

Der-p2 (*Dermatophagoides pteronyssinus*) allergen-like protein from the hard tick *Ixodes ricinus* – a novel member of ML (MD-2-related lipid-recognition) domain protein family

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SUMMARY

Objective. Expression of the gene encoding Der-p2 allergen-like protein in the castor bean tick *Ixodes ricinus* is induced by blood intake. Tick Der-p2 allergen-like protein belongs to a diverse family of ML proteins that includes major allergens of house dust mites, human MD-2 or similar proteins from *Drosophila melanogaster*. In ticks, genes encoding proteins belonging to the ML protein family were identified, but their protein products have not been characterized yet. **Methods.** A gene encoding tick Der-p2 allergen-like protein was amplified from cDNA of engorged *I. ricinus* female using the gene-specific primers designed on a basis of partial sequences of related allergen-like genes. The tissue and state specific patterns of expression of the gene were analysed. The IgE binding activity of the produced recombinant protein was studied by use of ELISA. **Results.** Analysis of the expression pattern showed that the gene encoding the tick Der-p2 allergen-like protein is strongly induced by the bloodmeal in gut and haemolymph throughout all tick developmental stages. Der-p2 allergen-like protein possesses a putative lipid-binding site, according to the comparisons with the related proteins. The ability of tick Der-p2 allergen-like protein to bind immunoglobulin E (IgE) was revealed. **Discussion.** The presence of a putative lipid-binding domain in Der-p2 allergen-like protein and its ability to interact with IgE might indicate the involvement of the protein in the tick's immune response.

Key words: Der-p2 allergen-like protein, *Ixodes ricinus*, tick, ML protein family, IgE-binding activity.

INTRODUCTION

While the occurrence of allergens in mites (phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acari) is quite often well documented, the knowledge of similar proteins in ticks is rather limited. Allergens of house dust mites can be divided into several groups (1–19). It has been reported that their homology within single groups is quite high (70–80%) but they differ in molecular weight, level of glycosylation, content of lipids or in biochemical function (Thomas *et al.* 2002).

Hard ticks belong to the phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acari, order Ixodida, and family Ixodidae. They are well-known blood-feeders, vectors of many pathogenic microorganisms, causing diseases both in animals and humans. While taking a bloodmeal ticks encounter a large diversity of pathogens from hosts, their organisms undergo dramatic changes on morphological, physiological and molecular levels. In the complex response of the gene expression towards

the presence of the pathogenic agent, it is possible to expect the production of proteins that represent the molecular factors of innate immunity either involved in defence reactions or pathogen pattern recognition (Ribeiro, 1995; Mans and Neitz, 2004; Sonenshine and Hynes, 2008). During blood feeding, ticks secrete different bioactive molecules into their host. The set of secreted molecules depends on the length of blood feeding or on the developmental stage of the tick, as well as on the immune response of the host (Lawrie and Nuttall, 2001). The majority of molecules are secreted by salivary glands and their description is an object of many recent studies, for example on *Argas monolakensis* (Mans *et al.* 2008), *Dermacentor andersoni* (Alarcon-Chaidez *et al.* 2007) or *Ixodes ricinus* (Leboulle *et al.* 2002; Rudenko *et al.* 2005; Chmelař *et al.* 2008).

Allergens or allergen-like proteins were identified and characterized in the hard tick *I. ricinus* and in the soft tick *Argas reflexus* (Jacot, *personal communication*; Hilger *et al.* 2005). An *alerg 1* gene of the castor bean tick encodes an allergen-like protein that is a member of the ML (MD-2-related lipid-recognition) domain protein family as well as group 2 of mite allergens. The ML protein family is

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comprised of 4 groups and is common to animals, plants and fungi. Its members contain an N-terminal signal peptide and conserved cysteine residues that form disulfide bridges (Wright *et al.* 2000). Often, their function is connected with innate immunity or with lipid binding and transport (Inohara and Nuñez, 2002). *I. ricinus* allergen-like protein shows an homology of about 30% to group 2 mite allergens and contains a domain with putative lipid-binding function (Jacot, *personal communication*). An allergen Arg r 1 from the pigeon tick *A. reflexus* has 4 conserved cysteine residues that form disulfide bridges, an N-terminal signal peptide, and other characteristics typical for a protein family of lipocalins including the G-X-W pattern. Based on the homology with other histamine-binding proteins of different tick species and due to possible antibody cross-reactivity with similar proteins from *Rhipicephalus appendiculatus* and *Haemaphysalis punctata* allergen Arg r 1 was grouped with histamine-binding proteins (Rolla *et al.* 2004; Hilger *et al.* 2005).

This is a report on the identification and characterization of a new tick gene encoding Der-p2 allergen-like protein from *I. ricinus*, analysis of protein structure, relation to other members of ML domain protein family and similar proteins from the other tick species. We defined the tissue and stage-specific expression pattern of the gene and analysed the Der-p2 allergen-like protein interaction with immunoglobulin E (IgE).

MATERIALS AND METHODS

Ticks

Hard ticks *I. ricinus* were provided by the Biology Centre, Institute of Parasitology, Academy of Sciences of the Czech Republic. Uninfected ticks were reared for several generations in the animal facilities of the institute and fed on guinea pigs that were raised to be free of infection under the strict hygiene regulations of the Central Commission for the Protection of Animals (§21, section 3e, law 246/1992). Blood-fed larvae, nymphs and females were obtained by attaching a container (cell) with ticks to a shaved area on the back of an adult uninfected guinea pig and left on the animal back until the specified engorgement stage. After the separation of the fed ticks from the guinea pig, RNA was isolated from them using TRI Reagent (Sigma, USA). The fully engorged females were kept at 4 °C for a further 3 days for blood digestion prior to RNA isolation.

First strand cDNA synthesis and polymerase chain reaction (PCR)

First strand cDNA was synthesized by use of RevertAid H Minus First Strand cDNA Synthesis

Kit (MBI Fermentas, Lithuania) according to the protocol provided by the manufacturer. Primers AL1F and AL1R were designed according to the known partial nucleotide sequence of the gene encoding *I. ricinus* allergen-like protein (AJ547805) and used for amplification of *I. ricinus* gene for the Der-p2 allergen-like protein (AL1F 5'-ATGTTCCGTTACGTTGTGTTCC-3'/AL1R 5-CATCC-CCACCGCTGTAAC-3'; Generi Biotech, Czech Republic).

RT-PCR analysis of stage specific and tissue specific patterns and time expression of the Der-p2 allergen-like gene

RT-PCR was performed using Enhanced Avian HS RT-PCR kit (Sigma, USA). An equal amount of total RNA isolated from unfed and engorged larvae, nymphs and females of *I. ricinus* and different tissues (salivary glands, gut, ovarium, Malphigian tubes, and haemolymph) of partially engorged females was used as a template in RT-PCR in order to reveal the Der-p2 allergen-like gene expression in different developmental and feeding stages. To determine time expression of the gene, an equal amount of total RNA from unfed, 1, 3, 5, 7 days fed, and totally engorged adult females were used as a template in RT-PCR. The primers, AL2F 5'-CACCATGTTCCGTTACGTTGTGTTCC-3', AL2R 5'-TCACTCGATGACGATGTCCGA-3' were used for the amplification with an annealing temperature of 55 °C. A constitutively expressed actin gene (AY333957) was chosen as a control. The control amplification was performed at the annealing temperature of 55 °C with the primers Act-F1 5'-CGTCTGGATCGGCGGCTCTAT-3' and Act-R1 5'-ACGCGCACTCTTTTCCACAATCTC-3'. All used RNAs were first checked for DNA contamination by adding them directly into the PCR reaction with specific primers.

Bioinformatics/sequence analysis

Sequences were compared against the GenBank non-redundant (NR) protein database using the BlastX program from the executable package at <ftp://ftp.ncbi.nlm.nih.gov/blast/executables> (Altschul *et al.* 1997) and searched against the Conserved Domains Database (CDD) (<ftp://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd>), which includes all Pfam (Bateman *et al.* 2000) and Smart (Schultz *et al.* 1998) protein domains, as well as against the VectorBase (Lawson *et al.* 2009) (<http://www.vectorbase.org>) and the Gene Index Project (Quackenbush *et al.* 2001) (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi>). Protein secondary structure prediction was performed by PSIPred program (<http://bioinf.cs.ucl.ac.uk/psipred>) (McGuffin *et al.* 2000). Putative signal peptide, possible sites of

O-glycosylation, N-glycosylation and phosphorylation were determined with the prediction servers Signal P 3.0 (<http://www.cbs.dtu.dk/services/SignalP>) (Bendtsen *et al.* 2004), NetOGlyc 3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc>) (Julenius *et al.* 2005), NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>) and NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos>) (Blom *et al.* 1999), respectively.

Tertiary structure prediction

For prediction of the 3-D structure of the Der-p2 allergen-like protein CPHmodel 2.0 Server was used (<http://www.cbs.dtu.dk/services/CPHmodels>) to find the homologues for the *I. ricinus* molecule. A pdb file of the molecule (according to the similar sequences of structures from the protein databank) was downloaded and used in Polyview-3D for protein visualization (<http://polyview.cchmc.org/polyview3d.html>) (Porollo and Meller, 2007).

Expression and purification of the recombinant protein

The gene encoding the Der-p2 allergen-like protein without signal peptide, prepared by PCR using the primers AL3F 5'CACCAGCGCAGAAAAGCCGT-3' and AL2R (sequence above), was expressed in pET100/D-TOPO (Invitrogen, USA) expression vector as recombinant fusion proteins with N-terminal 6xHis tag. Rosetta-gami competent cells (Novagen, Germany) were transformed by recombinant construct. All procedures were performed according to the manufacturer's protocol in Champion pET directional TOPO expression kits (Invitrogen, USA). Isopropyl- β -D-thiogalactopyranoside (IPTG) (1 mM final concentration) was used for the induction of recombinant protein expression. Recombinant Der-p2 allergen-like protein was purified by Co²⁺-affinity chromatography using cartridge Co-MAC (Novagen, Germany). Bacterial lysate was prepared using lysis buffer containing 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0. The purification under denaturing conditions was performed according to the manufacturer's protocol. The cartridge was equilibrated with 5 volumes of binding buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 0.005 M imidazole, pH 7.9), and then the sample in binding buffer was applied. The cartridge was washed with 10 volumes of binding buffer, 6 volumes of washing buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 0.06 M imidazole, pH 7.9) and the recombinant protein was eluted with 6 volumes of elution buffer (6 M urea, 0.5 M NaCl, 0.02 M Tris-HCl, 1 M imidazole, pH 7.9). The elution fractions were checked on SDS-PAGE and fractions containing recombinant protein given for further tests were dialysed against PBS (phosphate-buffered

saline) buffer, pH 7.4 with 6 M urea, 4 M urea, 2 M urea, 1 M urea, 0.5 M urea and PBS, respectively. The presence of the His-tagged Der-p2 allergen-like protein was verified by immunodetection with Ni-NTA conjugates (Qiagen, Germany).

Antimicrobial activity determination

A double-layer technique was used to estimate antimicrobial activity of the recombinant Der-p2 allergen-like protein (Čeřovský *et al.* 2008). The plates were incubated at 37 °C for 6 h with periodic checking for the growth of bacteria (*Escherichia coli*, *Micrococcus luteus*).

IgE binding reactivity

Purified recombinant Der-p2 allergen-like protein was diluted to 10 μ g/ml in PBS. Each well of a 96-well microplate (Maxisorp F96 MicroWell Plate, Nunc, Denmark) was coated with 50 μ l of the recombinant protein and incubated at 4 °C overnight. All further incubations were performed at room temperature with at least 5 washes with PBS containing 0.05% Tween 20 (PBST) between them. Then 50 μ l of human biotinylated IgE (BioPorto Diagnostic, Denmark) at different concentrations (0.1, 1, 3, 5, 10 μ g/ml) were added into a microplate and incubated for 1 h, after which 50 μ l of streptavidin-peroxidase (Sigma, USA) diluted 1 : 1000 in distilled water was added to each well and incubated for 30 min. Orthophenylenediamine (Sigma, USA) with hydrogen peroxide (50 μ l/well) was used as the substrate for the enzymatic colour reaction. The reaction was performed in the dark for 15 min and stopped by the addition of 50 μ l of 1 M H₂SO₄. Optical densities were measured at 490 nm with an ELISA spectrophotometer (Multiskan MCC 340, Labsystems Oy, Finland). Bovine serum albumin (BSA) and a recombinant protein produced in a prokaryotic expression system were used as controls.

Statistical analysis

The significance of any differences obtained between single protein groups and used different antibody concentrations was evaluated by the factorial ANOVA with a subsequent Tukey HSD test (Statistica 6.0, Statsoft, Inc. Tulsa, USA). The presented results represent 3 similar experiments.

RESULTS

A gene encoding Der-p2 allergen-like protein, a protein that contains the ML (MD-2-related lipid-recognition) domain was identified in the hard tick *I. ricinus*. The isolated cDNA sequence of

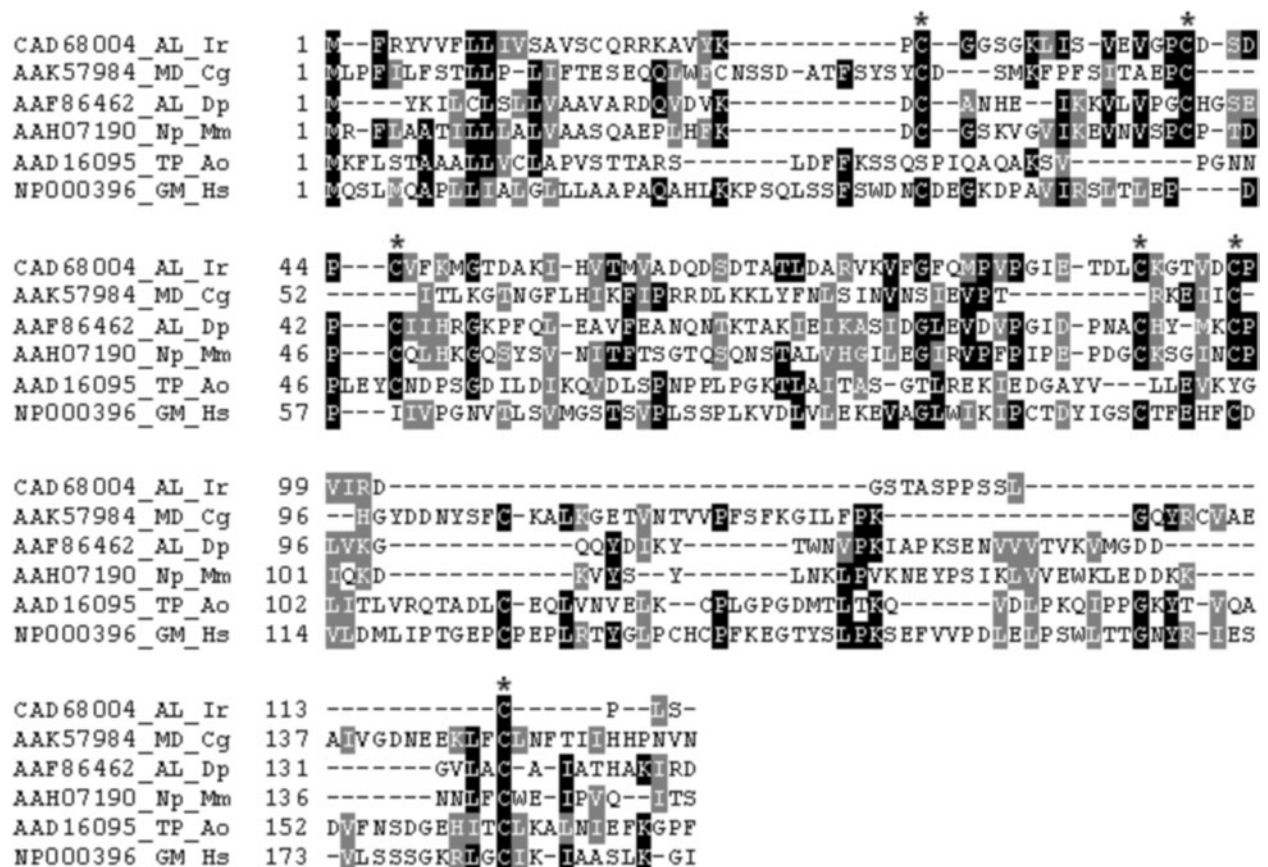


Fig. 1. Alignment of *Ixodes ricinus* Der-p2 allergen-like protein (CAD68004) and other members from all four groups of ML protein family. Group I: MD-2 protein of *Cricetus griseus* (GenBank AAK57984), group II: Der p 2 allergen from *Dermatophagoides pteronyssinus* (AAF86462), *Mus musculus* Npc2 protein (AAH07190), group III: PG/PI-TP of *Aspergillus oryzae* (AAD16095), group IV: GM2 activator from *Homo sapiens* (NP000396). Conserved cysteine residues are marked by asterisks. Black boxes with white letters show identical residues, and grey boxes with black letters show chemically similar residues.

Der-p2 allergen-like protein is 595 bp long and contains an open reading frame (ORF) encoding a protein of 144 amino acids with a predicted molecular mass of 15.57 kDa and isoelectric point 7.92 for the pre-protein and 13.77 kDa and pI 7.62 for the mature Der-p2 allergen-like protein. The protein carries a 16 amino acid long N-terminal signal peptide. Six conserved cysteine residues were detected at positions 26, 40, 45, 91, 96 and 136 of the pre-protein. Der-p2 allergen-like protein has a predicted helix at the site of the signal peptide followed by β -structures and coil regions only. While 5 putative phosphorylation sites (serines at positions 34, 65, 106, 118, 134) were defined in the Der-p2 allergen-like protein sequence, no sites of possible O-glycosylation and N-glycosylation were found.

Der-p2 allergen-like protein is a member of the ML domain protein family and includes a Der-p2-like domain (Marchler-Bauer and Bryant, 2004). Alignment of *I. ricinus* protein with several members from different groups of the ML protein family showed a low similarity, defining only a few regions with higher homology. Conserved cysteine residues

were revealed in aligned sequences at specific positions. The highest level of similarity of *I. ricinus* Der-p2 allergen-like protein was shown in group II of the ML domain protein family, while no similarity between it and the selected members from other groups (I, III, IV) was detected (Fig. 1).

BLASTP, GenBank, and Gene Index Project analyses of the translated product of the Der-p2 allergen-like protein gene revealed similarity to several tick proteins from *I. ricinus*, *I. scapularis* and *Rhipicephalus microplus* and to different mite allergens (members from the group II of ML protein family from organisms of the same subclass Acari, class Arachnida). The amino acid sequence of the *I. ricinus* Der-p2 allergen-like protein, submitted to the GenBank (ABL61513), showed the highest sequence identity to the allergen-like protein from *I. ricinus* (CAD68004) – 92% and to the Der-p2 allergen-like protein from *I. scapularis* – 88% (TC55436). Lower identities (42–58%) to other related proteins from *I. scapularis* and *R. microplus* were also revealed. An average similarity of 35% to allergens and their precursors from *Psoroptes ovis* (group 2 allergen Pso o 2 precursor, Q965E2),

ABL61513_Ir	1	-----MF-----RY
CAD68004_Ir	1	-----MF-----RY
TC55436_Is	1	AW-----SRPRLQSPTAMF-----RY
TC47248_Is	1	S-----CPARSW
EW945095_Is	1	P-----GPLGPRP
EEC13776_Is	1	-----MSRS
EEC06676_Is	1	-----MPGAV-----
TC18108_Rm	1	EQ-----ALKGHS
TC16878_Rm	1	T-----MIRF
TC22513_Rm	1	RF-----AVMNNS
CV443743_Rm	1	KSGFGEFPGFVPRVTCLTCCVSADYIVVPSFTGGATRIPISSPLTLVCTSTSLTDMLMRS
ABL61513_Ir	5	LVFLLIVGAVSCQRRKAVYKPCGGSGKLISVEVEPCDSDPCVFKRGTDVK-----VHV
CAD68004_Ir	5	VVFLLIVSAVSCQRRKAVYKPCGGSGKLISVEVGPCDSDPCVFKMGTDAK-----IHV
TC55436_Is	17	VVFLLIVSAVSCQRGKAVYKPCGGSGKLISVEVEPCDSDPCVFKMGTDAK-----IHV
TC47248_Is	9	SWSSASPPASDG-SSTTGDCGSTA-KLLAVEIEPCMSDPCVFKRGTDIK-----IYF
EW945095_Is	9	RLLPATEAQLQGLRKHHRKAPGCRD-RALQLR-----PLRLQRGTDIK-----IYF
EEC13776_Is	5	FLVLLVLGLASCQRRKLNRYDCGE-H--LQDSHCROAPCTAHRTTTSKTS SGGVRLFF
EEC06676_Is	6	-----IQSLIVTPCTSDPCVIPVGTQ-----INITF
TC18108_Rm	9	SSFS-----CDCAAPSAQT-GSEAKLLSFQIEPCDSEPCVFKRGNTTT-----IRF
TC16878_Rm	6	VACILLCGLAAARRDIIYEDCGSQAKILSACIEPCDSDPCVFKRGTKPK-----VYF
TC22513_Rm	9	VVIVLLVGLAFVQCRD IKYQDCGSEAKLLSVQIEPCDSEPCVFKRGNTTT-----IRF
CV443743_Rm	61	ALLFVAIGVAFGQIRDAVYEDCGSTAEIISLQVEPCMSDPCVMKRGTAAR-----IYF
ABL61513_Ir	58	TMVADQSDTATLDARVKVFGFQMPVPGIETDLCK-GTVECPVTKGRKESVTAIFPVPSL
CAD68004_Ir	58	TMVADQSDTATLDARVKVFGFQMPVPGIETDLCK-GTVDCPVIR---DGSTASPPSSL
TC55436_Is	70	TMVADQSDTATLDARVKVFGFQMPVPGIETDLCK-GTVECPVVKGRTYNATAVFPVPSL
TC47248_Is	59	EMISDQNSDVTTLDRVKMFGFDMFVPGIETDLCK-GVTKCPVVKGCTYKGTIIFFPVRAA
EW945095_Is	53	EMISDQNSDVTTLDRVKMFGFDMFVPGIETDLCK-GVTKCPVVKGFAYKGTIIFFPVRAA
EEC13776_Is	60	-LLADQNSDMVTTLDRVKMFGFDMFVPGIETDLCK-GVTKCPVVKGCTYKGTIIFFPVRAA
EEC06676_Is	32	EVESNQSDRVLFDPRVTVLGVQLPIPGVEKDACEGAWVCPVVKGLKLAGTISAYVYNF
TC18108_Rm	54	SVISDQSDTATLDARVKVFGFMLPIPGVEKDLCK-YAQCPVVKGNTYQGSIDVYVPWF
TC16878_Rm	59	TVTSDQDTEKATLDATIGVFGLGISVPLDKDLCE-NMVKCPISKGQTYSGVDEYVPPF
TC22513_Rm	62	SVISDQSDTATLDARVKVFGFMLPIPGVEKDLCK-YAQCPVVKGNTYQGSIDVYVPWF
CV443743_Rm	114	EMVSDQDSETAELKVTTKLFVSVVPGIETMCK-GVVTCPVVKGRTYKGVLTMPIASF
ABL61513_Ir	117	M-SLKTEVTFKVIQDKGLSVCQSDIVI--E
CAD68004_Ir	113	-----CPLS-----
TC55436_Is	129	M-SMKTEVTFKVIQDKGLSVCGIADVVI--E
TC47248_Is	118	SPQMKSVVTLKVLGEHGTSVCTKTDILV--Q
EW945095_Is	112	SPQMKSVVTL-----
EEC13776_Is	118	SPQV-----
EEC06676_Is	92	VPSLTVTTTWKVMVGAQGIACGATNVTVRK
TC18108_Rm	113	IPSVKTTAQIKLVGDEGVSVCIIRSKIIV--G
TC16878_Rm	118	APAMKTHVSLKVVGDKGISVCAKTPIMV--E
TC22513_Rm	121	IPSVKTTAQIKLVGDEGVSVCIIRSKIIV--G
CV443743_Rm	173	APAGKTSLNMKVKGDKGVSVCTMNSFLIL--E

Fig. 2. Proteins obtained by comparison of Der-p2 allergen-like protein with other tick proteins using blastp analysis against GenBank, VectorBase and Gene Index project databases (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; <http://iscapularis.vectorbase.org/index.php>; <http://compbio.dfci.harvard.edu/tgi/tgipage.html>). Der-p2 allergen-like protein (ABL61513) shows the highest similarity (96%) with allergen-like protein of *Ixodes ricinus* (CAD68004), lower similarity with proteins of *I. scapularis* – 60–95% (TC55436, TC47248, EW945095, EEC13776, EEC06676) and with proteins of *Rhipicephalus microplus* – 65–72% (TC18108, TC16878, TC22513, CV443743).

Lepidoglyphus destructor (type 2 allergens Lep d 2, AAQ73492, AAQ73491, AAQ73488, AAQ73490) or *Glycyphagus domesticus* (Gly d 2.03, AAQ54603) was detected (Figs 2 and 3).

The tertiary structure of ML proteins was defined only for a few members of the protein family. The highest homology of *I. ricinus* Der-p2 allergen-like protein to known structures was defined for the

ABL61513_Ir_AL	1	MFRYLIVLLIVGAVSCQRRKAVYKPCGSGKLIISVEVEPCDS-DPCVFKKGTDVKWHVTM
Q965E2_Pso_o_2	1	MMKTLIVLLAITLAVVSAG-KYKFCDCG-KGEVESLEVEGCSG-DYCVIHKGKKDLAISV
AAQ73492_Lep_d_2	1	MMKFIVLPAL-VAVASAG-KMKFKDCG-KGEVTELDITDCSG-DFCVLHRGKSVTLDAKF
AAQ54603_Gly_d_2	1	MMKFIVLPAL-VAVASAG-KMKFKDCG-KGEVTELDITDCSG-DFCVLHRGKSVTLDAKF
AAQ73491_Lep_d_2	1	MMKFIVLPAL-VAVASAG-KMKFKDCG-KGEVTELDITDCSG-DFCVLHRGKSVTLDAKF
CAI05848_Der_f_2	1	MISKILCLSLIVAAVVAD-QVDVKCA-NHEIKKMMVDGCHGSDPCIIHRGKPFNLEAIF
ABL61513_Ir_AL	60	VADQSDTATLDARVKVFGFQMPVPGIETDLCKGTVECPVIKGRKYSVTAIFPVPMSL
Q965E2_Pso_o_2	58	TSNQDSANLKLIDIVADINGVQIEVPGVDHDGCH-YVKCPIKKGQHFQVYKTYISIPAILPT
AAQ73492_Lep_d_2	57	VANQDSAKATIKVLAKVVGTOIQVPGFDTDGCK-IKCPICKGDPIDFKYSGTIPAITPK
AAQ54603_Gly_d_2	57	VANQDSAKATIKVLAKVVGTOIQVPGFDTDGCK-IKCPICKGDPIDFKYSGTIPAITPK
AAQ73491_Lep_d_2	57	VANQDSAKATIKVLAKVVGTOIQVPGFDTDGCK-IKCPICKGDPIDFKYSGTIPAITPK
CAI05848_Der_f_2	59	DANQNTKTAKIEIKANIDGLEVDVPGIDTNACH-YIKCPLVKGQYDAKYTNWVPKIAPK
ABL61513_Ir_AL	120	KT--EVTFKVIGDKGLSVCGQSD-IVIE-
Q965E2_Pso_o_2	117	TK-AKTIIAKIIGDKGLGECIVIN-GEIQD
AAQ73492_Lep_d_2	116	IK-AEVTAEIIGDHGILACGTVN-GTVE-
AAQ54603_Gly_d_2	116	IK-AEVTAEIIGDHGILACGTVN-GQVE-
AAQ73491_Lep_d_2	116	IK-AEVTAEIIGDHGILACGTVN-GQVE-
CAI05848_Der_f_2	118	SENVVVTVKLVGDMGVYLACAIATHAKIRD

Fig. 3. Comparison of *Ixodes ricinus* Der-p2 allergen-like protein with selected house dust mite proteins from group II of ML protein family. The similarity with allergens and their precursors of *Psoroptes ovis* (group 2 allergen Pso o 2 precursor, Q965E2), *Lepidoglyphus destructor* (type 2 allergens Lep d 2, AAQ73492, AAQ73491), *Glycyphagus domesticus* (Gly d 2.03, AAQ54603) and *Dermatophagoides farinae* (Der f 2, CAI05848) ranges from 30 to 35%.

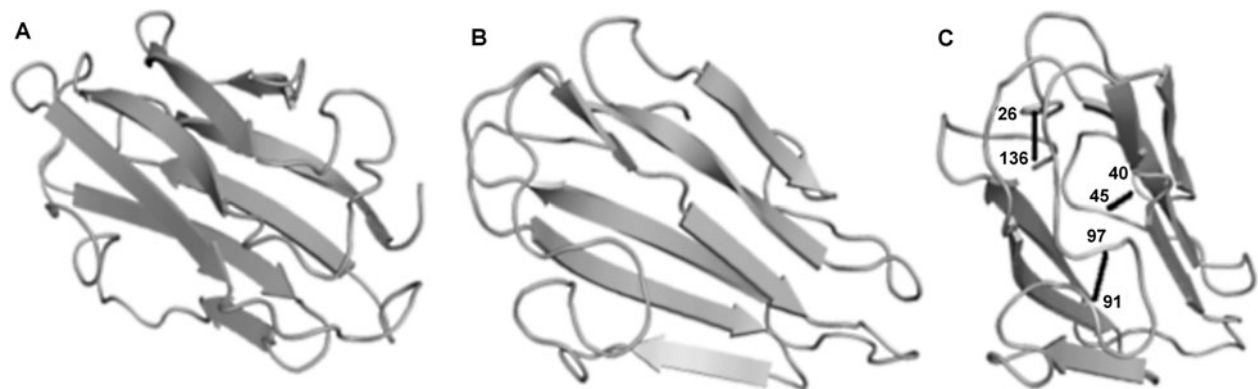


Fig. 4. Tertiary structure of Der f 2 allergen of *Dermatophagoides farinae* – 1 ahm (A) and predicted tertiary structure of Der-p2 allergen-like protein of *Ixodes ricinus* (B, C) with conserved cysteine residues marked.

major mite allergen from *Dermatophagoides farinae* Der f 2 (PDB 1 ahm) with 32.8% identity. Prediction of tick Der-p2 allergen-like protein structure revealed that it has an immunoglobulin fold (s-type). The tick allergen forms 2 plates of β -sheets, one is composed of 4 β -strands (residues 68–76, 79–83, 102–105, 121–129) and the other sheet consists of 3 β -strands (33–38, 53–61, 107–111). The mentioned structure is identical to that of Der f 2 (Bork *et al.* 1994; Ichikawa *et al.* 1998) (Fig. 4).

The results of RT-PCR on the detection of the stage expression pattern of the Der-p2 allergen-like protein gene show that its expression is strongly induced by a bloodmeal in all developmental stages (larva, nymph and adult) of the tick. This observation is confirmed by analysis of the time expression using total RNA from unfed and engorged females

at different feeding stages. Der-p2 allergen-like protein is slightly expressed in unfed ticks. The expression of Der-p2 allergen-like protein starts on the first day of feeding and reaches its maximum on the third day. The level remains stable until full engorgement (data not shown). Analysis of the tissue-specific pattern of the gene expression revealed that gut and haemolymph are the only sites of its expression. An expression level of the actin gene (positive control) was constant throughout the whole experiment (Fig. 5).

We produced a recombinant Der-p2 allergen-like protein in a prokaryotic expression system. The PCR product of 387 bp for Der-p2 allergen-like protein gene (without a signal peptide) was expressed as a 16 kDa 6xHis tag fusion protein (Fig. 6). MS analysis of the purified recombinant protein confirmed its

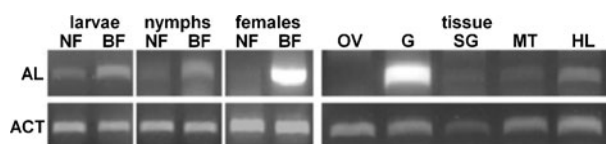


Fig. 5. Stage and tissue specific Der-p2-like gene expression analysis by RT-PCR. AL – Der-p2 allergen-like protein, ACT – actin; NF – unfed ticks, BF – engorged ticks; OV – ovary, G – gut, SG – salivary glands, MT – Malpighian tubes, HL – haemolymph.

identity to the predicted translated protein sequence of Der-p2 allergen-like protein gene from *I. ricinus* by 54.5% amino acid sequence coverage (data not shown).

Analysis of the antimicrobial activity of *I. ricinus* Der-p2 allergen-like protein against *E. coli* or *M. luteus* using the double-layer technique did not reveal any effect on bacterial growth.

Because of the relatively high similarity of Der-p2 allergen-like protein to Der f 2 and other mite allergens that interact with IgE, we tested the IgE-binding reactivity of the recombinant tick Der-p2 allergen-like protein by ELISA. BSA and a recombinant protein produced by *E. coli* were used as controls. After incubation of Der-p2 allergen-like protein and IgE, the detected signal increases according to the concentration of antibody used (Fig. 7). Statistically significant differences ($P < 10^{-5}$) were observed between all tested groups – protein samples, IgE concentrations and combination of proteins and used IgE concentrations. The results obtained after incubation of the Der-p2 allergen-like protein with IgE are significantly different from those of both control proteins in the case of 1 and 10 $\mu\text{g/ml}$ IgE concentrations; in the case of other concentrations of IgE the results significantly differ between Der-p2 allergen-like protein and BSA (0.1 $\mu\text{g/ml}$ IgE) and between the Der-p2 allergen-like protein and the control recombinant protein (3 and 5 $\mu\text{g/ml}$ IgE).

DISCUSSION

This is the first time that the gene encoding Der-p2 allergen-like protein from the hard tick *Ixodes ricinus* has been identified, isolated and characterized. Similar genes or their protein products have not yet been characterized in any other tick species. Protein homologues to *I. ricinus* Der-p2 allergen-like protein were found in *I. scapularis* EST database (Quackenbush *et al.* 2001), but have not yet been isolated or characterized. A similarity search of sequential databases from other available tick species revealed the existence of the related proteins in *I. scapularis* and *R. microplus*. All detected proteins contain the ML domain. The presence of N-terminal signal peptide and its gene expression in the tick's

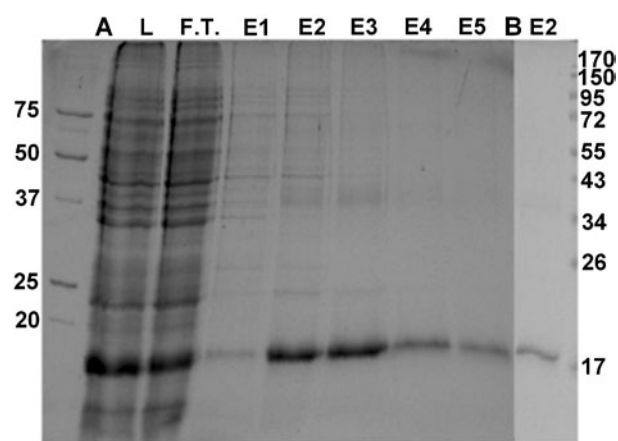


Fig. 6. Purification of recombinant Der-p2 allergen-like protein. A: L – bacterial lysate, F.T. – flow-through, E1-E5 – elution fractions, marker – Precision Plus Protein Standards (Bio-Rad, USA); B: fraction E2 in PBS used in further tests, marker – PageRuler Prestained Protein Ladder (Fermentas, Lithuania).

gut during blood intake could be an indicator of protein occurrence in secretory cells and its possible secretion into the gut lumen, as was observed in *I. ricinus* allergen-like protein (Inohara and Nuñez, 2002; Mullen *et al.* 2003; Jacot, *personal communication*). Visualization of the predicted tertiary structure of Der-p2 allergen-like protein on the basis of its similarity to Der f 2 allergen from *D. farinae* showed a structure with 2 anti-parallel beta-plated sheets typical for members of the ML protein family (Ichikawa *et al.* 1998; Derewenda *et al.* 2002; Marchler-Bauer *et al.* 2007). Three disulfide bridges stabilize the tertiary structure of ML proteins; Der-p2 allergen-like protein conserved cysteines are involved in disulfide bridge formation in the order of 1–6, 2–3, 4–5 as was observed in house dust mite allergens from group 2 (Mueller *et al.* 1998; Derewenda *et al.* 2002; Thomas *et al.* 2002). The presence of 3 disulfide bridges is considered to be important for recognition by IgE antibodies in the case of allergens (Mueller *et al.* 1998).

Tertiary structures of different ML protein family members were revealed only for allergens of house dust mites *D. pteronyssus* Der p 2 (Mueller *et al.* 1998; Derewenda *et al.* 2002), *D. farinae* Der f 2 (Ichikawa *et al.* 1998, 2005), bovine Npc2 protein (Friedland *et al.* 2003), human MD-2 (Ohto *et al.* 2007), and human ganglioside M-2 (GM-2) activator protein (Wright *et al.* 2000, 2005). The first 3 mentioned proteins belong to group II of the protein family. Similar to Der f 2, they are single-domain proteins of immunoglobulin fold. Proteins with such folding appeared to be involved in binding functions (Bork *et al.* 1994) that indicate also the presence of a putative lipid-binding site of the Der-p2 allergen-like protein. Ligands of Npc2, GM-2 activator or MD-2 proteins have been found,

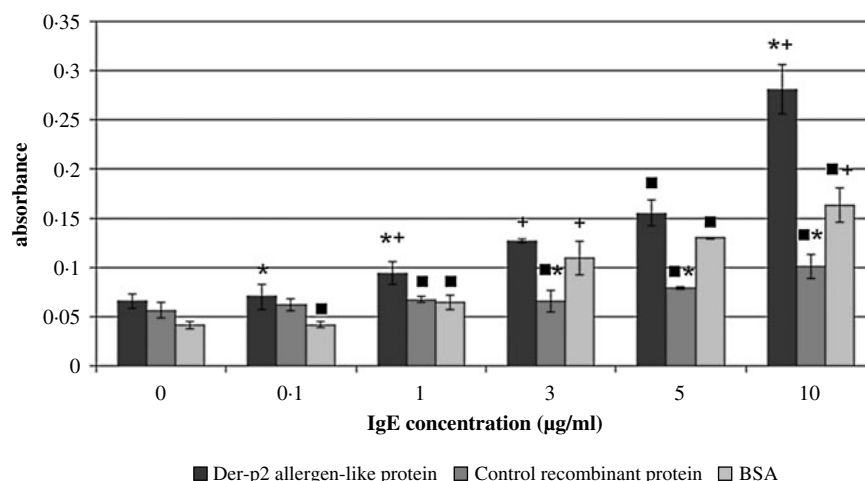


Fig. 7. IgE binding profiles to recombinant Der-p2 allergen-like protein, control recombinant protein produced in *E. coli* and BSA. It was confirmed that there are no statistically significant differences between proteins not incubated with antibody. Statistically significant differences were found in other groups: * statistical significant difference from BSA, + statistical significant difference from control recombinant protein, ■ statistical significant difference from Der-p2 allergen-like protein.

recognizing their role as lipid or lipidic molecule-interacting proteins (Mahuran, 1998; Shimazu *et al.* 1999; Friedland *et al.* 2003). This feature indicates the possible interaction with lipidic parts containing structures of different microorganisms as well as their possible roles in innate immunity.

The majority of allergens can cause allergic reactions with different levels of severity. They are able to interact with IgE antibodies, stimulate their production as well as induce T-cell responses. In mites, the major groups of allergens known to interact with IgE are those of groups 1 and 2, but this ability was also observed in some allergens from groups 4, 10, 11, 14, 15, 17, 18 and 19 (Thomas *et al.* 2002). IgE binding is known not only for mite allergens but also for arthropod allergens from other groups, such as insects or ticks (Rolla *et al.* 2004; Shin *et al.* 2009). Allergen Arg 1, identified in the tick *A. reflexus*, was characterized as the histamine-binding protein from the lipocalin protein family (Hilger *et al.* 2005). The Der-p2 allergen-like protein that we identified in *I. ricinus* belongs to the ML protein family that is similar to lipocalins by the presence of disulfide bridges, N-terminal signal peptide or by the ability to bind hydrophobic molecules. Several members of both protein families are known to be able to interact with compounds involved in the host immune reaction (Akerstrom *et al.* 2000; Inohara and Nuñez 2002; Beaufays *et al.* 2008). Unfortunately, more information is available about tick lipocalins than for the tick ML proteins. To date, lipocalins were identified and/or characterized in *A. reflexus*, *A. monolakensis*, *Ornithodoros moubata*, *O. savignyi*, *Rhipicephalus appendiculatus*, *Dermacentor reticulatus*, *I. scapularis*, and finally in *I. ricinus* (Keller *et al.* 1993; Paesen *et al.* 1999; Sangamnatdej *et al.* 2002; Mans *et al.* 2003, 2008;

Hilger *et al.* 2005; Beaufays *et al.* 2008). Members of the ML protein family were found in the ticks *O. parkeri*, *O. coriaceus*, *D. variabilis*, *R. appendiculatus*, *R. microplus*, *I. scapularis* and *I. ricinus* (Francischetti *et al.* 2009). Proteins similar to Der-p2 allergen-like protein were found in *I. scapularis* and in *R. microplus*. Their clear function is not yet known but it is possible that they are involved in the interaction with microorganisms entering the tick body during feeding. They can directly bind their surface structures carrying lipidic parts. In this way, they can label the microorganism that can be further processed by immune mechanisms of the tick.

Testing IgE binding by Der-p2 allergen-like protein, followed by comparison of the tertiary structure of the tick allergen to the house dust mite allergen of *D. farinae*, Der f 2, and also of *D. pteronyssinus*, Der p 2, that possesses 2 main localized IgE epitopes (Ichikawa *et al.* 1998; Mueller *et al.* 1998; Derewenda *et al.* 2002), was performed to reveal the possible existence of similar epitopes in the studied tick protein. Although not all residues showed similar biochemical character, we examined whether tick allergen binds IgE and revealed a detectable interaction. Expression of Der-p2 allergen-like protein gene in the gut and haemolymph defines the place of interaction. Der-p2 allergen-like protein could be involved in the interaction of different blood-transmitted compounds or microorganisms. Specific IgE was documented in patients after a bite by *I. ricinus*, *I. holocystus*, *I. pacificus*, *R. appendiculatus* or *A. reflexus* (Acero *et al.* 2003; Rolla *et al.* 2004). However, the studied tick Der-p2 allergen-like protein is not an allergen in the sense of triggering an allergic reaction. Showing high sequential and structural similarity to mite allergens (with 30–35% similarity) it might possess a different

functional activity that has not yet been defined. Der-p2 allergen-like protein could be involved in the neutralization of IgE antibodies received with blood from repeatedly tick-bitten people. This could be another mechanism of clearing the host antibodies by the tick rather than involving IgG-binding proteins, according to the mechanism known from *Rhipicephalus appendiculatus* (Wang *et al.* 1998; Wang and Nuttall, 1999). The predicted 3-D structure of *I. ricinus* Der-p2 allergen-like protein showed the presence of a cavity between the plates formed by β -sheets for lipid molecule binding. This feature was confirmed for several members of the ML protein family. Although characterization of Der-p2 allergen-like protein is not yet complete, the protein most probably participates in the recognition and transport of lipids from blood, or in the recognition of surface patterns created by lipidic molecules of microorganisms.

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