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 promotor 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-rRa-sHSPII antisera reacted specifically with purified rRa-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 *in vitro* 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 ellicit 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

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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 stands behind the suggestion of considering them as candidate anti-tick vaccines by [21] who reported the specific upregulation of a salivary sHSP of *Ixodis scapularis* tick during engorgement called FTSP-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 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 a possible candidate vaccine, may encourage further studies on the physiological and immunological role of sHSP in tick-host interaction.

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 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 $15,000 \times g$ 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 4.2×10^5 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. 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.

2.4. Expression in BL21 (DE3) and purification

The prokaryotic expression vector pET30b (Novagen Inc., Madison, USA) that carries the T7 promotor and Kanamycin resistance gene was used to express the *R. annulatus* small heat shock protein II gene (sHSPII). From the sequence of the clone of *R. annulatus* sHSPII, two primers (synthesized by Bioneer Corporation, Taejon, South Korea), FEcoRV (5′-CGG GAT ATC GTT CTG TGG TTC AAG TGA GTC T-3′) and RXhoI (5′-CGG CTC GAG GAT CCG TCG GCG AGC AAC AG-3′), were designed for PCR amplification of the ORF of *R. annulatus* sHSPII. 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), the colonies were picked and the plasmids were purified using the QIAprep spin plasmid kit (Qiagen, Hilden, Germany). Before expression, the fidelity and orientation of *R. annulatus* cDNA in the vector were confirmed by sequencing. For induction of recombinant *R. annulatus* sHSPII (rRa-sHSPII) expression, IPTG (Bio Basic, Inc., Ontario, Canada) was added to a final concentration of 1 mM and expression was induced for 5 h at 37 °C. After expression, the recombinant *R. annulatus* sHSPII 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 $10,000 \times g$ for 15 min and the supernatant (clear lysate) was immediately used or stored in $-20\,^{\circ}C$ 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 $4\,^{\circ}C$ and the empty sites were blocked by incubation with 2.5% BSA in coating buffer, pH 7.4 for 1 h at $37\,^{\circ}C$. 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 $37\,^{\circ}C$ 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 (Sigma–Aldrich, Saint Louis, MO, USA). Each of the previous steps was separated from the following one by three washes using washing buffer (0.01 M PBS, pH 7.4, containing 0.05% Tween). The color was finally developed by addition of 1 mg/ml of para-nitro phenyl phosphate (Bio Basic, Inc., Ontario, Canada) in substrate buffer (50 mM glycine buffer, pH 10.5 containing 0.5 mM MgCl$_2$). The reaction was stopped after 30 min with of 0.1 N NaOH. The absorbance values were determined at 405 nm using Bio-Tek *ELX* 800 ELISA reader A relationship between the antiserum dilution and the absorbance at 405 nm was plotted and the dilution that gives $A_{405} = 0.5$ was considered as the ELISA titer.

2.8. Immunoblotting

Immunoblot analysis was performed according to the method of Towbin et al. [27] with slight modifications using a NovaBlot semi-dry blotter (LKB, Bromma, Sweden). Briefly, 10 µg of the purified Ra-sHSPII and 30 µg of each of the *R. annulatus* whole tick, gut and larval protein extracts were allowed to run on 12% SDS-PAGE after boiling with reducing SDS-PAGE loading buffer. Similarly, 30 µg of each of *Hyalomma dromedarii* whole tick and larval and *Ornithodoros moubata* whole tick protein extracts were used for the same purpose. Gels were either stained with Coumassie Brilliant Blue stain (R-250) to determine the molecular weight of the corresponding bands as manifested by the low molecular weight marker (GE healthcare, United Kingdom) or soaked in transfer buffer (16 mM Tris–HCl, 120 mM glycine and 20% methanol) prior to transfer to Immobilon-P transfer membrane

(PolyVinylDimethylFluoride membrane, Millipore, Bedford, MA, USA). After transfer, membranes were incubated with blocking buffer (3% BSA in TBS, pH 7.5) for 1 h, washed three times with washing buffer (10 mM TBS containing 0.05% Tween 20) then incubated with gentle agitation overnight at room temperature with the polyclonal anti rRa-sHSPII in serum buffer (0.5% BSA in TBS containing 0.05% Tween 20, pH 7.5) 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% H$_2$O$_2$. 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 $100\,^{\circ}C$ for 20 min. After cooling, samples were centrifuged at $10,000 \times g$ 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 solublized 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 4.2×10^5 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 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 AAATAA 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

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                                        ▶
        gttctgtggttaaagtgagtctccaatcgttcgtcgtgcccgagagttgtacgtg   55

        atggctggtccaatgttcgcgaggttttctctgccatgccggcgtgccgtaatggcctgt  115
         M  A  G  P  M  F  A  R  F  S  L  P  C  R  R  A  V  M  A  C
        tcgcgacggaacctgtggagacgctacccggccaccatcggggatgtgttcggcgagatg  175
         S  R  R  N  L  W  R  R  Y  P  A  T  I  G  D  V  F  G  E  M
        gaccggcagctgcgacacttcgagcacgagatgtcgcgcactttccgcgacctcgaccga  235
         D  R  Q  L  R  H  F  E  H  E  M  S  R  T  F  R  D  L  D  R
        tcgggcgcgttcggaccggccttccgctggctgcgtgcgcgcgacgtgcccgtcgagtcc  295
         S  G  A  F  G  P  A  F  R  W  L  R  A  R  D  V  P  V  E  S
        ggcgcgggcgacaagttccaggtgcagttggacgtgagcaagttcagaccggaggacgtc  355
         G  A  G  D  K  F  Q  V  Q  L  D  V  S  K  F  R  P  E  D  V
        aaggtgtccctgtcgggcaaccagctcaccgtgcgcgcccgctccgaagtcaaggagggt  415
         K  V  S  L  S  G  N  Q  L  T  V  R  A  R  S  E  V  K  E  G
        aactcgacctacgttcgcgagttctctcgcactcggtgaccctgcccgaagacgtggacccc  475
         N  S  T  Y  V  R  E  F  S  H  S  V  T  L  P  E  D  V  D  P
                                  ◀
        gacacggtgcgatccctgttgctcgccgacggatcgctgtgcatcgaggctccccgcgcg  535
         D  T  V  R  S  L  L  L  A  D  G  S  L  C  I  E  A  P  R  A
        ctgcccgaacccaaggaggtacccaatcgagaagtcttccaccgaagcagccgcccaagt  595
         L  P  E  P  K  E  V  P  N  R  E  V  F  H  R  S  S  R  P  S
        tgaaactatgtgtgtgtgtgtgtgctacaaaaataagttggcgtaccttgtatggtgtta  655
         –
        aataaa                                                        661
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Fig. 1. cDNA sequence and deduced amino acid sequence of the tick *R. annulatus* small heat shock protein II gene (Ra sHSPII). Arrow-headlines indicate DNA primers locations 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).

of the identified gene and its deduced amino acid sequence are shown in Fig. 1.

The identified ORF encodes a polypeptide of 180 amino acids with calculated molecular weight of 20.507 kDa and calculated isoelectric point of 9.071. Analysis of the deduced amino acids sequence using the signal peptide prediction server of the CBS (http://www.cbs.dtu.dk/services/SignalP/) predicted that the molecule belongs to a non-secretory protein, lacking a signal peptide [29,30]. The absence of a signal peptide was predicted by using both neural network and hidden Markov Model (HMM) methods. In the neural network method, all the scores (the S-score predicting the signal peptide fragment at each amino acid position, the S-mean, the C-score predicting the cleavage sites, the Y-max which is a derivative of the S-score and C-score, and the D-score which is a simple average of the S-mean and Y-max score) were lower than the corresponding cut-off for signal peptides. The same result was obtained using the whole polypeptide sequence or the N-terminal 70 amino acid fragment only as recommended by the server. The deduced polypeptide sequence was also analyzed using the Scanprosite server (http://www.expasy.ch/tools/scanprosite/). This server scans any submitted sequence for particular protein domains or motifs conferring conserved functions to protein families. The protein families are grouped according to their sequence and functional similarity in the Prosite database that harbors, in addition to the conserved protein domain sequences, cognate documentation containing information about the structure and the function of each protein family. To improve the stringency of the scanning process, the ScanProsite is complemented by a group of manually created rules, the Prorules, that give more precise insights about critical amino acids (in term of structure and/or function) [31,32]. Accordingly, the ScanProsite tool predicted that the identified sequence belongs to the small heat shock protein 20 family and its conserved alpha crystallin domain ranges from amino acid D75 to R159. The polypeptide is thought to have one potential glycosylation site at N121 which may increase its predicted molecular weight by about 2.5 kDa.

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 rRa-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

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Ra sHSPII   MAGPMFARFSLPCRRAVMACSRRNLWRRYPA--TIGDVFGEMD--RQLRHFEHEMSRTFR  56
Is sal      ----------------MLVCPRRSFWRRYPVPRSVSDVFNEFD--HQLRSMERDMSRAFR  42
Op cry      -MLTRLRDFCL-FRRCALATCRRHIWGRPPFG-PLSDVFREFE--HQVRNMDREMSRTFR  55
Ce HSP      --------------------------------MSLYHYFR--PAQRSVFGDLMRDMA    23
Hs cry      --------------MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPTSTSLS  45

Ra sHSPII   DLDRS-GAFGPAFRWLRARDVPVESG-AGDKFQVQLDVSKRPEDVKVSLSGNQLTVRAR 114
Is sal      DLDSH-GYFGPTFRWLRTRDVPVETG-SAEKFQLQLDVAQRKPEDVKVSLSGNQLTVRAR 100
Op cry      DLDRATGGWSPAFRWLRARDVPVETAEGGEKFQLQLDLADRKPEEVRVSIMGNQVSVRAR 115
Ce HSP      LMERQ---FAPVCRISPSESSEIVNN--DQKFAINLNVSQRKPEDLKINLDGRTLSIQGE  78
Hs cry      PFYLRPPSFLRAPSWFDTGLSEMRLE--KDRFSVNLDVKHFSPEELKVKVLGDVIEVHGK 103

Ra sHSPII   SFVKEGN-STYVREFSHSVTLPEDVDPDTVRSLLLADGSLCIEAPR----ALPEPKEVPN 169
Is sal      AETKEGD-SSYVREFSHSVTLPEDVDPDTVRSVLQADGSLSIEAPRLRLEQAREPKEVP- 158
OP cry      SERKSSDGSNFVREFSHSVTLPDDVDPDTLRSVLNADGTLNIEGPRKHLPPPQEPKEVP- 174
Ce HSP      QELKTDH-GYSKKSFSRVILLPEDVDVGAVASNLSEDGKLSIEAPKK---EAVQGRSIPI 134
Hs cry      HFEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRKQVSGPERTIPITR 163

Ra sHSPII   REVFHRSSRPS-- 180
Is sal      IERADPDKQASQK 171
OP cry      IFRGDSAKKD--- 184
Ce HSP      QQAIVEEKSAE-- 145
Hs cry      EEKPAVTAAPKK- 175
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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* α 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.

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 antisera 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).
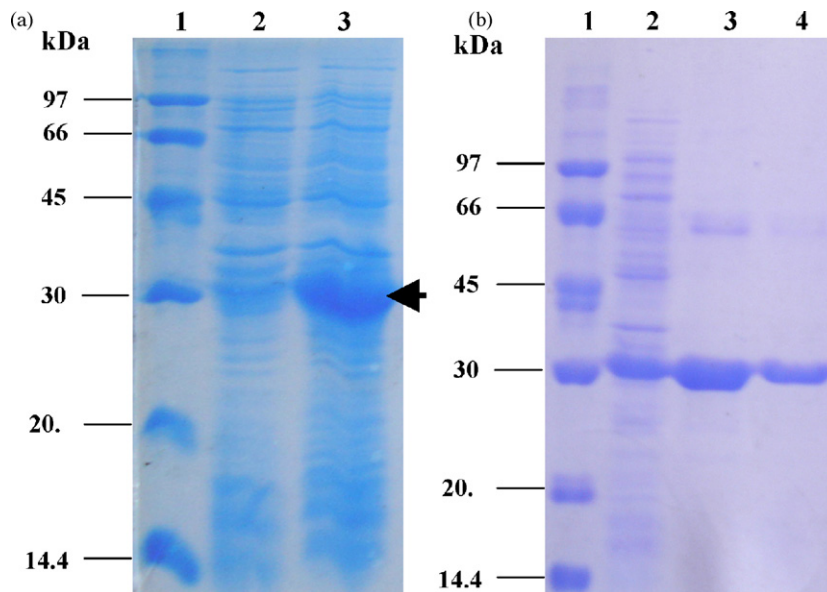

Fig. 3. (a) Analysis of *E. coli* BL21(DE3) lysate before and after protein expression. 12% SDS-PAGE of low molecular weight marker (Amersham, UK), lane (1); bacterial lysate prior to the addition of IPTG, lane (2); and bacterial lysate following the addition of IPTG, lane (3). The arrow shows the 30 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.
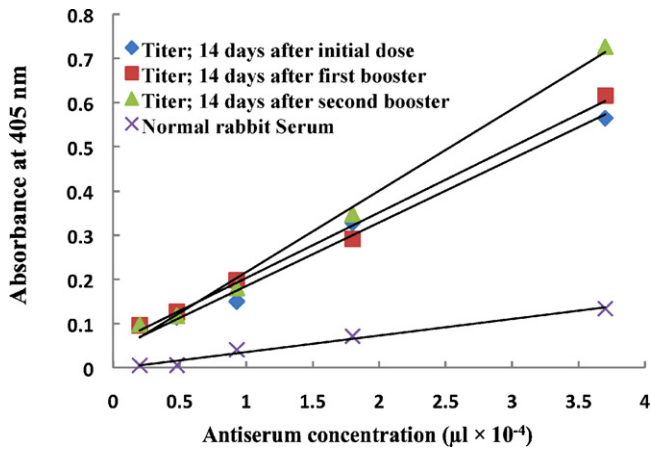
Fig. 4. Titration curve of the rabbit anti-rRa-sHSPII. Absorbance values at 405 nm are plotted versus anti-serum concentration after subtracting the blank values.

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 *H. dromedarii* whole tick and larvae in addition to *O. 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
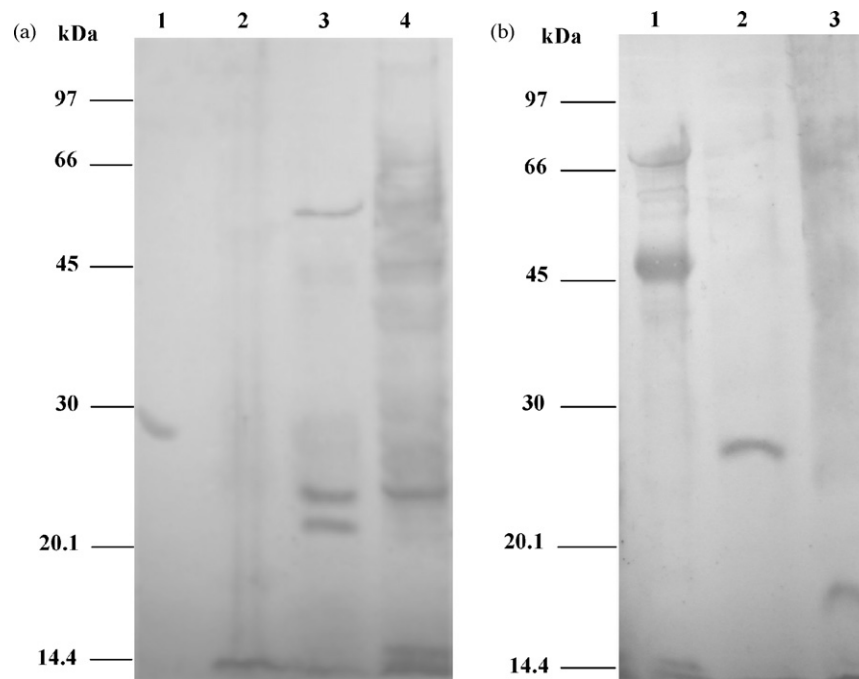

Fig. 5. (a) Immunoblotting of 12% SDS-PAGE of recombinant Ra-sHSPII, lane (1); whole *R. annulatus*, lane (2); *R. annulatus* gut, lane (3); and *R. annulatus* larval, lane (4) protein extracts. Samples were reacted with rabbit anti-rRa-sHSPII in a dilution of 1:1000. (b) Immunoblotting of 12% SDS-PAGE of whole *Hyalomma dromedarii* tick, lane (1); *H. dromedarii* larval, lane (2); and whole *Ornithodoros moubata* tick, lane (3) protein extracts. Samples were reacted with rabbit anti-rRa-sHSPII at a dilution of 1:1000.
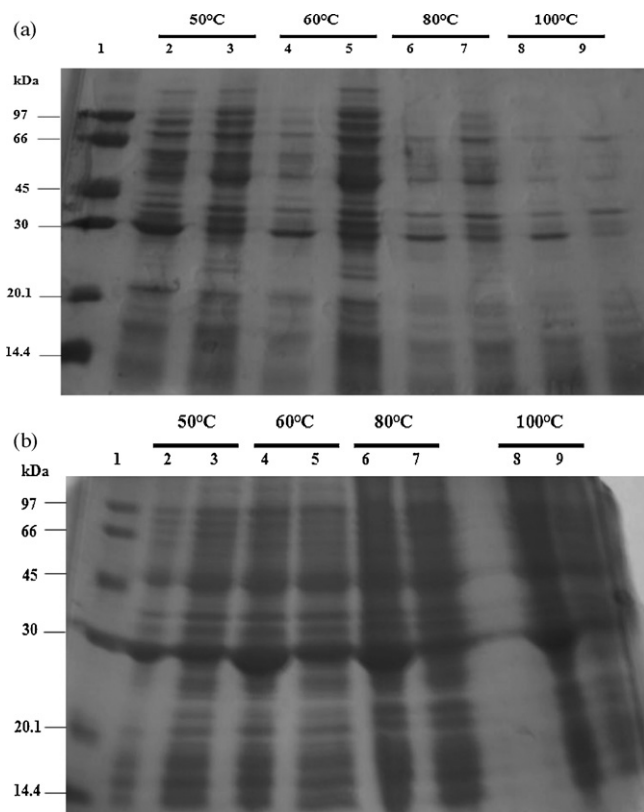
Fig. 6. Thermal stability of an *E. coli* crude extract expressing Ra-sHSPII. Extract of soluble *E. coli* proteins from induced BL21(DE3)/Ra-sHSPII and BL21(DE3)/Ba-GST cells expressing *Rhipicephalus annulatus* small heat shock protein II and *Rhipicephalus* (*Boophilus*) *annulatus* GST, respectively, were prepared as described in Section 2. Aliquots of 500 μl of cell extract (4 mg/ml) were heated for 20 min at various temperatures, as indicated. After being allowed to cool to room temperature, samples were centrifuged at 10,000 × g for 5 min. The supernatants (20 μl) were analyzed by SDS-PAGE. (a) Lane 1 is low molecular weight marker; lanes 2, 4, 6, and 8, BL21(DE3)/Ba-GST cell extract heated at 50, 60, 80, and 100 °C, respectively; lanes 3, 5, 7 and 9, BL21(DE3)/Ra-sHSPII cell extract heated at 50, 60, 80, and 100 °C, respectively. (b) Lane 1 is low molecular weight marker; lanes 2, 4, 6, and 8, BL21(DE3)/Ba-GST solubilized coagulated proteins heated at 50, 60, 80, and 100 °C, respectively; lanes 3, 5, 7 and 9, BL21(DE3)/Ra-sHSPII solubilized coagulated proteins heated at 50, 60, 80, and 100 °C, respectively.

immunity", to the use of crude tick protein extract for immunization [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 pastori* yeast [40].

Some drawbacks of the currently available vaccines encouraged scientists to search for new protective antigens, in an attempt to develop a universal polyvalent vaccine, effective against several 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 reservoir 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 understanding 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 Egyptian 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 661 bp sequence that forms a single 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 proteins 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% similarity 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 cellular compartments or even as a transmembrane protein cannot 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 compartment 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 pattern 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 associated 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 protein, 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 to play the essential role in substrate binding and stabilization [19] while the C-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 protective power of *Babesia bovis* HSP20 as a candidate vaccine [55]. They claimed that "the most immunostimulatory region of *B. bovis* Hsp20 for T helper cells from the cattle used in this study is 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 cellularly 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 Ba-GST, 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 termi-

nus 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.

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