International Journal of Biological Macromolecules 67 (2014) 7–15

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Molecular cloning of Ra-sHSPI, a novel member of the HSP20 family from *Rhipicephalus annulatus* salivary glands

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ARTICLE INFO

Article history:
Received 18 November 2013
Received in revised form 26 February 2014
Accepted 27 February 2014
Available online 10 March 2014

Keywords:
Rhipicephalus annulatus
sHSP
Thermal stability
Salivary glands
Cattle ticks

ABSTRACT

Infestation of cattle by ticks of *Rhipicephalus* spp. results in severe veterinary and economical losses. Identification of novel proteins from tick salivary glands will enhance our understanding of several aspects of tick physiology and will aid in the development of anti-tick vaccines. Small heat shock proteins (HSPs) have important roles in infection and immunity, especially between invertebrate vectors and mammalian hosts while initially performing their molecular chaperone activity. Here, we report the identification of a small HSP gene from the salivary glands of *Rhipicephalus annulatus* ticks through immunoscreening of the corresponding cDNA expression library. The identified cDNA contained a 742 bp sequence with 543 bp open reading frame. It was subsequently cloned, expressed and successfully purified under both native and denaturing conditions. Sequence analysis and functional investigations showed that the protein belongs to the HSP20 family, hence the annotated name Ra-sHSPI. Indeed, recombinant Ra-sHSPI showed two typical *in vitro* activities of holdase chaperones, including thermal protection of bacterial cellular extracts and the recombinant *Hind*III at elevated temperatures. Moreover, the recombinant Ra-sHSPI showed strong immunogenic effect in animal model. These results pave the way toward further investigation of Ra-sHSPI role in ticks feeding and its potential use as protective antigen.

1. Introduction

Hematophagous (blood feeding) arthropods include a wide range of disease vectors including mosquitoes, flies and ticks. Ticks are important blood feeding ectoparasites affecting several mammals including human, cattle and dogs [1]. Due to their particular physiology, their ability to attach to their host for prolonged time period and the relatively large volume of blood they ingest, ticks can cause numerous diseases while feeding on their host blood [1]. Cattle are one of ticks host, being parasitized by *Boophilus* (*Rhipicephalus*) spp., *Hyalomma* spp., *Ambylomma* spp. [2] and *Haemaphysalis* spp. [3]. They constitute one of the major livestock species in many countries, and are an important source of energy, food, raw material, leather and manure for crops in developing countries [4]. Cattle infestation by ticks results in severe veterinary problems such as irritation, anemia, leather damage and the transmission of protozoal, bacterial and viral pathogens [1,5]. The consequent tick-borne diseases result in devastating economical losses [5]. In addition, failure to control ticks and tick-borne disease is a major factor limiting livestock production world-wide [5].

Traditionally, the control of cattle ticks has relied on a chemical strategy, through the use of acaricides, the success of which had been challenged by the consequent human and animal hazards [6,7], the unsatisfactory effectiveness, the selection for resistant tick strains [8,9] and the resulting environmental pollution [10]. Instead, an immunological approach depending on the use of tick antigenic proteins to immunize cattle had proven its effectiveness in several countries, mainly against *Rhipicephalus microplus* ticks present in Australia and the USA [11,12].

To the best of our knowledge, there is no specific vaccine against *R. annulatus*, the endemic specie in several parts of Africa and Middle East. On the other hand, the current trend encourages the development of multivalent vaccines, simultaneously targeting several tick species [9]. Accordingly, the identification of new ticks antigens that may serve as vaccine candidate remain the rate limiting step for vaccine development [13]. Identification and characterization of those antigens will not only enable vaccine improvement, but also enhance our understanding of some physiological aspects of ticks, including its complex host–parasite relationship.

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http://dx.doi.org/10.1016/j.ijbiomac.2014.02.057
0141-8130/

Salivary glands, the largest glands in ticks, represent a cornerstone to shed light on tick physiology and potential protective antigens [14]. Besides their initial role in feeding, salivary glands play other major roles in excretion and osmoregulation [15]. Ticks salivary secretion encompasses a plethora of biologically active compounds; these facilitate the feeding process, pathogen transmission processes and minimize host rejection. They are particularly adapted to ensure adequate salivary secretion and to withstand the change in tick physiology concomitant with the feeding process [16,17]. Indeed, to accommodate the dramatic changes that accompany the ingestion of the blood meal, the salivary gland cells of feeding ticks undergo extensive remodeling during feeding [18], for example, by increasing the expression of some genes and the induction of new gene activity [19]. Moreover, successful feeding is essential to ensure ticks fertility and reproduction, a special attribute of ticks biology and physiology [20].

One of the interesting proteins that become upregulated in fed ticks salivary glands are small heat shock protein (sHSP) [14]. Small HSP are low molecular weight (12–43 kDa), ATP-independent chaperones [21]. They are known to enable the cells to withstand an otherwise lethal thermal stress [22] and are currently investigated as important player in several parasites as malaria [23] and filaria [24]. On the other hand, they were reported to be upregulated following feeding in *Ixodes scapularis* ticks [14] and were previously identified by our group upon screening of a cDNA expression library in *R. annulatus* ticks [25].

Due to the rising scientific interest about sHSP and the potential role of salivary gland proteins as vaccine candidates, we were motivated to characterize another sHSP from *R. annulatus* salivary glands. In this study, we report the identification of a novel sHSP with different sequence characteristics and enhanced immunogenic properties than our previously identified Ra-sHSPII. The molecular cloning, protein expression and functional investigation of this novel sHSP from the salivary glands of the Egyptian cattle ticks *R. annulatus* are described.

2. Materials and methods

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

Whole tick, salivary and larval antigens of *R. annulatus* were prepared according to the method of Ghosh and Khan [26] and Ghosh et al. [27]. In brief, laboratory reared, clean, 5–6-day-old unfed ticks, larvae or salivary glands isolated by dissecting from adult ticks, were homogenized in cold buffer A which includes, 0.15 M phosphate-buffered saline (PBS) and 1 mM disodium EDTA, pH 7.2, containing cocktail protease inhibitors, and were then filtered, sonicated, and centrifuged at 15,000 × g for 60 min at 4 °C. The supernatant was designated as whole tick, salivary or larval antigen. The protein concentrations of the antigens were estimated according to the method of Bradford [28]. Gut antigen was prepared according to the method of Das et al. [29]. In brief, midguts from the partially fed ticks were dissected out and homogenized in extraction buffer A, sonicated, and centrifuged. Supernatants were then collected as gut antigen.

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 (Sigma, USA) were injected on day 0. The rabbit was boosted by 50 μg of the same antigens mixed with Freund's incomplete adjuvant 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 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 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 [30]. 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). 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) 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) 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) at a dilution of 1:10,000. The positive phages were cloned, and the inserts were excised with the ExAssistTM Helper Phage (Stratagene). 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) carries the T7 promotor and Kanamycin resistance gene was used to express the *R. annulatus* small heat shock protein I gene (sHSPI). From the sequence of the clone of *R. annulatus* sHSPI, two primers, F-*Eco*RV (5′-CGG GAT ATC ACT CAG CCA ATT CGC AAG CAT CAT-3′) and R-*Xho*I (5′-CGG CTC GAG CGC AAG CGA AAT ACA AAC ACA GGA-3′), were designed for PCR amplification of the ORF of *R. annulatus* sHSPI. The primers F-*Eco*RV and R-*Xho*I contained *Eco*RV and *Xho*I restriction sites, respectively. These sites were also present as unique sites in the cloning region of the pET30b expression vector, ensuring correct orientation of the insert. To ensure fidelity, PCR was performed using platinum pfx-DNA polymerase (Gibco) that has proofreading capacity. PCR product and vector were digested with *Eco*RV and *Xho*I before ligation. The ligated construct was transformed into BL21 (DE3) and colonies were picked and the plasmids were purified using the QIAprep spin plasmid kit (Qiagen, Germany). Before expression, the fidelity and orientation of *R. annulatus* cDNA in the vector were confirmed by sequencing. For induction of recombinant *R. annulatus* sHSPI (rRa-sHSPI) expression, IPTG was added to a final concentration of 1 mM and expression was induced at 30 °C for 24 h or at 37 °C for 5 h. After expression, the recombinant *R. annulatus* sHSPI was affinity purified under both native and denaturation conditions using the