subjected to protein sequencing by N-terminal analysis using the Edman degradation method. The results obtained are summarized in Table 3.8 in which are also indicated homologies for sequences suggested. Each homologue sequence was obtained by insertion of protein sequences suggested in protein database UniProt.

Table 3.8. Sequences determined in purified protein from cuticle of tibia

SAMPLE	MOLECULAR MASS	SUGGESTED SEQUENCE	HOMOLOGUES	
II (His-rich)	a. b. c. d. e.	~ 30 kDa	${}^1\text{A D V A M N V A G A}^* \text{A Y N F G Q}^*{}_{16}$ ${}^1\text{S N V G M N V A G G}^* \text{G}^* \text{Y N F F}^* \text{Q}^*{}_{16}$ ${}^1\text{S D V A M N V A G A}^* \text{A Y N F N}^* \text{Q}^*{}_{16}$ ${}^1\text{A D V A M N V A G A}^* \text{A Y N F A}^* \text{Q}^*{}_{16}$ ${}^1\text{A D V A M N V A G A}^* \text{A Y N F Y}^* \text{Q}^*{}_{16}$	69 to 73% of identity with Adult-specific rigid cuticular protein of <i>Araneus diadematus</i> .
V (Cys-rich)	~ 14 kDa	${}^1\text{A D V A M N V A G A}_{10}$	No homology found.	
VII (Gly- Ala-rich)	a. b. c.	~ 4 kDa	${}^1\text{S}^* \text{I V L A R}_5$ ${}^1\text{G}^* \text{I V L A R}_5$ ${}^1\text{Y}^* \text{I V L A R}_5$	No homology found.

* Identification of amino acid is uncertain

3.2.4. Occurrence of glycosylations in proteins

Crude extracts of abdominal, leg (tibia) and fang cuticle protein from *C. salei* in double distilled water and in 8 M urea buffer were studied in order to identify presence of glycosylated proteins. Respective products of purification (of crude extracts in urea) were subjected to the same study. The results are shown in Figure 3.9 and indicate that glycosylated proteins occur mostly in cuticle of tibia and fangs. The same results give the idea that proteins purified from cuticle of tibia and fangs do not contain glycosylations. Another interesting aspect of these results is the more frequent occurrence of glycoproteins of low molecular weight as observed also in Figure 3.9.

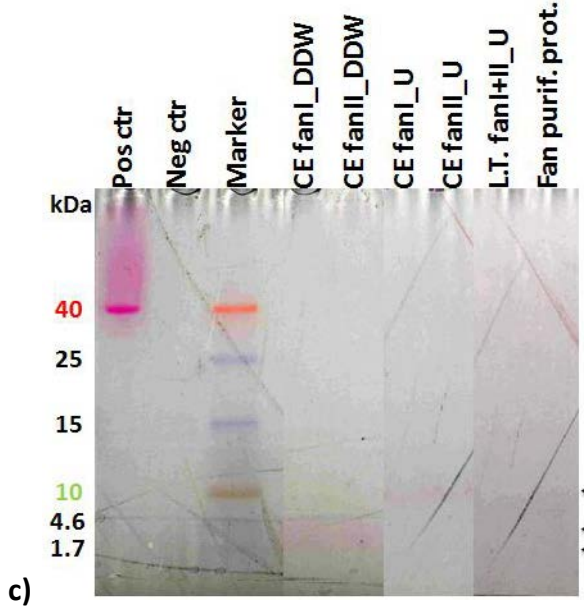
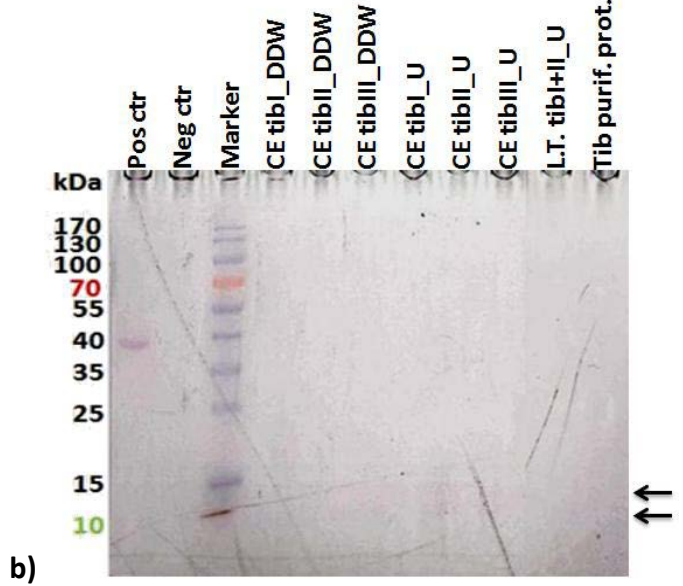
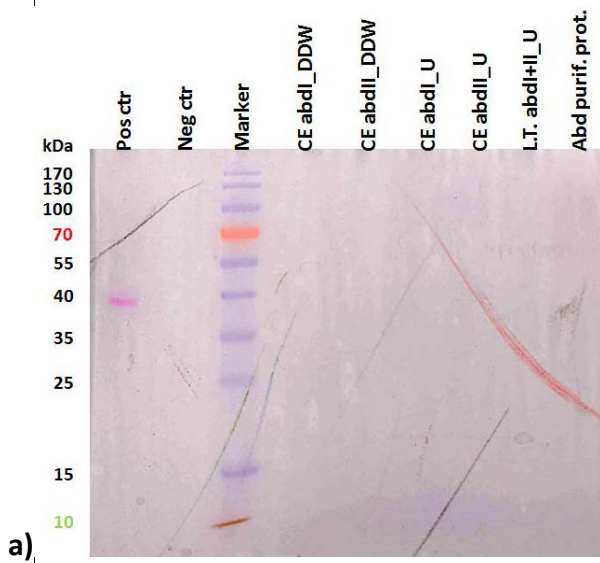


Fig. 3.10. Glycoproteins detection in gels with protein profiles from cuticle of abdomen, tibia and fangs of *C. salei*. a) 12% SDS gel showing cuticular glycoproteins from abdomen. b) 12% SDS gel showing cuticular glycoproteins from tibia. c) 15% SDS gel showing cuticular glycoproteins from fangs. (Pos ctr) positive control; (Neg ctr) negative control); (CE abd) crude extracts of abdominal cuticle in DDW and in urea; (CE tib) crude extracts of tibia cuticle in DDW and in urea; (CE fan) crude extracts of fangs cuticle in DDW and in urea; (L.T. abd) Low-throw abdomen; (L.T. tib) Low-throw tibia; (L.T. fan) Low-throw fangs; (Abd purif. prot.) abdominal purified proteins; (Tib purif. prot.) tibia purified proteins; (Fan purif. prot.) fangs purified proteins. Arrows indicate bands where glycoproteins were detected.

3.3. The chitin and protein ratios in cuticle of different anatomical structures of *C. salei*

By the fact that protein extraction procedures from arthropod cuticles are described in literature as a process not always completely successful, the efficiency of the extraction process was evaluated by infrared spectroscopy. This approach was applied to cuticular powder material, before and after extraction, allowing to obtain differences between initial and final material. The

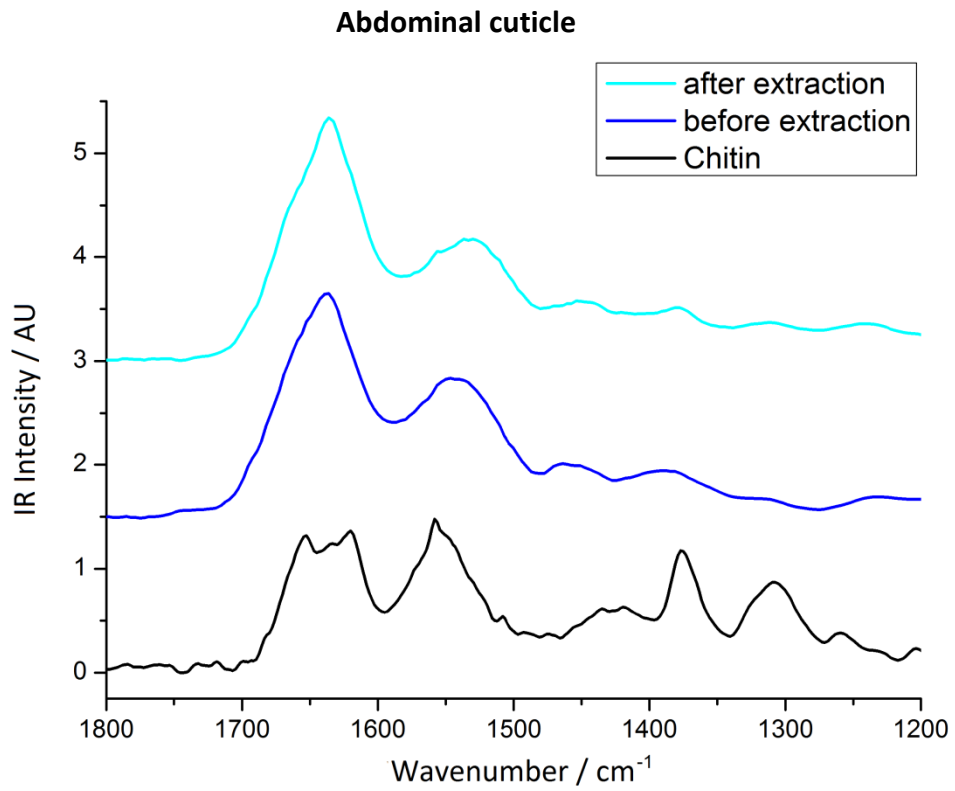
result provides information about present chemical groups in the sample, once the spectrum at the IR region represents the vibration of existent molecules.

Another important aspect in this kind of study is the determination of total amounts of chitin and protein present in different cuticles. This information allows a better understanding of cuticle compositions and helps in assessing the efficiency of protein extraction process.

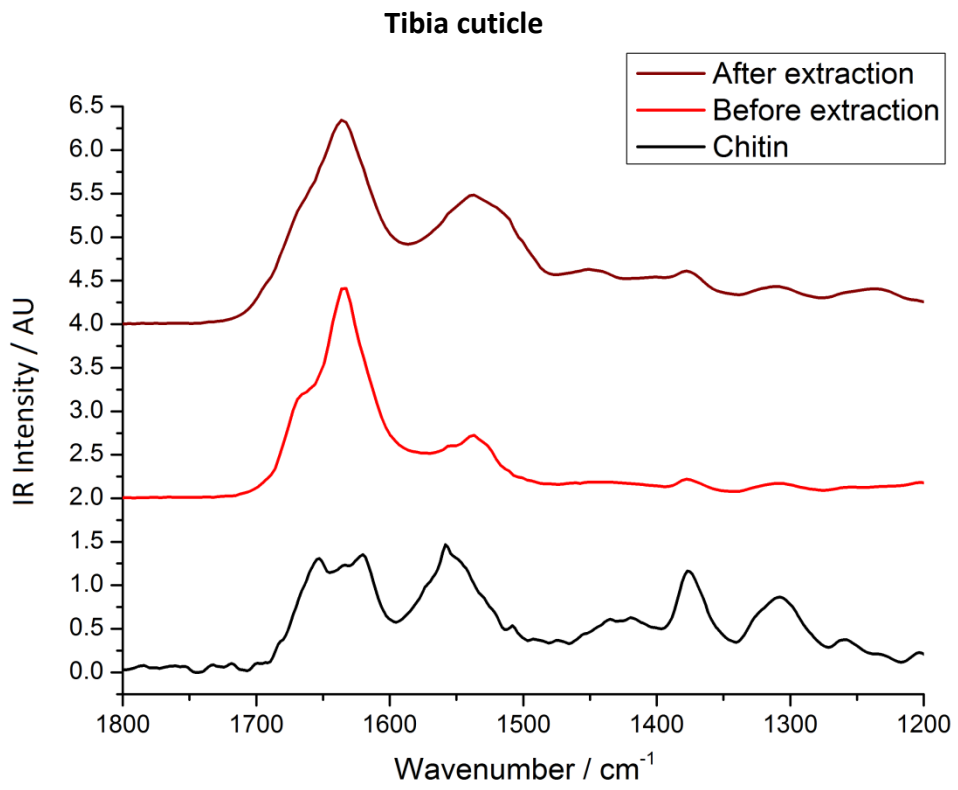
3.3.1. ATR-FTIR of cuticle before and after protein extraction

In order to monitor the protein extraction process, ATR-FTIR measurements were performed on cuticular powder material of abdomen, tibia and fangs, before and after protein extraction (Figure 3.11). The material here considered is the same than that which product of extraction was afterwards subjected to chromatography and each IR spectrum corresponds to the average of spectra for each type of cuticle.

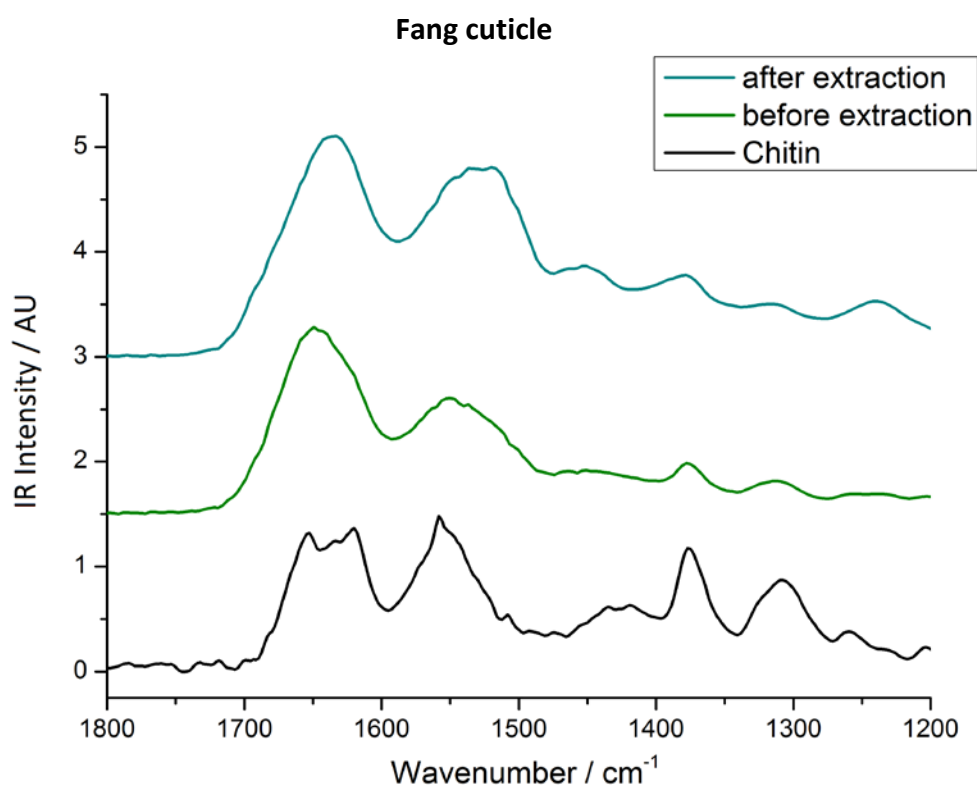
The spectra, which present representation comprises only wavenumbers between 1800 cm^{-1} and 1200 cm^{-1} , were normalized at 3257 cm^{-1} and show clear difference in two main absorption peaks at the region between 1700 cm^{-1} and 1500 cm^{-1} . More exactly, the qualitative information provided is based on difference of intensities of the peaks at 1650 cm^{-1} and 1550 cm^{-1} . So as higher is the first peak in comparison with the second one, larger is the amount of protein present in the considered cuticle. Due to the complexity of the sample, quantitative analysis is not possible.



a)



b)



c)

Fig. 3.11. General relation between protein and chitin contents in cuticles of *C. salei*, before and after protein extraction. a) Remaining material from abdominal cuticle; b) Remaining material from tibia cuticle; c) Remaining material from fang cuticle. Spectra normalization was done at 3257 cm⁻¹.

As expected, chitin:protein ratio decreases from “before protein extraction” to “after protein extraction”. However, it is clear that after extraction there is still protein attached to chitin what is concluded by the intensity contribution still verified around 1550 cm⁻¹, making the spectrum is far from resembling to pure chitin reference spectrum (black).

3.3.2. The chitin content

To determine total amounts of chitin and protein present in cuticles of *C. salei* considered during this work, bleaching of cuticles was performed in 0.3% sodium hypochlorite. This process allowed the complete protein removal from the cuticles of tibia and fangs, what is also seen in the IR spectra (Figure 3.12). For abdomen the IR spectrum shows non-complete oxidation of the

protein. The IR spectra in figure 3.12 are normalized at 3257 cm⁻¹ and comprising wavenumbers only between 1800 cm⁻¹ and 1200 cm⁻¹.

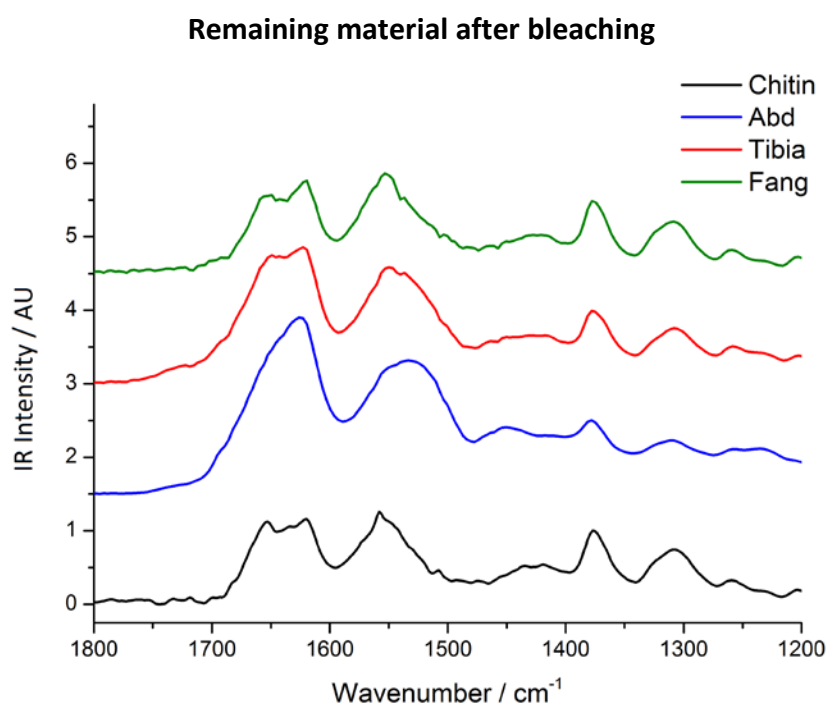


Fig. 3.12. Relation between protein and chitin contents in cuticles from abdomen, tibia and fangs of *C. salei*, before and after bleaching. Spectra normalization was done at 3257 cm⁻¹.

The initial and final dry weights as well as percentages of chitin and protein ratios for each one of the anatomical structures considered are summarized in Table 3.9.

Table 3.9. Initial and final dry weight of cuticle of different anatomical structures of *C. salei* subjected to bleaching in 0.3% of sodium hypochlorite and their chitin:protein ratios.

Cuticles from:	Initial dry weight (mg)	Final dry weight (mg)	Chitin (%) ⁵	Protein (%) ⁵
• abdomen	4.14	1.01	24	76
• tibia	2.88	1.28	44	56
• fangs	1.04	0.33	32	68

The result shows that the abdominal cuticle has the largest amount of protein followed by fangs and, lastly, by tibia.

⁵ Percentage of chitin and protein are relative to the starting material.

Chapter 4

Discussion

4. DISCUSSION

Mechanical properties of cuticle in arthropods are intrinsically correlated with chitin arrangement, degree of sclerotization and hydration. Nevertheless, cuticle properties are also determined by the precise combination of proteins in cuticular matrix (Willis, 1987; Andersen *et al.*, 1995), establishing with chitin different types of interactions, building and determining different structures with specific properties (Willis *et al.*, 2005).

The presence of metallic ions is one of the features associated to cuticular proteins, that greatly contribute to the stability and stiffness of the cuticle, for instance in anatomical structures like-jaws. Among them, divalent transitions ions have received special attention from few years now, the zinc ion being one of the most studied examples (Lichtenegger *et al.* 2005; Bromell *et al.*, 2008; Cribb *et al.*, 2009).

Creation of new generation materials can be supported by this kind of knowledge. Therefore, extensive biomolecular studies on cuticles of arthropods are needed and this was the purpose with which the present project was devised.

The aim of this work was to understand the molecular composition along the cuticle of fangs from *Cupiennis salei*, highlighting the protein content. Thus, the starting point comprised the establishment of a protocol for efficient protein extraction from cuticular anatomical structures – opisthosome (abdomen) and tibia – showing soft and hard characteristics, respectively. Once concluded the protein extraction optimization, the product was subjected to purification following parameters that are in accordance to general features known about fang cuticular proteins: zinc-dependence and histidine-rich content. Reproducible work conditions used in abdomen and tibia were then applied in fangs. Throughout the work, remaining products of extraction were also monitored and analyzed in order to understand the efficiency of the process used, providing information of the remaining material composition.

The first idea becoming clear during this work was the possibility to extract cuticular proteins from *C. salei* in enough quantities to apply then in different approaches (section 3.1). Such can be explained by chitin-protein interactions that is mostly suggested to occur due to cross-linked proteins in antiparallel β -sheet half-barrel structures (between protein-protein and protein-chitin), stabilized by hydrophobic ligations and hydrogen bonds and resulting in helicoidal architectures

(Iconomidou *et al.*, 2001; Hamodrakas *et al.*, 2002; Iconomidou *et al.*, 2005). Depending on protein sclerotization level, this stable structure can be disturbed when the system is exposed to denaturing conditions, such as those created by urea and guanidine leading to cleavage of protein-protein/chitin-protein interactions.

The protocol principle, established through part of the experimental approaches described above, led to the extraction of a wide range of proteins from cuticle. Gel electrophoresis of the extractions from abdomen and tibia showed variable intense proteins bands with two of them around 35 and 25 kDa which occurred in the two cuticles (Figure 3.3b). Thus, the protocol established provides a way to efficiently extract cuticular proteins of different molecular weights (Figure 3.3) and comprising characteristics that make them soluble in different conditions. Considering the differences in amount of protein extracted in urea and in guanidine as well as the desired conditions of the further experiments intended to perform on extracted proteins, the crude extracts of cuticular proteins chosen for the following methodologies were those containing urea.

The established protocol principle clarified that, for abdominal and tibia cuticles, protein extraction in DDW is not efficient – suggesting the absence of water-soluble proteins in the cuticle of these anatomical structures – and emphasized a clear difference between individual and sequential extractions: the exposition of the cuticular powder material to guanidine only shows wide efficiency when the material is not previously exposed to urea. Another aspect that was possible to conclude was the fact of dialysis step causes rapid protein precipitation, situation created by the dialysis conditions applied. For that reason, this procedure was excluded from the protocol although it is known that its use would confer an advantage.

Since the principal aim of this project was to state a biochemical approach to study and characterize Zn-binding cuticular proteins from fangs, at the step of protein purification, it was necessary to create conditions that would prefer the binding of possible proteins of interest. For this reason, protein purification was performed using HiTrap™ IMAC HP loaded with 0.1 M ZnCl₂, the compound which provides the metal ion that occurs physiologically in some cuticular proteins and that specifically attaches to histidine-rich proteins, such as those present in fangs.

The reason for the differences obtained in chromatograms, referring to protein purification from abdominal and tibia cuticles, is mainly due to the amino acid composition of each one of these anatomical structures. Specificity of tibia's cuticular proteins for zinc (appendix Figure I c and d) is not due to physiological occurrence of this metal ion in cuticle of tibia but to amino acid

compositions characteristic to this structure (Figure 3.7) which here shows high content in cysteine, and histidines residues that may bind zinc ions readily. It is known that presence of cysteine residues is not a common feature of cuticular proteins in arthropods but its occurrence might be related with non-cuticular chitin-binding domains (Rebers and Willis, 2001). From what is observed in *C. salei*, these cysteine residues can be either related with this kind of domains or represent a new type of proteins that has not been identified so far in arthropods. Histidine residues are mostly involved in protein sclerotization of hard cuticles (Iconomidou *et al.*, 2005), interacting through intermolecular hydrogen bonding with adjacent proteins side chain – the cross-links (Rebers and Willis, 2001; Politi *et al.*, 2012). Glycine and alanine content can be associated with hydrophobicity level of cuticular proteins – as higher the content of these residues, much hydrophobic the protein is (Andersen *et al.*, 1995).

Furthermore, proteins purified from tibia, to which sequences were determined (Table 3.8), give some promising information:

- Established homology suggests that histidine-rich protein (II) is structural and, as in its homologue, it makes part of the rigid cuticle of the spider. Another important point is that the homologue found has a R&R conserved sequence, what, whether its presence is also possible to prove in this protein identified in *C. salei*, then it could be added to protein databases as a structural cuticular protein identified in a new organism;
- Since the sequenced determined for cysteine-rich protein (V) starts as in histidines-rich proteins (II), this might be another example of structural protein.

In cuticular proteins, both purified from abdomen or attached to chitin after protein extraction process (Table 3.5 and Figure 3.6), characteristics resembling cuticular proteins from locust are revealed. Similarly to what was concluded in previous works, these proteins show:

- High glycine content, usually associated to proteins in the soft intersegmental membranes (Hojrup *et al.*, 1986a);
- High alanine content, which in association with proline and valine (also frequents in abdominal cuticles previously studied), compose the AAPA/V motif (Andersen *et al.*, 1995)
- Presence of glycine, proline and tyrosine, which levels can be associated with protein hydrophobicity.

The last amino acid with relative high percentage in cuticle of abdomen is serine that is not common in cuticular proteins and, when occurs is associated with proteins dominated by disordered regions (Andersen, 2011).

In contrast with our expectations, Table 3.4 and Figure 3.5 show lower signal for stained protein purified from fangs, in comparison with the signal obtained from protein purified from tibia (the other example of hard cuticle analyzed in this context). This aspect, on one hand is explained by the low amount of protein extracted and on the other hand is a result of inefficient protein detection in gel after electrophoresis. As a consequent it was also impossible to transfer these proteins to PVDF membrane for subsequent studies (amino acid analysis and sequencing). For this reason, after extraction process, the only study possible with fangs was the amino acid analysis of the remaining chitin, as shown on Table 3.7 and Figure 3.9. These results show a high level of glycine and alanine residues what is in agreement with what occurs in fangs like-jaws of other arthropods that have been studied so far (Broomell *et al.*, 2008; Miserez *et al.*, 2008). The low amount of protein extracted from fangs gives also idea about the chitin-protein arrangement in the cuticle, suggesting covalent interactions, between proteins and between proteins and chitin, involving sclerotizing cross-linking agents with participation of zinc ions (Fraenkel and Rudall, 1947; Willis, 1999; Iconomidou *et al.*, 2005; Politi *et al.*, 2012).

It is important to note that glycoproteins identification (Figure 3.10) shows that stiff cuticles present glycosylations in their proteins – those extracted in urea and, interestingly, also extracted in DDW, but not for those purified. Studies in the same direction were performed in insects (Willis, 1987; Andersen, *et al.*, 1995) and in crustaceans (Compère *et al.*, 2002) and from what it is seen here, in general, results present in this work follow a different understanding than the one suggested for insects. For example, in insects, glycoproteins are associated with soft cuticles and here there was no detection of glycoproteins from abdomen (the soft cuticle studied). In short, this kind of protein post-translational modifications, that in shell of crustaceans are mostly related to calcification process and in insects are described as a way to modulate binding and recognition between molecules, will need extensive studies in future.

Protein extraction processes, concerning material extracted according to principle protocol established, showed different efficiencies (Figure 3.11). Results are mainly dependent on type of cuticle (soft or hard) due to mechanical properties inherent to each one of them. Note that make straight conclusions about characteristics of each cuticle taking into account the product of extraction might be, most of the times, erroneous. Such happens because the proteins which are extracted from cuticle may not be exactly representative for the total mixture of proteins secreted

from the epidermal cells. For that reason, it is important to stress that the properties reported for proteins extracted from various cuticles may not give a complete picture of the proteins present.

The last important result of this work that in one hand allows a brief summary of this work and in the other hand leaves new open questions, is the determination of chitin and protein ratios in each type of cuticle (Table 3.9). This result revealed differential amounts of chitin and protein in abdominal, tibia and fang cuticles of *C. salei* and, surprisingly, the one with highest amount of protein was cuticle from abdomen. This aspect is not in agreement with what is known about soft cuticles which are usually described as those containing high amount of chitin and low amount of protein (Shawky and Vincent, 1978). Still about this large amount of proteins, they can be related with non-cuticular proteins involved in chitin-binding domains (Rebers and Willis, 2001) or might represent a new idea about soft cuticles, characteristic for spiders. So, this comparison of chitin:protein ratios in different types of cuticles in *C. salei* is another topic that might deserve more attention in order to better characterize cuticles of this organism.

Despite all these promising results, as already mention, protein concentration of crude extracts from fangs didn't allow an extensive study of this anatomical study. Low protein concentration of fractions purified from abdominal cuticle was also the factor that made impossible to determine protein sequences from this anatomical structure.

One of the strategies to overcome this problem would be concentrating the protein amount (by vacuum or lyophilization). However, presence of urea is an obstacle to these techniques, making necessary a prior dialysis of the crude extract or purified proteins. For this reason, optimization of conditions for carrying out dialysis will be imperative to continue these studies.

Chapter 5

Conclusion and Future Perspectives

5. CONCLUSION AND FUTURE PERSPECTIVES

In general, this project contributed to obtain important information regarding soft and hard cuticles of *C. salei* and establishing important notion related to the cuticular protein content of abdomen, tibia and fangs. The results obtained also emphasize how difficult these kinds of studies can be mainly due to the mechanical properties of the materials themselves. The protein content from fangs is especially difficult to analyze what brings new challenges in order to improve the study of this anatomical structure.

Based on data here gathered and in some information obtained so far from *C. salei* and other arthropods, the next approaches should focus on several points. Primarily improve the conditions for protein extraction from fangs, since the protocol established for abdomen and tibia revealed to be not enough for the characteristics of the fangs. The use of ethylenediaminetetraacetic acid (EDTA), prior to protein extraction, might allow zinc removal from the cuticle of the fang allowing easier protein extraction process. Once obtained protein from fang in quantity enough for working conditions, the protein content will be characterized, if possible, taking into account the differential protein distribution along the fang, since it is known that fang has different protein composition along its structure. In order to overcome the problem of low protein bioavailability (in fangs), as soon as partial sequences of proteins of interest are known, their localization *in situ* can be estimated, their correspondent DNA/mRNA can be identified and purified and, with it, it will be possible to make a cDNA library available for further biotechnological studies .

Cupiennius salei is everyday providing more and better information relevant for science and with potential to the humanity. Here it is presented the beginning of a promising study with some details to explore and with multiple doubts to answer.

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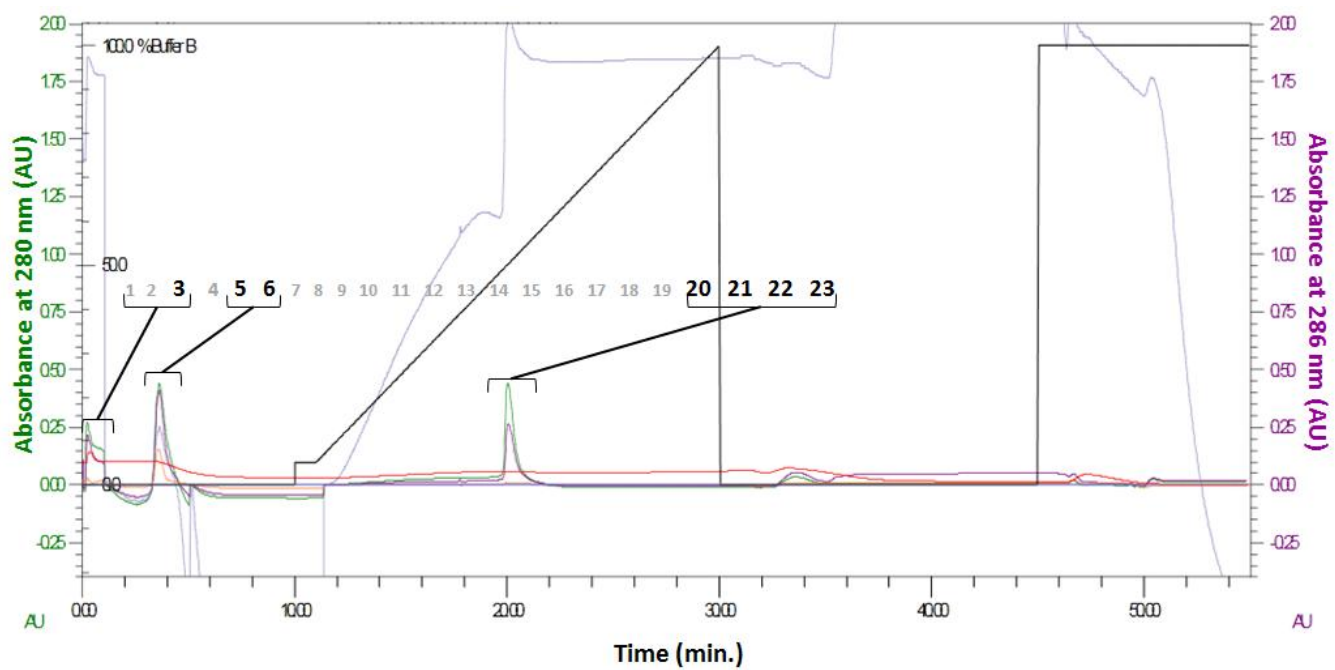
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APPENDIX

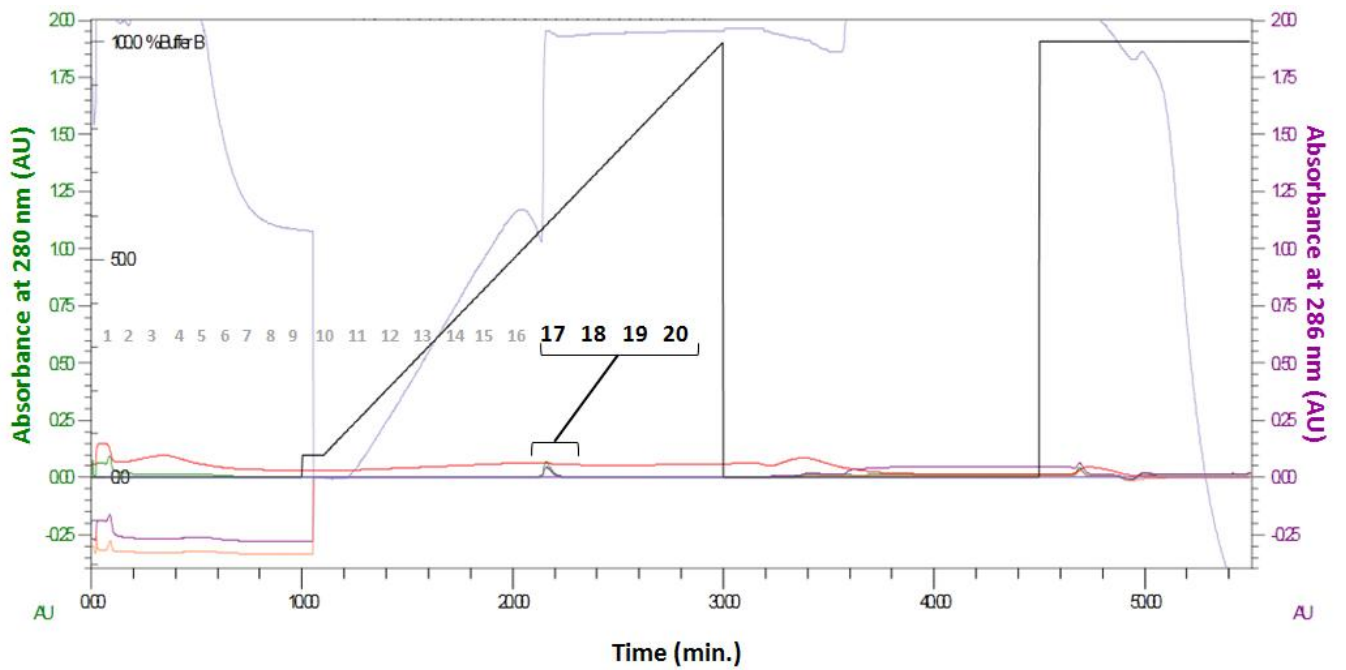
- Appendix I

Chromatogram from crude extract of abdominal cuticle in 8 M urea buffer



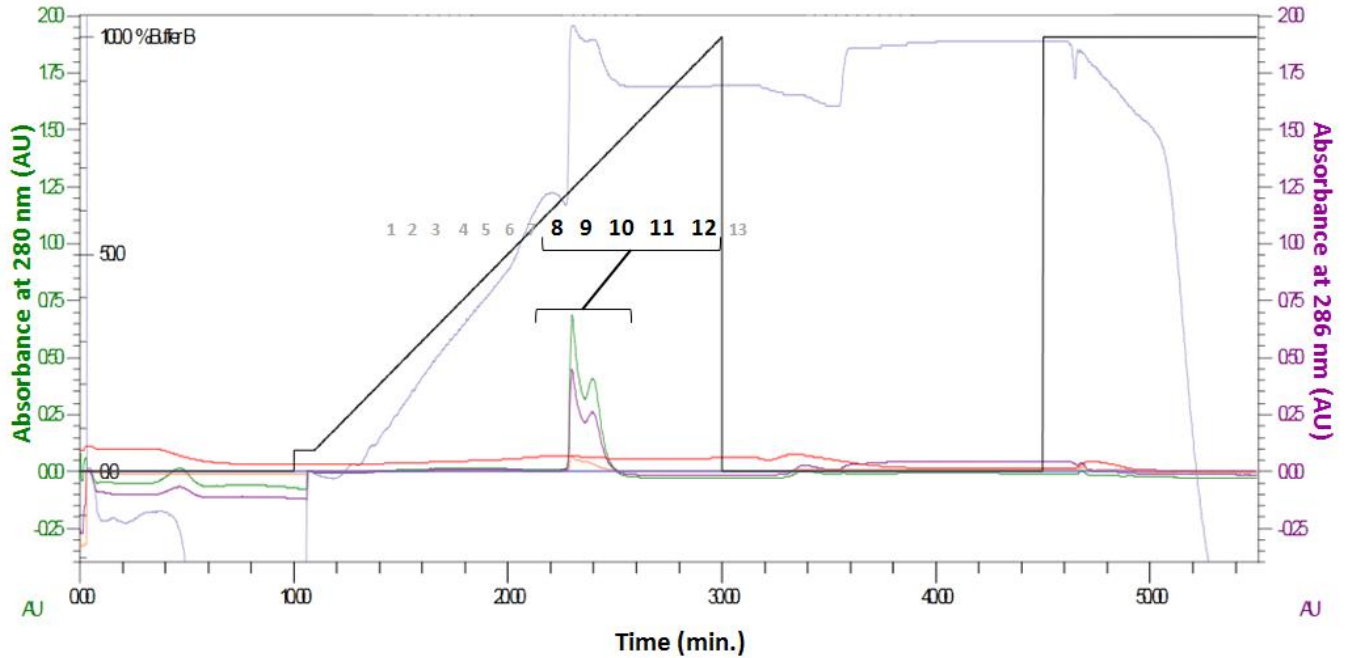
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Chromatogram from crude extract of abdominal cuticle in 8 M urea buffer



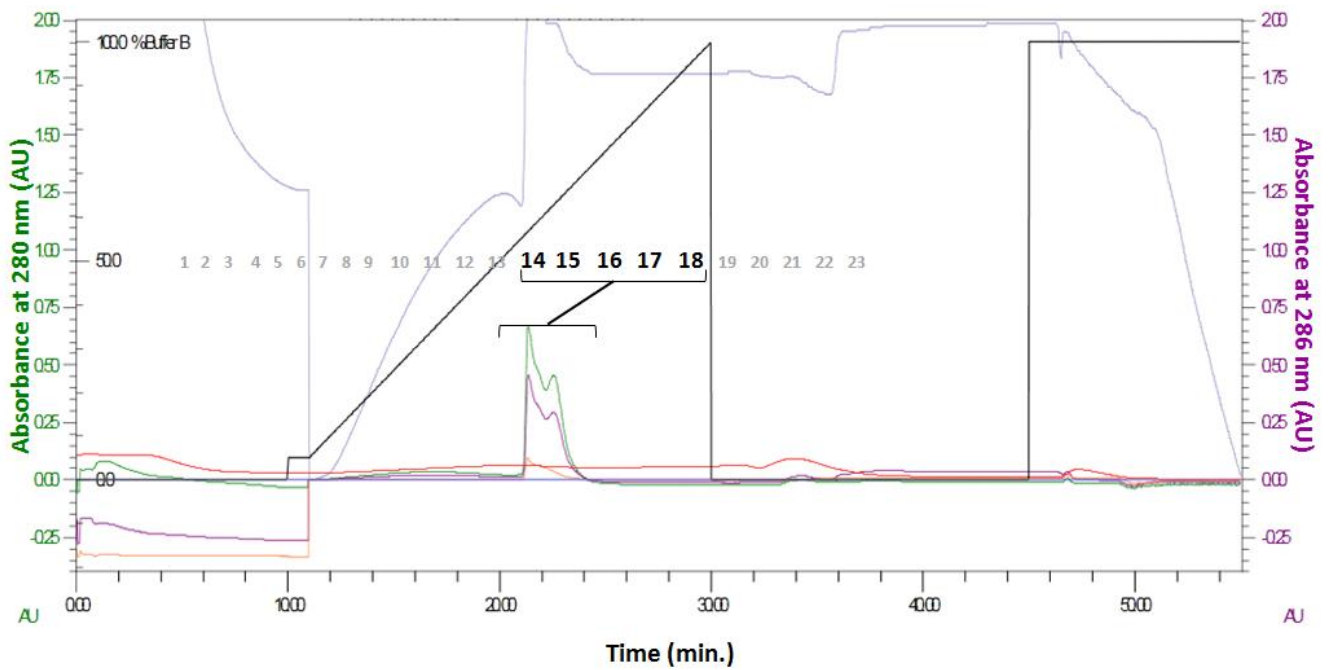
b)

Chromatogram from crude extract of tibia cuticle in 8 M urea buffer



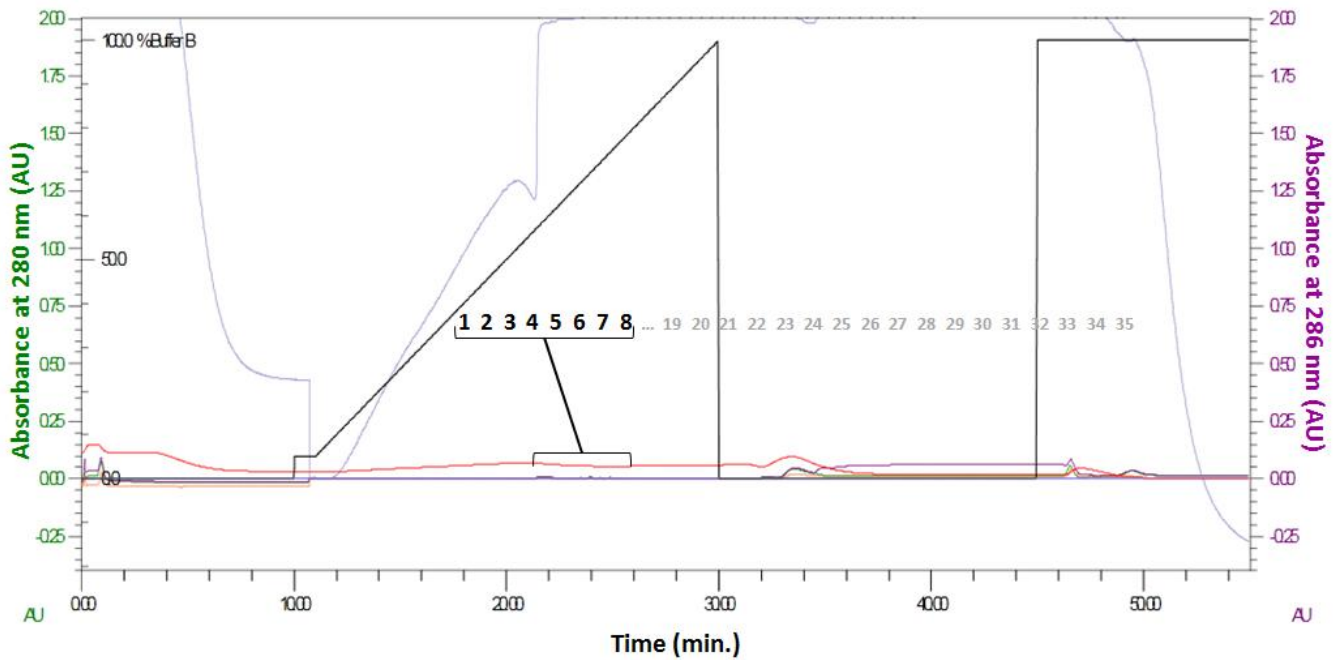
c)

Chromatogram from crude extract of tibia cuticle in 8 M urea buffer



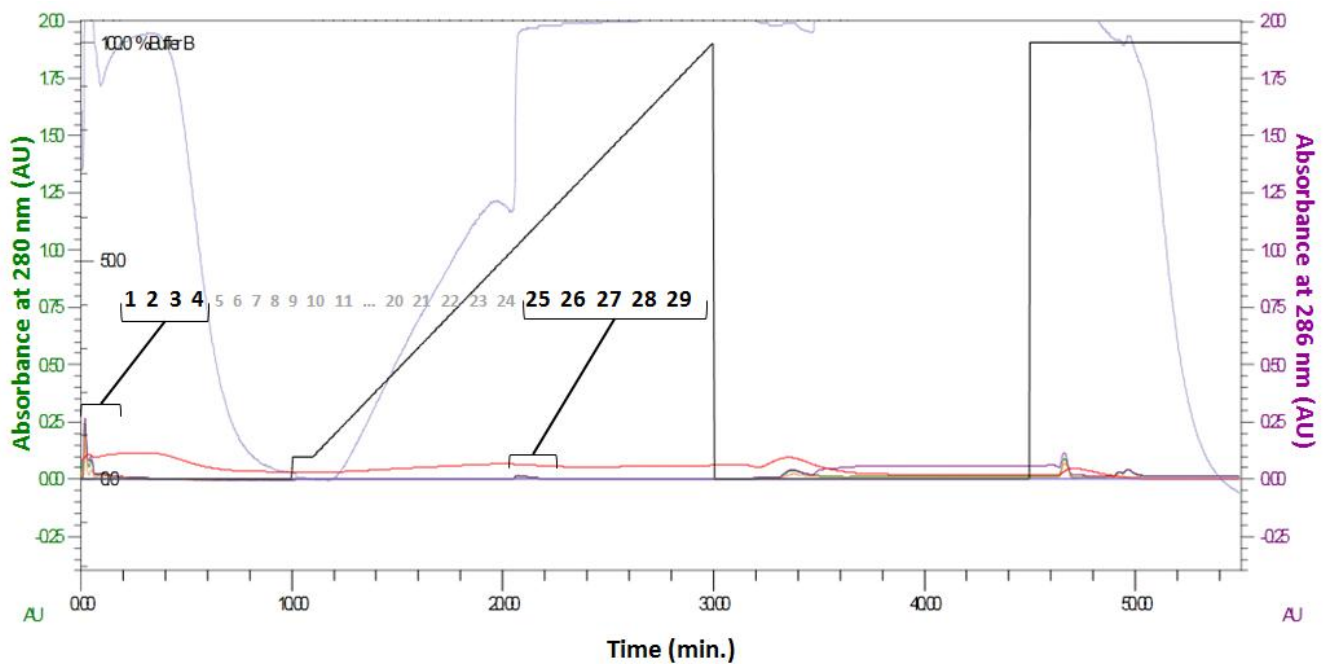
d)

Chromatogram from crude extract of fang cuticle in 8 M urea buffer



e)

Chromatogram from crude extract of fang cuticle in 8 M urea buffer



f)

Fig. 1. Affinity chromatography of crude extracts of abdomen, tibia and fangs in 8 M urea purified in HiTrap™ IMAC HP loaded with 0.1 M ZnCl₂. a/b) Purification of cuticular protein from abdomen; c/d) Purification of cuticular protein from tibia; e/f) Purification of cuticular protein from fangs. Numbers indicated in each chromatogram correspond to elution fractions collected (black – fractions considered relevant; grey – fractions considered irrelevant).

