



Pergamon

Insect Biochemistry and Molecular Biology 33 (2003) 63–71

*Insect
Biochemistry
and
Molecular
Biology*

www.elsevier.com/locate/ibmb

The major salivary gland antigens of *Culex quinquefasciatus* are D7-related proteins

Rosely dos Santos Malafronte ^{a,b}, Eric Calvo ^{b,c}, Anthony A. James ^c,
Osvaldo Marinotti ^{b,c,*}

^a Instituto de Medicina Tropical, Universidade de São Paulo, São Paulo, Brazil

^b Departamento de Parasitologia, ICB, Universidade de São Paulo, São Paulo, Brazil

^c Department of Molecular Biology and Biochemistry, McGaugh Hall, University of California, Irvine, CA 92697-3900, USA

Received 21 December 2001; received in revised form 11 July 2002; accepted 15 July 2002

Abstract

The sera of persons with strong allergic responses to the bites of the mosquito, *Culex quinquefasciatus*, contained IgE antibodies reactive with two major salivary gland proteins with molecular weights of 35 and 28 kDa. These antigens were purified, their amino termini sequenced, and the sequences were used to search for similar sequences in public databases. Two cDNAs, CuQu-D7Clu1 and CuQu-D7Clu12, which encode D7-related proteins, were identified as containing predicted amino acid sequences identical to the 35 and 28 kDa antigens, respectively. These proteins are expressed specifically in adult female salivary glands and, their predicted tertiary structures are consistent with a role as carriers of hydrophobic molecules in mosquito saliva.

© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Culex quinquefasciatus*; Salivary glands; Mosquito bite allergy

1. Introduction

The main focus of attention on mosquito salivary glands is their role in blood feeding and the transmission of diseases. The female mosquito introduces her proboscis during probing into the skin of the host and salivates a mixture of molecules containing antihemostatic and immunomodulatory activities (Ribeiro, 1995). The active movement of the insect mouthparts associated with the antihemostatic compounds of saliva enables mosquitoes to locate within seconds to minutes after contact a vessel or hemorrhagic pool from which to suck blood. Among the antihemostatic factors present in the saliva are anticoagulants, vasodilators and platelet aggregation inhibitors (Ribeiro, 2000).

The immunomodulatory activity of mosquito saliva has been documented as well (Cross et al., 1994). The infectivity of Cache Valley virus and La Crosse virus is

likely enhanced by mosquito saliva, indicating that the vertebrate immune reaction to the pathogen is somehow modulated by mosquito salivary components (Osorio et al., 1996; Edwards et al., 1998). The mechanisms involved in the immunomodulation are unknown, although several components of hematophagous arthropods saliva that interact with vertebrates immune system have been described (Owhashi et al., 2001; Nuttal et al., 2000; Charlab et al., 2001; Wikel et al., 1996).

Another aspect of mosquito saliva that has been investigated is its involvement in allergic responses in humans and animals. Mellamby (1946) first proposed the general natural history of hypersensitivity to mosquito bites. Naive hosts develop no reaction or a delayed-type hypersensitivity reaction when first exposed to bites, evolving to immediate-type hypersensitivity and desensitization as exposure continues. Although in most cases mosquito bites elicit mild symptoms, allergic patients may present severe reactions that can include urticaria and anaphylaxis (Simons and Peng, 2001). In cases of severe reactions, treatments have been based on the use of antihistamines and immunotherapy with mosquito whole-body extracts (Beaudouin et al., 2001; McCormack et al.,

* Corresponding author. Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, CA 92697-3900, USA. Tel.: +1-949-824-3210; fax: +1-949-824-2814.

E-mail address: omarinot@uci.edu (O. Marinotti).

1995). Studies have correlated the incidence of skin reactions to mosquito bites with the occurrence of specific anti-mosquito saliva antibodies (IgG and IgE) in the sera of allergic individuals (Peng et al., 1996; Peng and Simons, 1998; Shan et al., 1995). The frequency of these allergic reactions emphasizes the need for the characterization of mosquito salivary allergens that may facilitate the development of better and specific diagnostic tools and efficient desensitization protocols based on the use of recombinant antigens.

Nascimento et al. (2000) began analyzing the salivary antigens of *Culex quinquefasciatus* by showing that sera from mice immunized by bites of mosquitoes contain antibodies to polypeptides of 35 and 28 kDa. In this paper we show that these antigens also are recognized by antibodies present in human sera and discuss possible functions for these proteins.

2. Materials and methods

2.1. Human subjects and sera

This project was approved by the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo and all participants, after a brief explanation of the research project, gave their written consent to be included in this study. Blood was taken from 47 adult (aged 23–42 years) and 23 children (aged 3–13 years) from São Paulo city (Brazil). These individuals claimed to have had no previous allergic reactions after mosquito bites (control group). RAST-positive sera (above 0.35 U/ml) were obtained from 17 subjects (adults and children). Twelve sera were positive for antibodies to *Aedes communis* antigen and 5 were positive for antibodies to other arthropods (*Solenopsis invicta* and *Apis mellifera*). The IgE values of the sera ranged from 0.4 to 17.5 U/ml.

2.2. Mosquitoes and antigen preparations

Culex quinquefasciatus mosquitoes (PIN strain) collected at the margins of the Pinheiros River, São Paulo City, Brazil, were reared in a local insectary, as previously described (Nascimento et al., 2000). Adult mosquitoes were anesthetized with CO₂ and transferred to a chilled plate until dissection. Salivary glands were dissected under a stereomicroscope in 20 mM phosphate buffered saline, and the salivary gland extracts (SGE) were prepared according to Peng et al. (1995). Proteins were quantified according to Bradford (1976), using bovine serum albumin as a standard for proteins concentration.

2.3. IgE ELISA

ELISA procedures and standardization followed the protocols described by Peng et al. (1995) and Konishi (1990) with modifications. Polystyrene plates (Corning high binding) were coated with 50 µl SGE (0.05 µg/ml) diluted in 0.05 M carbonate buffer, pH 9.6 (Sigma), and incubated overnight at 4 °C. Sera diluted 1:20 for adult and 1:80 for children were incubated in the coated plates overnight at 4 °C. The secondary antibody (antihuman IgE e-chain specific-peroxidase conjugated) (Sigma) also was incubated overnight (1:1000 adult; 1:1500 children) at 4 °C. The enzyme substrate (o-phenylenediamine, 5 mg, Sigma) diluted in phosphate–citrate buffer (Sigma) was added to the wells and incubated for 30 min at 37 °C. Reactions were stopped by addition of 25 µl of 4 N HCl and the absorbances were read at 492 nm using a Labsystem Multiskan MS microplate reader. The cut-off values (0.172 for adults and 0.164 for children) were determined by addition of two standard deviations to the mean of the absorbances obtained with the sera from control group. Absorption of the sera with SGE was conducted as described by Peng et al. (1995).

2.4. Immunoblotting

SDS-PAGE and blotting to nitrocellulose membranes were carried out as described previously by Nascimento et al. (2000), using a Bio-Rad MiniProtean II and a Bio-Rad Semi-dry blotting apparatus. The membranes were incubated overnight with sera diluted 1:5, followed by washings and another overnight incubation, at this time with the goat anti-human IgE-peroxidase diluted 1:50 (Sigma). Color was developed by incubation of the membranes with the substrates, 4-chloro-1-naphtol and hydrogen peroxide.

2.5. Recombinant protein expression

The CuQu-D7Clu1 and CuQu-D7Clu12 cDNAs were amplified using the following primers:

CuQu-D7Clu1KPN: 5'-ggggtaccccgatggatcccgaagag-3';
CuQu-D7Clu1ECO: 5'-gggaattcttactcaacacttagcttcg-3';
CuQu-D7Clu12KPN: 5'-ggggtacctgaggcggacaaaatc-3';
CuQu-D7Clu12ECO: 5'-gggaattccaactaacagcctgcattcc-3'.

These primers were designed to amplify the complete coding region of the cDNAs, except for those nucleotides encoding the signal peptides. The amplification products were digested with *EcoR* I and *Kpn* I restriction endonucleases and cloned into expression vectors. The CuQu-D7Clu1 PCR product was cloned into the pRSET B plasmid (Invitrogen life technologies) while the CuQu-D7Clu12 PCR product was cloned into pRSET C. Following transformation, BL21(DE3)pLysS *E. coli*

containing the recombinant vectors were grown in SOB medium and recombinant protein synthesis induced by the addition of 1 mM IPTG to the cultures.

2.6. Amino acid sequencing

Culex quinquefasciatus salivary glands proteins were fractionated by SDS-PAGE under reducing conditions, transferred to PVDF membrane (Bio-Rad Laboratories Inc.) by semidry blot, stained with Ponceau-S and air-dried. The stained proteins were excised from the membrane and submitted to automated Edman degradation (Edman, 1950) for N-terminal sequencing (Protein Sequence Analysis, Macromolecular Structure Analysis Facility, University of Kentucky, USA).

2.7. RT-PCR

Total RNA from larvae, pupae, adults or dissected salivary glands was isolated using TRIZOL reagent (GIBCO-BRL) and was treated with RNase-free DNase (RQ1, Promega) prior to RT-PCR assays. Gene-specific primers and the one-step RT-PCR kit (Invitrogen) were used and the amplified products were submitted to electrophoresis in a 0.8% agarose gel. Primer sequences were:

18S FOR: 5'-GTCTCAAAGGTTAAGCCATGCATGTCTAAG-3';
18S REV: 5'-CGGAATCGAACCCTGATTCCCCGTACCCG-3';
CuQu-D7Clu12 FOR: 5'-AAAAATCAGCGTGTATGCCAAGTGC-3';
CuQu-D7Clu12 REV: 5'-GGTAATGCCAGTTTCTCAATGTGC-3';
CuQu-D7Clu1 FOR: 5'-GGGTATATCGATTTCCGCTTCTGTGG-3';
CuQu-D7Clu1 REV: 5'-GCATTGGCATCGTACTTGGAGGTTG-3'.

2.8. Data analysis

Protein and DNA database searches were performed using BLAST at the National Center for Biotechnology Information (NCBI, Altschul et al., 1997) and FASTA3 at European Bioinformatics Institute (Pearson and Lipman, 1988). All alignments were obtained by the Clustal W method using the program MEGALIGN (DNA-STAR). Nucleotide positions were readjusted manually to maximize the similarities in the final alignment.

Three-dimensional structure predictions for the *C. quinquefasciatus* proteins were obtained using the algorithm, 3D-PSSM Web Server v 2.6.0 (Three-Dimensional Position Specific Scoring Matrix; Kelley et al., 2000), at <http://www.bmm.icnet.uk/~3dpssm>.

3. Results

Three groups of human sera from individuals from São Paulo city were tested by ELISA assays for the presence of antibodies specific to *C. quinquefasciatus* salivary gland extract (SGE) antigens. The control group

was composed of sera from individuals (adults and children) that claimed to not have allergic reactions to mosquito bites. The experimental group was composed of sera from patients that tested positive for allergic responses to mosquito bites in a RAST test and that had previously complained about allergic reactions to mosquito bites. A third group was formed by sera of individuals with allergic clinical histories, but not to mosquito bites. The results indicate that the levels of the specific anti-mosquito IgE in mosquito allergic patients are higher than in the other groups (Fig. 1). However, it was noticed that some sera from the control group presented antibodies levels as high as those of RAST-positive individuals. IgE levels higher than the cut-off value were found in 11% (5/47) of control adults and 21% (5/23) of control children. Levels of anti-mosquito IgE in sera from individuals allergic to other arthropods were similar to the levels found in the control group.

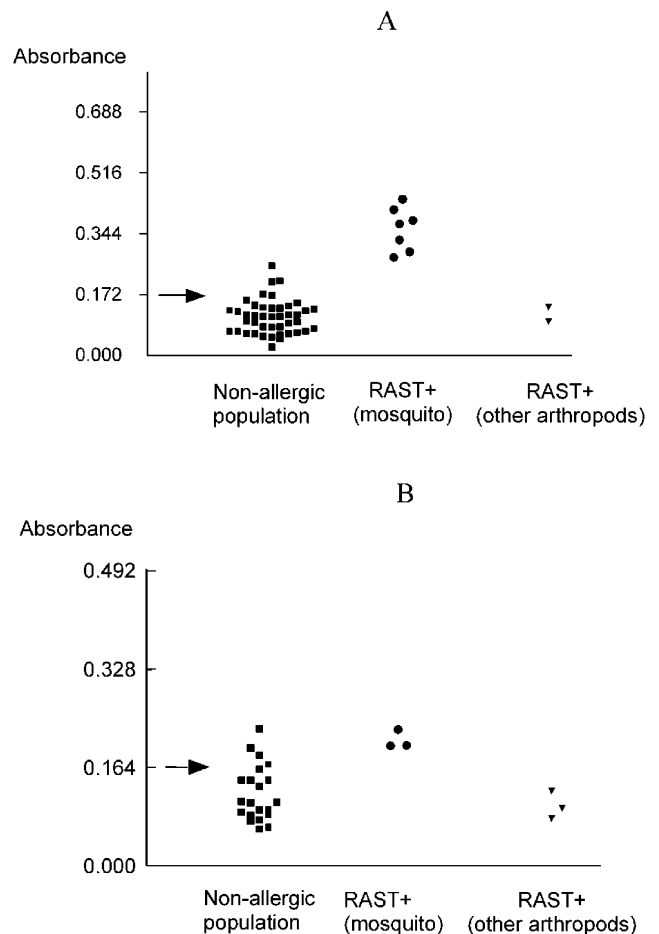


Fig. 1. Antimosquito SGE IgE levels in the sera of adults (A) and children (B). ELISA tests were performed with the sera of individuals with no allergic reaction to mosquito bites, control group (■), individuals with previous RAST- positive results to mosquito allergens (●), or individuals RAST-positive for allergens derived from arthropods other than mosquito (▼). The arrows indicate the cut-off values (0.172 for adult and 0.164 for children) determined by addition of 2 standard deviations to the mean of the obtained absorbances.

Absorption of the sera with *C. quinquefasciatus* SGE was performed as described by Peng et al. (1995) in order to test for the specificity of the antibodies present in the sera of non-allergic individuals. The absorbance values obtained by ELISA assays were reduced after absorption assays, demonstrating the SGE binding specificity of the immunoglobulins present in the sera of those individuals (result not shown).

Immunoblots were performed to identify the salivary antigens recognized by the antibodies present in the sera of six patients allergic to mosquito bites and nine of those from the control group presenting high levels of IgE (Figs. 2, 3 and 4). All sera recognized the 35 and 28 kDa polypeptides. Some of the sera also recognized the 45 and 20 kDa polypeptides. Sera from individuals giving absorbance below the cut-off values in the ELISA tests were used as negative controls and, as expected, did not react with any protein in the SGE.

The 35 and 28 kDa polypeptides were purified by SDS-PAGE and submitted to aminoterminal sequencing. The sequences obtained for them were DEWSPMDPEE-VALEE (35 kDa) and AWKPFSPPEETLF (28 kDa). These amino acid sequences were used to search public databases for similar or identical protein sequences. The 35 and 28 kDa proteins N-termini amino acid sequences are identical to parts of the predicted proteins encoded by CuQu-D7Clu1 (NCBI accession number AF420269) and CuQu-D7Clu12 (NCBI accession number AF420270) cDNAs, respectively. These cDNA clones represent mRNAs present in adult *C. quinquefasciatus* salivary glands (Valenzuela et al., 2002).

CuQu-D7Clu1 and CuQu-D7Clu12 recombinant pro-

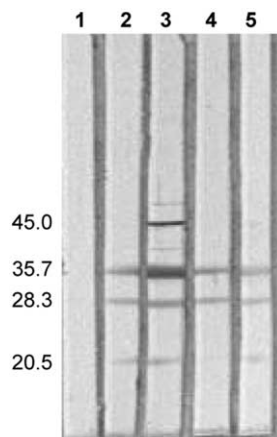


Fig. 2. Immunoblot analysis of anti-mosquito SGE IgE present in the sera of non-allergic adults. Salivary gland proteins were submitted to SDS-PAGE in a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The identification of reactive polypeptides was performed as described in Section 2. Numbers to the left of the figure indicate the molecular weight of the reactive polypeptides and numbers at the top indicate sera from different individuals. 1, sera from a non-allergic adult with ELISA result below the cut-off value (negative control); 2 to 5, sera from four different non-allergic adults with ELISA results above the cut-off value.

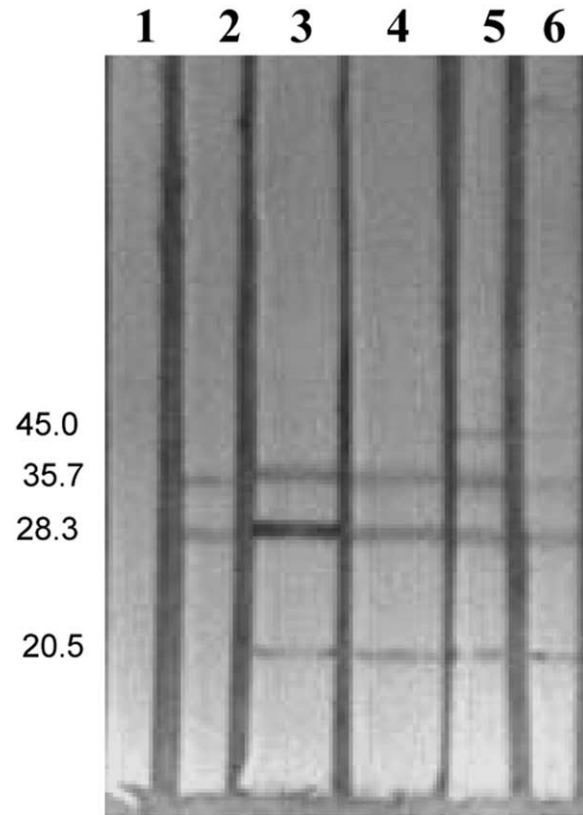


Fig. 3. Immunoblot analysis of anti-mosquito SGE IgE present in the sera of non-allergic children. 1, sera from a non-allergic child with ELISA result below the cut-off value (negative control); 2 to 6, sera from five different non-allergic children with ELISA result above the cut-off value.

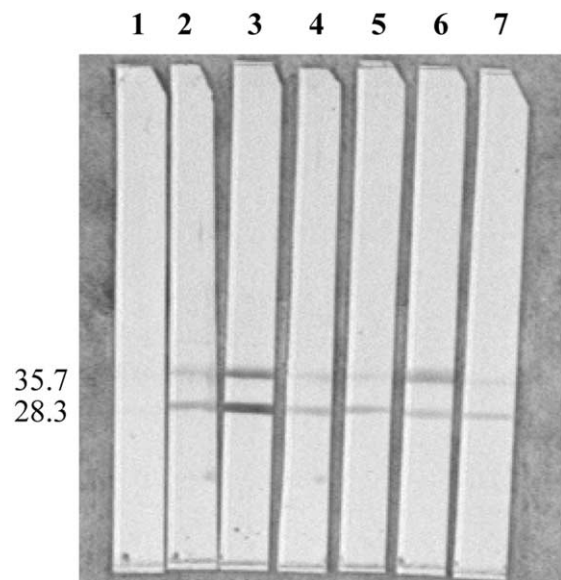


Fig. 4. Immunoblot analysis of anti-mosquito SGE IgE present in the sera of individuals allergic to mosquito bites (RAST-positives). 1, sera from non-allergic individual (negative control); 2 to 7, sera from allergic (RAST-positive) individuals with ELISA result above the cut-off value. Lanes 2, 4, 5 and 7 correspond to the sera of four different children; 3 and 6 correspond to the sera of two adults.

teins were expressed in *E. coli* and immunoblots revealed that both recombinant polypeptides are recognized by the IgE of allergic individuals and are not recognized by antibodies present in the sera of the control group with low levels of anti mosquito antibodies (Fig. 5). Based on the cDNAs sequences, gene specific primers were designed and RT-PCR analysis demonstrated that the mRNAs corresponding to the 35 and 28 kDa polypeptides are present exclusively in the adult female salivary glands (Fig. 6).

In order to determine possible biological functions, the amino acid sequences of CuQu-D7Clu1 and CuQu-D7Clu12 translated protein sequences were submitted to 3D-analysis using the 3D-PSSM program (Kelley et al., 2000). Strong structural similarity was observed between the two *C. quinquefasciatus* proteins, and among them and a *Tenebrio molitor* hemolymph protein (THP12) that belongs to the odorant and pheromone-binding protein family. The similarity in the predicted tertiary structures of these three proteins, even when they show a weak sequence similarity (less than 14% of identity), suggests that they could share the same biological function, that is the transport of small hydrophobic molecules (Fig. 7).

Despite the overall lack of identity, amino acids were conserved in positions important for the maintenance of the structure of these molecules. The hydrophobic amino

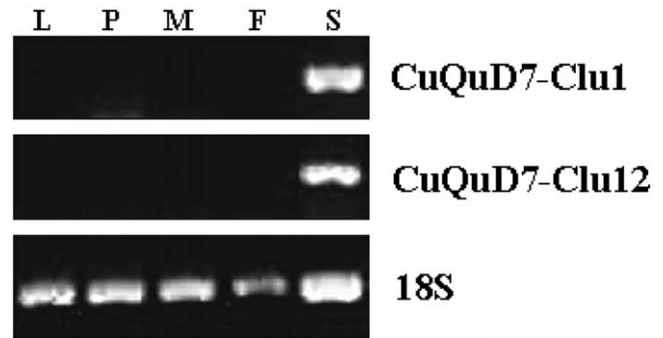


Fig. 6. Expression analysis of *Culex quinquefasciatus* CuQu-D7Clu1 and CuQu-D7Clu12 genes. Reverse transcription-PCR amplification was performed with gene-specific primers for the CuQu-D7Clu1, CuQu-D7Clu12 and the 18S ribosomal RNA. The templates were total RNA extracted from: L, last instar larvae; P, pupae; M, adult males; F, adult females minus salivary glands; SG, adult female salivary glands.

acids involved in the binding of hydrophobic ligands, residues Phe52, Phe68, Phe99 and Phe100 of THP12, are substituted conservatively (in equivalent steric position) by Trp93, Leu209, Tyr239 and Tyr240 in CuQu-D7Clu1 (35 kDa) and Trp93, Leu209, Tyr239 in CuQu-D7Clu12 (28 kDa). One non-conservative substitution was observed. In CuQu-D7Clu12, the Asn240, which is a small hydrophilic uncharged amino acid, replaces the hydrophobic residues. The four Cys are conserved absolutely among the three proteins analyzed, indicating the possibility of formation of disulfide bonds in D7-related protein of *Culex* mosquitoes. In THP12 the disulfide bonds Cys14-Cys45 and Cys85-Cys102 contribute to the proximity of helices A and B and helices E and F, respectively (Rothemund et al., 1999).

The position of the α -helix breaking residue Pro37 of *T. molitor* THP12 protein is conserved in CuQu-D7Clu1 and Gly57 is found at the same equivalent position in both proteins of *C. quinquefasciatus*. Two α -helices predicted for the D7-related proteins of *Culex* mosquitoes, overlap well with helices D and E of THP12. We could not find predicted α -helices in the portions of *Culex* proteins that correspond to the helix F of THP12, however, two extra α -helices are predicted at the carboxyl termini of the proteins encoded by clones CuQu-D7Clu1 and CuQu-D7Clu12.

4. Discussion

Human hypersensitivity to mosquito bites has been the subject of many investigations, however, the antigens related to the allergic reactions are unknown for most mosquito species. In order to identify the major antigens of *C. quinquefasciatus* saliva, ELISA and immunoblot analysis were conducted on sera of individuals from São Paulo City (Brazil).

In the majority of cases, levels of anti-mosquito SGE

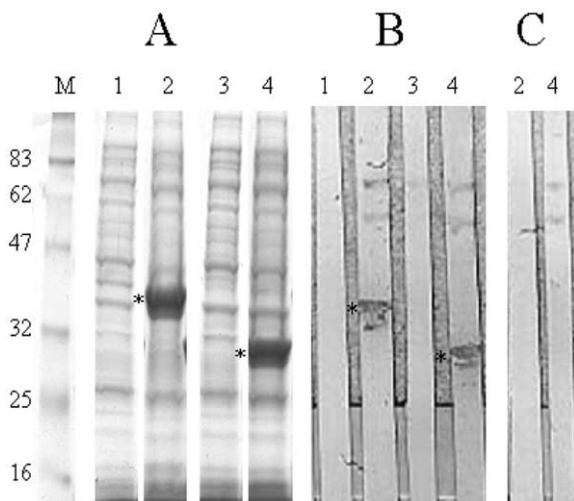


Fig. 5. SDS-PAGE and immunoblot analysis of recombinant D-7 proteins. (A) Coomassie blue stained SDS-PAGE of crude extracts of *E. coli* expressing recombinant *C. quinquefasciatus* D7 related proteins. Lane 1, pRSET-CuQu-D7Clu1 before IPTG induction; lane 2, pRSET-CuQu-D7Clu1 after 3 hr of induction with 1mM IPTG at 37°C; lane 3, pRSET-CuQu-D7Clu12 before IPTG induction; lane 4, pRSET-CuQu-D7Clu12 after 3 h of induction with 1 mM IPTG at 37 °C. M indicates molecular weight markers in kDa; (B) Immunodetection of recombinant D-7 proteins using a pool of sera from allergic adults (see Section 2). The numbers on the top of the figure indicate samples identical to those in (A). (C) Immunodetection of recombinant D-7 proteins using a pool of sera from non-allergic adults (control group with low level of anti mosquito IgE). The numbers on the top indicate samples identical to those in (A). * indicates the positions of the recombinant proteins.

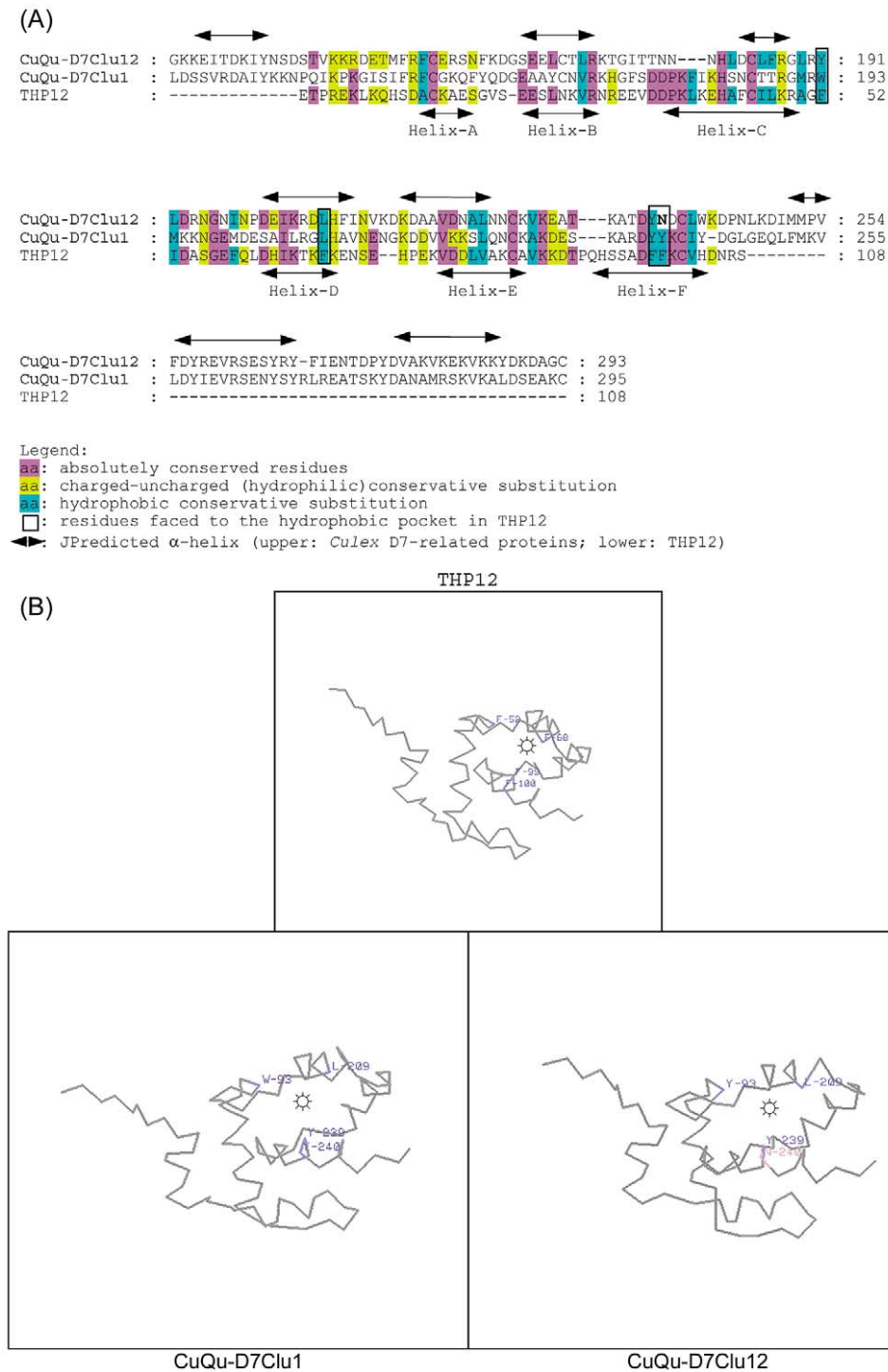


Fig. 7. Comparisons between the predicted primary (A) and tertiary (B) structures of the *Culex quinquefasciatus* D7 related proteins and the *Tenebrio molitor* THP12. The putative hydrophobic pocket is marked with ⚡ in the 3D-structure.

reactive IgE were higher in the sera of adult and children testing RAST-positive for mosquito antigens, than for those sera of the self-reported, non-allergic control group. It was interesting to note that some members of the control group had high levels of mosquito SGE reactive IgE (comparable to that found in allergic individuals) in their sera. It is possible that some of the

so-called “normal” or control individuals may present delayed skin reactions, which they may not relate to mosquito bites. Furthermore, other aspects of their immune response may modulate the severity of their reactions to mosquito antigens. Immunoblots revealed that the sera from all allergic individuals and the control individuals with high levels

of anti-mosquito IgE contain antibodies that bind to 28 and 35 kDa proteins of *C. quinquefasciatus* salivary glands. Some, but not all of the tested sera recognized 45 and 20 kDa antigens.

We noticed that the antibodies present in sera from all self-reporting non-allergic individuals with elevated IgE recognized the 20 kDa protein. None of the RAST-positive sera, however, recognized this protein. Sera from non-allergic individuals were screened with ELISA tests using a homologous antigen (*C. quinquefasciatus* salivary glands extracts) while RAST-positive individuals were identified with a heterologous antigen (*Aedes communis* mosquito extract). These results indicate that development of ELISA in which homologous antigens are used may provide a more sensitive test for mosquito bite allergies. Furthermore, ELISA tests would prevent the inoculation of crude mosquito extracts used for skin tests, a procedure that may induce non-specific intradermic reactions.

The search for molecules with sequence similarity to the ones determined for 35 and 28 kDa polypeptides in the NCBI database identified two *C. quinquefasciatus* cDNA clones. Both of them encode D7-like proteins (Valenzuela et al., 2002). The first D7 protein was identified in the salivary glands of *Aedes aegypti* mosquitoes (James et al., 1991) and more recently proteins with similar amino acid sequences have been identified in the salivary glands of several hematophagous insects (Valenzuela et al., 2002; Arcá et al., 1999, 2002). While the 35 and 28 kDa proteins of *C. quinquefasciatus* salivary glands are major components of mosquito saliva, no function has been determined for the molecules. However, their sex- and tissue-specific expression patterns suggest that they are involved with the blood feeding capabilities of female mosquitoes.

Putative phosphorylation sites (Kuenzel et al., 1987) are present in both predicted proteins, while N-glycosylation sites (Marshall, 1972) are present only in the polypeptide encoded by clone CuQu-D7Clu1. Since the predicted molecular weights of the mature proteins encoded by CuQu-D7Clu1 and CuQu-D7Clu12 are similar, and calculated as 33 and 34 kDa, respectively (Valenzuela et al., 2002), differences in post-translational modifications may account for the different gel mobility and therefore apparent molecular weights determined by SDS-PAGE for the 28 and 35 kDa proteins.

Arcá et al. (2002) and Valenzuela et al. (2002) suggested that D7-related proteins could act as carriers of small hydrophobic ligands in the saliva of mosquitoes, due their amino acid sequence similarity with insect odorant-binding proteins. Insect odorant and pheromone binding proteins are expressed mainly in the insect antennae and they are believed to bind small hydrophobic odorant molecules, carry and present them to the olfactory receptors. Structurally related proteins have been identified in other non-sensory organs and hemo-

lymph of insects, where they might perform other carrier functions (Biessmann et al., 2002). A hemolymph protein with sequence and structural characteristics similar to odorant binding proteins, named THP12, was described in *T. molitor* as a carrier protein associated with the transport of small hydrophobic ligands through the hemolymph (Rothmund et al., 1999; Graham et al., 2001). In *T. molitor*, the THP12 is a nonbundle α -helical structure consisting of six α -helices with loops between the helices and disordered N-terminus. The hydrophobic core is formed by helices D, E and F and the residues Phe52, Phe68, Phe99 and Phe100 clustered in a hydrophobic pore that functions as a binding site (Rothmund et al., 1999; Graham et al., 2001).

The amino acid alignment and three-dimensional modeling of *C. quinquefasciatus* D7-related proteins (clones CuQu-D7Clu1 and CuQu-D7Clu12) and the *T. molitor* THP12 supports the proposal that they may share a similar function. However, it is evident that only the C-terminal regions of D7-related proteins align with THP12. We assume that these amino acids must be the ones essential for the putative function. The N-terminal domain of the molecules could play a different function. It is interesting that THP12 is a secreted protein found in high concentration in the hemolymph of *T. molitor*, whereas the D7-related proteins are secreted in abundance by the salivary glands of *Culex* and *Aedes* mosquitoes (James et al., 1991; Nascimento et al., 2000). The high concentration of these proteins would facilitate their proposed function as carrier of trace quantities of hydrophobic ligands through aqueous fluids where they act. In this way, D7-related proteins could act as a carrier of hydrophobic molecules, which could be released into the vertebrate host, helping the blood feeding of mosquitoes. However, further studies are needed to elucidate their function or functions in the saliva of hematophagous insects.

Isawa et al. (2002) recently demonstrated that one of the *Anopheles stephensi* D7-related salivary proteins, designated hamadarin, inhibits activation of the plasma contact system and subsequent release of bradykinin, a mediator of the inflammatory reaction. The existence of multiple D7-related proteins in the saliva of *An. stephensi* (Suwan et al., 2002) as well as in the saliva of several other mosquitoes (Valenzuela et al., 2002) supports the possibility that each one of the D7 proteins performs a different antihemostatic function. In the bug *R. prolixus*, another blood-sucking insect, although no D7-related proteins have been found, seven proteins belonging to the lipocalin family were identified, each one of them having a different antihemostatic activity. Among these activities, vasodilation, inhibition of platelet aggregation, inhibition of coagulation and reduction of inflammation and immune response have been reported (Monfort et al., 2000). It is possible that the D7-related proteins evolved in a similar way as the *R.*

prolixus lipocalins, resulting in a diverse set of anti-hemostatic factors that help mosquito blood feeding activity.

By characterizing the reactions of allergic individuals to mosquito salivary antigens, we successfully identified two proteins with strong negative predicted values for mosquito allergy. Individuals who did not react to these proteins did not report mosquito bite allergy. All the allergic individuals had antibodies reactive to them. This work could provide the basis for the development of diagnostic test for mosquito (*C. quinquefasciatus*) allergy. Also, recombinant mosquito salivary proteins could be tested for their ability to desensitize allergic individuals or could be used in the development of immunotherapeutic agents to treat severe allergic reactions to mosquito bites.

Acknowledgements

The authors would like to thank Dr. Anete S. Grumach (Faculdade de Medicina-USP) and Dr. Sonia Regina Silva Siciliano (Setor de proteínas-Laboratório Fleury) for providing some of the human sera used in this work, Dr. J.M.C. Ribeiro for helpful discussions, Mara Williams for critical reading of the manuscript, Kailash Sachdeva, Regina Célia Leal and Susana Pessoa de Lima for technical assistance, and Lynn Olson for help in typing the manuscript. This work was supported by grants from FAPESP and NIAID (AI29746).

References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Arcá, B., Lombardo, F., Capurro, M., della Torre, A., Dimopoulos, G., James, A.A., Coluzzi, M., 1999. Trapping cDNAs encoding secreted proteins from salivary glands of the malaria vector *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA* 96, 1516–1521.
- Arcá, B., Lombardo, F., Lanfrancotti, A., Spanos, L., Veneri, M., Louis, C., Coluzzi, M., 2002. A cluster of four D-7-related genes is expressed in the salivary glands of the African malaria vector *Anopheles gambiae*. *Insect Mol. Biol.* 11, 47–55.
- Beaudouin, E., Kanny, G., Renaudin, J.M., Moneret-Vautrin, D.A., 2001. Allergen-specific immunotherapy to mosquitoes. *Allergy* 56, 787.
- Biessmann, H., Walter, M.F., Dimitratos, S., Woods, D., 2002. Isolation of cDNAs encoding putative odorant-binding proteins from the antennae of the malaria-transmitting mosquito *Anopheles gambiae*. *Insect Mol. Biol.* 11, 123–132.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72, 248–254.
- Charlab, R., Valenzuela, J.G., Andersen, J., Ribeiro, J.M.C., 2001. The invertebrate growth factor/CECR1 subfamily of adenosine deaminase proteins. *Gene* 267, 13–22.
- Cross, M.L., Cupp, E.W., Enriquez, F.J., 1994. Differential modulation of murine cellular immune responses by salivary gland extract of *Aedes aegypti*. *Am. J. Trop. Med. Hyg.* 51, 690–696.
- Edman, P., 1950. Method for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* 4, 283.
- Edwards, J.F., Higgs, S., Beaty, B.J., 1998. Mosquito feeding-induced enhancement of Cache Valley Virus (Bunyaviridae) infection in mice. *J. Med. Entomol.* 35, 261–265.
- Graham, L.A., Tang, W., Baust, J.G., Liou, Y.C., Reid, T.S., Davies, P.L., 2001. Characterization and cloning of a *Tenebrio molitor* hemolymph protein with sequence similarity to insect odorant-binding protein. *Insect Biochem. Mol. Biol.* 31, 691–702.
- Isawa, H., Yuda, M., Orito, Y., Chinzei, Y., 2002. A mosquito salivary protein inhibits activation of the plasma contact system by binding to factor XII and high molecular weight kininogen. *J. Biol. Chem.* 277, 27651–27658.
- James, A.A., Blackmer, K., Marinotti, O., Goshn, C., Racioppi, J.V., 1991. Isolation and characterization of the gene expressing the major salivary gland protein of the female mosquito *Aedes aegypti*. *Molec. Biochem. Parasitol.* 44, 245–254.
- Kelley, L.A., MacCallum, R.M., Sternberg, M.J.E., 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* 299, 499–520.
- Konishi, E., 1990. Distribution of immunoglobulin G and E antibody levels to salivary gland extracts of *Aedes albopictus* (Diptera: Culicidae) in several age group of Japanese population. *J. Med. Entomol.* 27, 519–522.
- Kuenzel, E.A., Mulligan, J.A., Sommercorn, J., Krebs, E.G., 1987. Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. *J. Biol. Chem.* 262, 9136–9140.
- Marshall, R.D., 1972. Glycoproteins. *Annu. Rev. Biochem.* 41, 673–702.
- McCormack, D.R., Salata, K.F., Hershy, J.N., Carpenter, G.B., Engler, R.J., 1995. Mosquito bite anaphylaxis: immunotherapy with whole body extracts. *Ann. Allergy Asthma Immunol.* 74, 39–44.
- Mellamby, K., 1946. Man's reaction to mosquito bites. *Nature* 158, 554.
- Monfort, W.R., Weichsel, A., Andersen, J.F., 2000. Nitrophorins and related antihemostatic lipocalins from *Rhodnius prolixus* and other blood-sucking arthropods. *Biochem. Biophys. Acta* 1482, 110–118.
- Nascimento, E.P., Malafronte, R.dos S., Marinotti, O., 2000. Salivary gland proteins of the mosquito *Culex quinquefasciatus*. *Arch. Insect Biochem. Physiol.* 43, 9–15.
- Nuttal, P.A., Paesen, G.C., Lawrie, C.H., Wang, H., 2000. Vector-host interactions in disease transmission. *J. Microbiol. Biotechnol.* 2, 381–386.
- Osorio, J.E., Godsey, M.S., Defoliart, G.R., Yuill, T.M., 1996. La Crosse viremias in white-tailed deer and chipmunks exposed by injection or mosquito bite. *Am. J. Trop. Med. Hyg.* 54, 338–342.
- Ohashi, M., Harada, M., Suguri, S., Ohmae, H., Ishii, A.I., 2001. The role of saliva of *Anopheles stephensi* in inflammatory response: identification of a high molecular weight neutrophil chemotactic factor. *Parasitol. Res.* 87, 376–382.
- Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. USA* 85, 2444–2448.
- Peng, Z., Yang, J., Simons, F.E.R., 1995. Measurement of mosquito *Aedes vexans* salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. *Ann. Allergy Asthma Immunol.* 74, 259–264.
- Peng, Z., Yang, J., Simons, F.E.R., 1996. Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens. *Ann. Allergy Asthma Immunol.* 77, 238–244.
- Peng, Z., Simons, F.E.R., 1998. Immunoblot analyses of salivary allergens in 10 mosquito species with world-wide distribution, and the human IgE responses to these allergens. *J. Allergy Clin. Immunol.* 101, 498–505.

- Ribeiro, J.M.C., 1995. Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infect. Agents Dis.* 4, 143–152.
- Ribeiro, J.M.C., 2000. Blood-feeding in mosquitoes: probing time and salivary gland anti-haemostatic activities in representatives of three genera (*Aedes*, *Anopheles*, *Culex*). *Med. Vet. Entomol.* 14, 142–148.
- Rothmund, S., Liou, Y.C., Davies, P.L., Krause, E., Sonnichsen, F.D., 1999. A new class of hexahelical insect proteins revealed as putative carriers of small hydrophobic ligands. *Structure* 7, 1325–1332.
- Shan, E.Z., Taniguchi, Y., Shimizu, M., Ando, K., Chinzei, Y., Suto, C., Ohtaki, T., Ohtaki, N., 1995. Immunoglobulins specific to mosquito salivary gland proteins in the sera of persons with common or hypersensitive reactions to mosquito bites. *J. Dermatol.* 22, 411–418.
- Simons, F.E.R., Peng, Z., 2001. Mosquito allergy: recombinant mosquito salivary antigens for new diagnostic tests. *Int. Arch. Allergy Immunol.* 124, 403–405.
- Suwan, N., Wilkinson, M.C., Crampton, J.M., Bates, P.A., 2002. Expression of D7 and D7-related proteins in the salivary glands of the human malaria mosquito *Anopheles stephensi*. *Insect Mol. Biol.* 11, 223–232.
- Valenzuela, J.G., Charlab, R., Calvo, E., Miranda-Santos, I.K.F., Marinotti, O., Francischetti, I., Ribeiro, J.M.C., 2002. The D7 family of salivary proteins in blood sucking diptera. *Insect Mol. Biol.* 11, 149–155.
- Wikel, S.K., Rachamandra, R.N., Bergman, D.K., 1996. Arthropod modulation of host immune responses. In: Wikel, S.K. (Ed.), *The Immunology of Host–Ectoparasitic Arthropod Relationships*. CAB International, Wallingford, pp. 107–130.