Molecular cloning of a small heat shock protein (sHSPII) from the cattle tick *Rhipicephalus* (*Boophilus*) *annulatus* salivary gland

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**Abstract**

Immunoscreening of a cDNA expression library of the *Rhipicephalus* (*Boophilus*) *annulatus* tick with purified rabbit anti-*R annulatus* salivary glands antigens polyclonal antibodies led to the identification of a 661 bp sequence. The sequence includes an open reading frame of 543 bp encoding a protein of 180 amino acids with calculated molecular weight of 20.51 kDa, isoelectric point of 9.071 and with no signal sequence. Comparison of the deduced amino acids with protein data bank showed that the identified polypeptide belongs to the alpha crystallin small heat shock proteins superfamily and shows sequence similarity of 62% and 55% to *Ixodes scapularis* fed tick salivary gland protein and *Ornithodoros parkeri* alpha-crystallin protein, respectively. Accordingly, this protein was called Ra-sHSPII. The Ra-sHSPII protein was expressed in *E. coli* under T7 promoter of the pET-30b vector, purified under denaturation conditions and the immunogenicity and cross-reactivity of the recombinant Ra-sHSPII were evaluated. Direct ELISA showed that the Ra-sHSPII is a strong immunogen. In immunoblotting assay the anti-Ra-sHSPII antiserum reacted specifically with purified Ra-sHSPII, with several proteins in *R. annulatus* whole tick, larval and gut protein extracts in addition to *Hyalomma dromedarii* and *Ornithodoros moubata* whole tick protein extracts, as examples of hard and soft tick species, respectively. The rRa-sHSPII protein confers thermal protection to other proteins as found in other sHSPs. *E. coli* cell extracts containing the protein were protected from heat-denatured precipitation when heated up to 100 °C, whereas extracts from cells not expressing the protein were heat-sensitive at 60 °C.

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1. Introduction

Ticks represent the second most important vectors of human diseases (mosquitoes being the first) [1] and the most important vector–transmitting diseases in animals [2] since they are able to transmit more pathogens than any other hematophagous arthropods [3].

Immunization of cattle with tick-protective antigens offers a promising and attractive alternative as it causes no health risks, is environmentally safe and cost efficient [4]. However, identification of new antigens from other tick species is predicted to contribute to tick control by developing a more effective vaccines or polyvalent vaccines effective against numerous tick species [5,6]. Identification, cloning and expression of new protective antigens from ticks remain the rate-limiting step for vaccine development.

Tick salivary glands represent a challenging field of study for protective antigen identification; they play an essential role in blood ingestion and blood meal concentration. Both tick feeding process and pathogen transmission to cattle are facilitated by the numerous components of ticks saliva including anti-haemostatic, anti-inflammatory and immunomodulatory factors [7,8] that prevent tick rejection by the host [8] and blood loss through the feeding lesion [7] in addition to rendering pathogen transmission much more efficient through the lesion [9,10]. Vaccine development from tick salivary glands antigens may adopt one of two main strategies: either by neutralizing the activity of salivary immunomodulatory molecules (also called secretory proteins) [11], or by immunization of cattle by salivary molecules that elicit an appropriate immune response (called non-secretory proteins) [11] that may affect tick life cycle [5,12], fecundity or feeding potential.

Small heat shock proteins (sHSP) are small molecular mass heat shock proteins (monomer ranging from 12 to 43 kDa), that function, in their oligomeric state, as molecular chaperones [13]. Their sequence shows considerable similarity to the alpha-crystallin vertebrate eye lens [14–17]. They are usually characterized by the presence of an evolutionary conserved alpha-crystallin domain (formed of about 100 amino acids) [15,18] in the middle of an N-terminal arm (of variable length) and a poorly conserved C-terminal...
tail/extension [13,19]. To our knowledge, sHSPs in arthropods, including ticks, are not frequently investigated. However, these proteins are intensively studied and characterized in plants and bacteria.

sHSPs perform many cellular functions and help the cell to withstand many types of stresses such as high temperature and oxidative stress [13]. sHSPs, when accomplishing their role as molecular chaperones, are thought to hold unfolded proteins in a soluble, folding-competent state until the intervention of high molecular weight HSPs for subsequent refolding in an ATP-dependant manner [13]. In addition, sHSPs contribute in other important cellular processes, particularly, in shaping and activating the mammalian immune response [20]. These roles of sHSPs stand behind the suggestion of considering them as candidate anti-tick vaccines by [21] who reported the specific upregulation of a salivary shSP of *Ixodes scapularis* tick during engorgement called FTSPS-5 [21].

We aim, through this study and others, to take a preliminary step in identification of possible protective tick antigens and provide some insights about biomolecules involved in tick pathogenesis. Here, we report cloning, expression, and purification of a *Rhipicephalus annulatus* salivary shSP from a cDNA expression library using purified polyclonal rabbit immunoglobulins raised against ticks salivary glands or midgut isolated by dissection from adult *R. annulatus* using purified polyclonal rabbit immunoglobulins raised against tick salivary proteins. Also we show the ability of this protein to protect most proteins in *E. coli* cell extracts from thermal aggregation at a very high temperature. This molecule, besides presenting some insights about biomolecules involved in tick pathogenesis, in identification of possible protective tick antigens and provide vaccines by [21] who reported the specific upregulation of a salivary antigen. The antigens dissolved in 0.5 ml of saline (0.9% NaCl) were injected on day 0. The rabbit was boosted by 50 μg/ml chromatographically purified polyclonal rabbit anti-*R. annulatus* salivary antigens in PBS containing 5% dry milk following the immunoscreening protocol described by Sambrook and Russell [24]. Prior to sequencing, the cloned cDNAs resulting from immunoscreening were subjected to dot blot hybridization in order to put them in homogenous groups. Briefly, cloned cDNAs were amplified by PCR, and subsequently the product was denatured by boiling for 5 min. The denatured products were blotted onto 0.45 μm (Nytran1-0.45 from Schleicher and Schuell, Dassel, Germany). The membranes were dried at room temperature for about 30 min and subsequently fixed in an oven at 80°C for 2 h. The longest cDNAs obtained from immunoscreening were Dig-labeled using Digoxigenin DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) and used as a probe. Hybridization was performed in a buffer containing 50% deionized formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0), 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS and 2% (w/v) blocking agent, overnight at 42°C. The membranes were washed twice 15 min each to a final stringency of 0.5× SSC and 0.1% (w/v) SDS, at 65°C and then detection was carried out using anti-Digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:10,000. To isolate a full sequence cDNA, the library was further subjected to plaque hybridization screening, using the longest cloned cDNA resulting from immunoscreening as a probe. Dig-labeling of the probe and hybridization were carried out as described above. The positive plaques were detected after incubation of filters with anti-Digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:10,000. The positive phages were cloned, and the inserts were excised with the ExAssistTM Helper Phage (Stratagene, Corporation La Jolla, CA, USA). The sizes of the inserts were determined by PCR amplification of the plasmids using M13 forward and reverse primers, followed by agarose gel electrophoresis, and phagemid DNA of positive clones was sequenced.

### 2. Materials and methods

#### 2.1. Preparation of whole tick, larval, salivary and gut antigens

Whole tick, salivary, larval and gut antigens of *R. annulatus* were prepared according to the methods described by Shahein et al. [22]. In brief, laboratory reared, clean, 5–6-day-old unfed ticks, larvae, salivary glands or midgut isolated by dissection from adult ticks were homogenized in cold buffer A which includes, 0.15 M NaCl, 10 mM phosphate-buffered saline (PBS), 1 mM disodium EDTA, pH 7.2, and protease inhibitors cocktail (Sigma–Aldrich, Saint Louis, MO, USA). The extracts were then filtered, sonicated and centrifuged at 15,000 × g for 60 min at 4°C. The supernatants were designated as whole tick, salivary, larval or gut antigen. The protein concentrations of the antigens were estimated according to the method of Bradford [23].

#### 2.2. Preparation of rabbit anti-salivary antigens

For raising anti-salivary antibodies of *R. annulatus*, a male rabbit (3 kg) was immunized by intramuscular injection with 100 μg of salivary antigen. The antigens dissolved in 0.5 ml of saline (0.9% NaCl) and mixed with an equal volume of Freund’s complete adjuvant (DIFCO Laboratories Detroit, Michigan, USA) were injected on day 0. The rabbit was boosted by 50 μg of the same antigens mixed with Freund’s incomplete adjuvant (DIFCO laboratories Detroit, Michigan, USA) on day 14 by the same route. Seven days after boosting, the rabbit was bled from the marginal ear vein, the serum was pooled, and the immunoglobulins were purified by affinity chromatography using protein G-sepharose CL-4B (GE healthcare, UK) according to the instructions of the manufacturer.

#### 2.3. Immunoscreening of *R. annulatus* cDNA library

A titer of 4.2 × 10⁵ plaque-forming units (pfu) from *R. annulatus* λZAP Express II cDNA library were immunoscreened with 0.5 μg/ml chromatographically purified polyclonal rabbit anti-*R. annulatus* salivary antigens in PBS containing 5% dry milk following the immunoscreening protocol described by Sambrook and Russell [24]. Prior to sequencing, the cloned cDNAs resulting from immunoscreening were subjected to dot blot hybridization in order to put them in homogenous groups. Briefly, cloned cDNAs were amplified by PCR, and subsequently the product was denatured by boiling for 5 min. The denatured products were blotted onto 0.45 μm (Nytran1-0.45 from Schleicher and Schuell, Dassel, Germany). The membranes were dried at room temperature for about 30 min and subsequently fixed in an oven at 80°C for 2 h. The longest cDNAs obtained from immunoscreening were Dig-labeled using Digoxigenin DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) and used as a probe. Hybridization was performed in a buffer containing 50% deionized formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0), 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS and 2% (w/v) blocking agent, overnight at 42°C. The membranes were washed twice 15 min each to a final stringency of 0.5× SSC and 0.1% (w/v) SDS, at 65°C and then detection was carried out using anti-Digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:10,000. The positive plaques were detected after incubation of filters with anti-Digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:10,000. The positive phages were cloned, and the inserts were excised with the ExAssistTM Helper Phage (Stratagene, Corporation La Jolla, CA, USA). The sizes of the inserts were determined by PCR amplification of the plasmids using M13 forward and reverse primers, followed by agarose gel electrophoresis, and phagemid DNA of positive clones was sequenced.

#### 2.4. Expression in BL21 (DE3) and purification

The prokaryotic expression vector pET30b (Novagen Inc., Madison, USA) that carries the T7 promoter and Kanamycin resistance gene was used to express the *R. annulatus* small heat shock protein II gene (shHSPII). From the sequence of the clone of *R. annulatus* shHSPII, two primers (synthesized by Bioneer Corporation, Taejon, South Korea), FecoRV (5′-CGG GAT ATC GTT CTG TGG TTC AAG TGA-3′) and RXhoI (5′-CGG CTC CAG GAT CCG TCG GCC AGC AAC AG-3′), were designed for PCR amplification of the ORF of *R. annulatus* shHSPII. The primers FecoRV and RXhoI contained EcoRV and XhoI restriction sites, respectively. These sites were also present as unique sites in the cloning region of the pET30b expression vector (Novagen Inc., Madison, USA), ensuring correct orientation of the insert. To ensure fidelity, PCR was performed using platinum pfu-DNA polymerase (Fermentas, Burlington, ON, Canada) that has proofreading capacity. PCR product and vector were digested with EcoRV and XhoI (Fermentas, Burlington, ON, Canada) before ligation. The ligated construct was transformed into BL21 (DE3) and expression was induced for 5 h at 37 °C. After expression, the recombinant *R. annulatus* shHSPII was affinity purified under denaturation conditions using the Qiagen Ni-NTA agarose matrix (Qiagen, Hilden, Germany). Following the instructions of the manufacturer. Briefly, bacterial cell pellets were thawed on ice for 15 min and...
resuspended in 1–3 ml of denaturing lysis/washing buffer (100 mM HEPES, 10 mM imidazole, pH 7.5 and 8 M urea). The mixture was incubated with agitation for 1 h at room temperature. Then, the lysate was centrifuged at 10,000 × g for 15 min and the supernatant (clear lysate) was immediately used or stored in −20 °C until subsequent use.

The histidine tagged protein was eluted using the elution buffer containing 8 M urea, 100 mM HEPES, and 1 M imidazole, pH 8.

2.5. DNA sequencing and data analysis

DNA sequencing was performed on an ABI-PRISM 310 automated DNA sequencer (Perkin-Elmer, Foster City, CA) at the DNA Sequencing Facility, VACSERA, Cairo, Egypt. Sequences were analyzed using the analysis software from the Expasy website (http://www.expasy.org) and the centre for biological sequence analysis (http://www.cbs.dtu.dk).

2.6. Polyacrylamide gel electrophoresis

The electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA). Preparation of gels, samples, and electrophoresis were performed according to the conditions described by Laemmli [25].

2.7. Enzyme-linked immunosorbent assay

Direct ELISA was performed according to the method of Ricoux et al. [26]. ELISA plate was coated with 1.5 μg/well of the purified rRa-sHSP II in 100 μl of coating buffer (50 mM carbonate buffer, pH 9.6). The plates were incubated overnight at 4 °C and the empty sites were blocked by incubation with 2.5% BSA in coating buffer, pH 7.4 for 1 h at 37 °C. Serial dilutions ranging from 3.7 to 0.2 × 10⁻⁴ of the preimmune (normal rabbit serum) and rRa-sHSPII immune rabbit serum in serum buffer (0.5% BSA in PBS, pH 7.4 containing 0.05% Tween 20) were added and incubated at 37 °C for 1 h. Anti-rabbit IgG alkaline phosphatase conjugate (Sigma–Aldrich, Saint Louis, MO, USA) at a dilution of 1:5000 was used for 1 h incubation. Biotinylated goat anti-rabbit IgG antibody (Sigma–Aldrich, Saint Louis, MO, USA) at a dilution of 1:1000. Then, the antigen–antibody reaction was detected by incubating the membranes with anti-rabbit IgG peroxidase conjugate (Sigma–Aldrich, Saint Louis, MO, USA) at a dilution of 1:2000 in serum buffer for 1 h with gentle agitation at room temperature. The protein bands were visualized by incubating the membranes for 15–30 min in freshly prepared 4-chloro 1-naphthol (4C–1N) developing solution (30 mg 4C–1N) (MP Biomedicals, Inc., Fountain Pkwy, OH, USA) in 10 mM TBS containing 20% methanol and 0.06% H₂O₂. After color development, the membranes were washed twice with distilled water for about 30 min to stop the reaction, air-dried, and then photographed.

2.9. Thermal stability

Molecular chaperones and sHSPs are known to confer stability to other soluble proteins following thermal stress. Accordingly, thermal stability of bacterial protein extracts expressing Ra-sHSPII was assessed according to the method of Kim et al. [28]. Briefly, a bacterial culture of BL21 (DE3) transformed with recombinant pET30b-Ra-sHSPII was induced using 100 mM IPTG at OD 0.9–1.1 for 2 h. After centrifugation, the bacterial pellet was washed twice and then resuspended in buffer B (25 mM Tris-base, 10% glycerol, 2 mM DTT and 1 mM EDTA). The same protocol was followed for BL21(DE3) E. coli culture transformed with recombinant pET30b-BaGST [22], as a possible control expressing a protein that does not confer thermal stability.

Following sonication and centrifugation as described earlier, 500 μl aliquots of the supernatants were heated to 50, 60, 80 and 100 °C for 20 min. After cooling, samples were centrifuged at 10,000 × g for 5 min to precipitate coagulated proteins and the protein content in the clear supernatant was determined according to Bradford’s method. Coagulated proteins were solubilized in 50 μl of reducing SDS-PAGE loading buffer. Volumes corresponding to 20 μl of either soluble proteins or coagulated solubilized proteins of both Ra-sHSPII and Ba-GST were loaded on 12% SDS-PAGE that was subsequently stained with Coomassie blue stain.

3. Results

3.1. Immunoscreening of Rhipicephalus annulatus cDNA expression library and cDNA sequence analysis

Immunoscreening of a λZAP Express II cDNA library previously constructed from adult R. annulatus whole ticks, salivary glands, gut, ovaries and eggs with purified polyclonal rabbit anti-R. annulatus salivary glands antigens revealed several positive clones from a total of 4.2 × 10⁶ pfu screened. All clones were isolated and sequenced in their entirety. The sequencing results revealed that 4 positive clones contained 661 bp with a start codon (ATG) from the 5′-UTR to position 56 in frame and a stop codon (TGA) at position 596 downstream of the 5′ cDNA end, forming a single open reading frame (ORF) of 540 bp. The nucleotide sequence carried 63 bp at the 3′-untranslated region (3′-UTR) which, in turn, showed two possible sites of polyadenylation signals AAATAAA and AAATAAA at 626 and 656 bp respectively. The polyA tail, however, was not included within the cloned and sequenced fragment. The 3′-UTR showed also a dinucleotide (TG) Simple Sequence Repeat (SSR), i.e. a microsatellite starting from position 604. The complete nucleotide sequence
shown in Fig. 1.

of the identified gene and its deduced amino acid sequence are used for the subsequent gene amplification by PCR. The start ATG and stop TGA codons are in bold italic format. Underlined bold characters denote the two possible sites of polyadenylation signal. The grey highlighted sequence is a microsatellite of dinucleotide repeat. This sequence has been submitted to GenBank and assigned the accession number (HM149782).

Homologues of the deduced amino acid sequence were searched for in GenBank database using the BLAST algorithm. The amino acids sequence showed high degree of similarity to previously identified heat shock proteins (particularly the alpha crystallin heat shock proteins) including those of some tick species (I. scapularis and Ornithodoros parkeri), Caenorhabditis elegans and Homo sapiens' B chain of α crystallin in a percentage of 62.2%, 55%, 20% and 17.2%, respectively. The deduced amino acid sequence was subjected to alignment with these peptides using the Dialign software of the Expasy website (www.expasy.org) and the high score observed reinforced the suggestion that this sequence codes for an alpha crystallin member of the heat shock protein family. Fig. 2 shows multiple sequence alignment of the amino acid sequence of Ra-sHSPII with other heat shock proteins in the databases. The sequence was thus named Ra-sHSPII (for R. annulatus small Heat Shock Protein II), and its cDNA sequence was submitted to GenBank and assigned the accession number (HM149782).

However, despite the absence of both N-terminal signal peptide sequence and strong hydrophobic regions within the Ra-sHSPII protein, the protein was found to have a NN-score (Neural Network score) of 0.718 according to the Secretome P-2 server of the CBS [33]. To the best of our knowledge, this server is the only one that predicts non-classically secreted protein. Since these proteins are devoid of signal motifs, the Secretome P-2 server uses an artificial neural network that predicts non-classical secretion based on other sequences known to follow this pathway. Sequences exceeding the threshold value (0.5) are predicted to be non-classically secreted. Since the NN score of Ra-sHSPII exceeded the threshold by 0.218, it may represent a “leaderless” secretory protein, i.e. the Ra-sHSPII may follow an unusual pathway for secretion.

3.2. In vitro expression and purification of the Ra-sHSPII gene

The expression of Ra-sHSPII was carried out in E. coli BL21 (DE3), using the prokaryotic expression vector pET30b. 510 bp of the coding region of the Ra-sHSPII cDNA was introduced and expression was induced using IPTG at a final concentration of 1 mM at 37 °C.

Analysis of the bacterial lysate by 12% SDS-PAGE before and after addition of the IPTG showed an overexpressed protein having a molecular weight of 30 kDa approximately (Fig. 3a). The purification of the Ra-sHSPII protein was performed using the IMAC system of Qiagen Ni-NTA agarose matrix. The Ra-sHSPII protein was successfully eluted when purified under denaturing conditions.
Fig. 2. Alignment of the deduced amino acid sequence of *R. annulatus* sHSPII with *Ixodes scapularis* fed tick salivary protein 5 (AY788855), *Ornithodoros parkeri* alpha crystallin (EF633915), *C. elegans* heat shock protein 16.2 (AC006774) and *Homo sapiens* alpha crystallin of the basic type (NM001885). Highlighted amino acids represent similarities between species while the squared F residue is conserved in the metazoan alpha crystallin domain but not in the non-metazoan.

Fig. 3. (a) Analysis of *E. coli* BL21(DE3) lysate before and after protein expression. 12% SDS-PAGE of low molecular weight marker (Amersham, UK), lane (1); bacterial lysate prior to the addition of IPTG, lane (2); and bacterial lysate following the addition of IPTG, lane (3). The arrow shows the 30 kDa band corresponding to the recombinant Ra-sHSPII protein. (b) Purification of the recombinant Ra-sHSPII protein using Ni-NTA column under denaturing conditions. 12% SDS-PAGE of low molecular weight marker, lane (1); column flow through, lane (2); and elution fractions of the purified sHSPII, lanes (3) and (4). Elution was performed using 100 mM HEPES, 8 M urea and 1 M imidazole in elution buffer.

using 8 M urea as denaturing agent in all buffers and 1 M imidazole in elution buffer (Fig. 3b).

Prior to rabbit immunization, fractions containing the highest recombinant Ra-sHSPII protein concentration (as estimated by Bradford method) were pooled and dialysed against 0.1 M PBS, pH 7.

3.3. Immunogenicity analysis of rRa-sHSPII

3.3.1. Production of anti-rRa-sHSPII protein and antibody titration

A 3 kg Chinchilla bastard rabbit was immunized with purified rRa-sHSPII (20 μg as an initial dose and 15 μg in two booster doses at 2 weeks intervals). Sera were collected before injection and 2 weeks after each injection (Fig. 4).

Antibodies titers of rabbit anti-rRa-sHSPII were 1:3100, 1:3320 and 1:4000 after the first, second and third immunization doses, respectively while the normal rabbit serum cross-reacted with rRa-sHSPII giving a titer of 1:746.

3.3.2. Ra-sHSPII immunogenicity and its cross-reactivity with other tick species

To confirm the immunogenicity of the rRa-sHSPII protein, rabbit anti-rRa-sHSPII antiserum were reacted with the purified rRa-sHSPII protein employing the immunoblotting technique at the expected molecular weight of about 30 kDa (Fig. 5a-lane 1).
In a similar way, rabbit anti-rRa-sHSPII antisera were used to detect the presence of Ra-sHSPII similar or related proteins in R. annulatus whole tick, gut and larval protein extracts. Several cross-reactive proteins were detected in each of the previous extracts. In R. annulatus whole tick protein extract, only a small molecular weight band was cross reacted with the anti-Ra-sHSPII antisera, with molecular weight around 13 kDa. In respect to the gut protein extract, four cross-reactive protein bands were detected with molecular masses of 55, 25, 22, and 13 kDa, while in the larval protein extract the cross-reactive protein bands were mainly located between 25 and 13 kDa (Fig. 5a).

In order to detect the presence of similar or related proteins in other tick species, protein extracts from Hyalomma dromedarii whole tick and larvae in addition to Ornithodoros moubata whole tick protein extracts were reacted with rabbit anti-rRa-sHSPII (using the same dilutions as previous). Rabbit anti-rRa-sHSPII antibodies detected four strongly cross-reactive protein bands ranging from about 85 to 13 kDa in H. dromedarii whole tick extract while in H. dromedarii larval protein extract two cross-reactive protein bands were detected at about 25 and 13 kDa. On the other hand, two protein bands around 17 and 13 kDa in the O. moubata whole tick protein extract were detected (Fig. 5b).

### 3.4. Thermal stability

Upon expression of the Ra-sHSPII and the Ba-GST in BL21 (DE3) cells and the subsequent heat treatment of the extracts, bacterial extracts expressing the rRa-sHSPII showed higher thermal tolerance than those expressing Ba-GST. Several bacterial proteins were thermally degraded or precipitated in the insoluble fraction at temperatures 50, 60 and 80 °C (Fig. 6a). These results are especially prominent regarding the high molecular weight fraction. No important differences were observed between extracts expressing Ra-sHSPII and Ba-GST when heated to 100 °C. SDS-PAGE analysis of the coagulated proteins showed higher bacterial protein and higher recombinant protein concentration in case of Ba-GST than in the case of Ra-sHSPII (Fig. 6b), thus confirming the above results.

### 4. Discussion

Cattle infestation by ticks, one of the most dangerous blood sucking ectoparasites, represents a major veterinary problem not only in tropical and subtropical areas, but also in many temperate areas of the world [34]. In all areas where ticks infest cattle, affection of growth, anemia, decreased milk production, and leather damage are recorded. In addition, the transmission of a wide variety of pathogenic organisms seriously affects livestock industry in affected areas. Therefore, infestation by ticks does not only impair an important source of animal protein but also results in subsequent important economical losses.

Numerous approaches have been adopted for the control of cattle ticks. Among the most successful was the immunological approach, i.e. vaccination. This approach evolved from the simple observation that repeated infestation by ticks resulted in ‘tick
immunity", to the use of crude tick protein extract for immuniza-
tion [35–38], until the use of molecular cloning techniques for
production of recombinant protective antigens, the pioneer being
the rBm86 protein produced in E. coli [39] and Pichia pastorii yeast
[40].

Some drawbacks of the currently available vaccines encour-
aged scientists to search for new protective antigens, in an attempt
to develop a universal polyvalent vaccine, effective against sev-
eral tick species [41–44]. Besides necessitating the identification
of new protective antigens, development of new anti-ticks vaccines
requires a thorough understanding of tick's general physiology,
particularly the feeding process. Ticks' salivary glands are a reser-
voir of a wide range of biologically active molecules that affect
ticks' feeding, life cycle and infectivity. Studying the structural and
biochemical properties of these molecules may enhance our under-
standing of the feeding process in ticks and will provide extensive
insights about promising protective antigens.

In the present study, specific cDNA library of the local Egypt-
tian ticks R. annulatus (generated from the genetic material of the
gut, salivary glands, eggs and whole ticks), was immunoscreened
using purified polyclonal rabbit anti-R. annulatus salivary glands
antigens. Four clones contained a 861 bp sequence that forms a sin-
gle open reading frame (ORF) of 540 bp. The identified ORF encodes
a polypeptide of 180 amino acids, of calculated molecular weight
of 20.507 kDa. According to the GenBank database BLAST algorithm,
this polypeptide belongs to the alpha crystallin heat shock pro-
teins superfamily as confirmed by the Scanprosite server of the
"PROSITE database of proteins domains, families and functional
sites" [31,32]. It shares with many shSP a conserved region called
alpha-crystallin domain extending from amino acid D75 to amino
acid R159. The predicted protein sequence shows 62% and 55% sim-
ilarity to I. scapularis fed tick salivary gland protein and O. parkeri
alpha crystallin protein, respectively. According to the prediction
server of the CBS, the Ra-shSPII polypeptide lacks an N-terminal
signal sequence and is, therefore, a non-secretory protein.

Thus, it can be inferred that Ra-shSPII may be a cytosolic non-
secretory protein. However, presence of the Ra-shSPII in other
acellular compartments or even as a transmembrane protein can-
not be ruled out. This contradictory fact is reported by several
authors for other shSP. shSPs are known to be "ubiquitous in
term of cellular localization as well as in the biological world"
[13]. Some shSPs appear to be membrane-associated in Toxoplasma
gondii [45]. More interestingly, membrane-associated shSPs do not
contain the characteristic hydrophobic transmembrane domains
or signal sequences that usually target nascent peptides towards
the cell membrane [13]. These findings encouraged analysis of the
Ra-shSPII by the Secretome P-2 server of the CBS, that predicts
secretory proteins lacking the appropriate signal sequence [33].
Indeed, the score of the Ra-shSPII indicated that it may represent
a "leaderless" secretory protein, i.e. a protein following a non-
classical secretory pathway. Several examples of non-classically
secreted proteins have been identified and are known to escape the
endoplasmic reticulum–Golgi network complex [46,47]. This fact
may outline the ability of shSP to move from one cellular compart-
ment to another. shSP of Drosophila salivary glands are cytoplasmic
after mild to moderate heat shocks and nuclear after severe heat
shocks [48]. However, if confirmed to be true for Ra-shSPII, the
non-classical secretion pathway challenges the glycosylation pat-
tern predicted for Ra-shSPII; if the protein is not secreted via the
endoplasmic reticulum, it would not be subjected to glycosylation
[33].

As previously described, one of the main and most studied role of
shSPs is their role as molecular chaperone that maintain unfolded
or disfolded proteins in a folding-competent state until adequate
refolding. Considering that the feeding process in ticks is associ-
ated with a rise in temperature [49]; and that accumulation of HSPs
results in thermotolerance, which is the "cell's ability to withstand
an otherwise lethal thermal challenge" [50], the role of shSP as
molecular chaperone in tick salivary gland can easily be predicted;
they may act to confer protection to the whole organism or to the
salivary glands in particular in order to withstand the increase in
temperature and other stresses following ingestion of the blood
meal. This assumption supports Xu et al. [21] observation of the
specific upregulation of a salivary shSP of I. scapularis tick during
engorgement called FTSP-5 [21]. Furthermore, some recent studies
outline an important immunological role for some members of the
HSP 20 family [51,52].

In order to study and characterize Ra-shSPII protein, a 510 bp
fragment of the Ra-shSPII gene was cloned in the IPTG-inducible
pET-30b vector and subsequently transformed into BL21 (DE3) E.
coli cells. The protein was expressed as a six-histidine tagged pro-
tein, having the histidine-tag at its N-terminal and had an apparent
molecular weight of 30 kDa. rRa-shSPII protein was successfully
eluted only under denaturing conditions, possibly due to the high
expression levels of the protein and its resulting aggregation in
cytoplasmic inclusion bodies.

The rRa-shSPII protein includes 162 amino acids from the ORF
of the Ra-shSPII gene (starting from the N-terminal). This means
that, the rRa-shSPII protein carries the N-terminal of the native
non-recombinant protein in addition to the conserved alpha crystallin domain (that extends to about the 159th amino acid, as indicated by the ScanProsite server). Both the N-terminal and the alpha crystallin domain are of special “immunological importance”. Generally, in sHSP, the N-terminal is thought only to act as solubilizer of the complex formed between the sHSP and its substrate or as “spacer” in preventing sHSP-substrate complex from aggregating together in insoluble aggregates [53]. Also, it is a widely accepted concept that the C-terminal extension of sHSPs undergoes numerous modifications, including truncation [54]. In vitro molecular chaperone function of C-terminal truncated HSP 16.2 of C. elegans is equivalent to the wild-type protein although less soluble [19].

Therefore, to raise polyclonal antibodies against a sHSP, the N-terminal region will preferentially be targeted. This fact is supported by observation of Norimine et al. [55] who studied the N-terminal region spanning amino acid 11 to 62. ’

Regarding the alpha crystallin domain, it offers a challenging suggestion about the possible efficiency of the rRA-sHSPII protein against many tick species due to the wide spread nature of the alpha crystallin heat shock proteins superfamily from one side [13] and the conservation of this domain from the other side [15,18].

The immunogenicity of the rRA-sHSPII protein was assessed by raising the corresponding polyclonal antibodies in rabbit. After the initial dose and two booster doses, sera were collected and the anti-rRA-sHSPII titers were assessed by ELISA. A satisfactory titer was obtained after the third immunization indicating that the rRA-sHSPII is strongly immunogenic.

Using the immunoblot technique, polyclonal rRA-sHSPII antibodies were shown to strongly and specifically react with the purified rRA-sHSPII that was used for immunization, thus confirming its immunogenicity. Cross-reactive proteins with polyclonal rRA-sHSPII antibodies were detected in R. annulatus whole tick, gut and larval protein extracts. These may be due to either the wide distribution of sHSP [13,15,18] or to the highly cross-reactive nature of polyclonal antibodies. The same applies to the results obtained with H. dromedarii (whole tick and larval protein extracts) and O. moubata whole tick protein extracts. In all cases, further studies are needed to confirm the identity of the protein bands involved in cross-reactivity.

Although being naturally and cellurally widely distributed, our knowledge about sHSPs remains in its infancy. However, it is generally accepted that sHSPs act as molecular chaperones by binding their “clients” through oligomeric complexes either to protect them from aggregation or to maintain them in a “folding-competent state”; this state keeps cellular proteins prone to easy refolding by the action of large HSPs [56]. The latter action was recently described as “holdase” action [57].

Typically, the action of sHSP has been investigated in vitro through thermal stability, thermal protection and rescuing of heat-sensitive enzyme activity following thermal stress [58]. Regarding rRA-sHSPII, our results suggest its ability to protect E. coli protein extracts at temperatures up to 80°C. This effect cannot be attributed to the simple presence of a heterologous protein in the pET 30b plasmid since another protein of similar molecular weight, the BAGST, failed to confer such stability to the same E. coli extracts. The coagulation of the Ba-GST itself under high temperatures in contrast to the rRA-sHSPII that remained in the soluble fraction, provides further evidence regarding the nature of the molecular chaperone action of the rRA-sHSPII; it simultaneously withstands high temperatures and confers protection to associated proteins. It can be also inferred from our results that a complete carboxyl terminus is not essential for the thermal stability effect of sHSP, since the rRA-sHSPII lacks a carboxyl terminus fragment. Recently, it has been hypothesized that high temperatures expose the hydrophobic sides of monomeric sHSP, which promote their interaction while binding to their clients to perform their action. Further functional and structural studies are needed to provide insights about the detailed mechanism of action of the sHSP in general and their role in ticks’ feeding and development.

References
