

Proteome analysis of abundantly expressed proteins from unfed larvae of the cattle tick, *Boophilus microplus*

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Received 15 April 2004; received in revised form 26 October 2004; accepted 26 October 2004

Abstract

Protein expression in unfed larvae of the cattle tick, *Boophilus microplus*, was characterized using gel electrophoresis and mass spectrometry in an effort to assemble a database of proteins produced at this stage of development. Soluble and insoluble proteins were extracted and resolved by two-dimensional (2D) gel electrophoresis. Twenty abundantly expressed larval proteins were selected for peptide mass mapping and for peptide sequencing by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) and quadrupole time-of-flight (Q-ToF) tandem mass spectrometry (MS), respectively. Only one protein, tropomyosin, was unequivocally identified from its peptide mass map. Ten proteins were assigned putative identities based on BLAST searching of heterologous databases with peptide sequences. These included a cytoskeletal protein (troponin I), multiple cuticular proteins, a glycine-rich salivary gland-associated protein and proteins with a presumed housekeeping role (arginine kinase, a high-mobility group protein and a small heat shock protein). Eight additional proteins were identified by searching translated open reading frames of a *B. microplus* EST database (unpublished): putative fatty-acid binding protein, thioredoxin, glycine-rich salivary gland protein and additional cuticular proteins. One remaining protein was not identifiable, suggesting it may be a novel molecule. The ongoing assembly of this database contributes to our understanding of proteins expressed by the tick and provides a resource that can be mined for molecules that play a role in tick-host interactions.

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Keywords: *Boophilus microplus*; Two-dimensional gel electrophoresis; Mass spectrometry; Proteome; Larvae

1. Introduction

Rapid advances in genomic and proteomic technologies have stimulated a revolution in molecular entomology, as reflected in the increasing number of publications on arthropods utilizing such approaches. These include studies on the fruit fly, *Drosophila melanogaster* (Vierstraete et al., 2003), as well as vectors of disease, including tsetse flies that spread African trypanosomes (Haddow et al., 2002; Haines et al., 2002), *Ixodes scapularis*, the main vector of Lyme disease

(Valenzuela et al., 2002) and *Rhodnius prolixus*, the vector of Chagas disease (Ribeiro et al., 2004).

The cattle tick, *Boophilus microplus*, is an ectoparasitic arthropod that is a vector of *Babesia spp* and *Anaplasma marginale*, the causative agents of bovine babesiosis and anaplasmosis, respectively. *B. microplus* was essentially eradicated from the United States in 1943 (Graham and Hourrigan, 1977). However, *B. microplus* is prevalent in Mexico and hundreds of thousands of cattle are imported annually from Mexico into the United States (Bram et al., 2002). A quarantine zone is maintained along the Texas-Mexico border to prevent the re-entry of the tick into the US, and a stringent quarantine program at border import facilities involves the physical inspection and dipping of cattle in vats of an acaricide

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prior to their importation. The prolonged and intensive use of acaricides for the control of the tick population has contributed to the development of acaricide resistant populations of ticks (Ortiz Estrada et al., 1995; Villarino et al., 2002; Li et al., 2003), necessitating the design of novel control strategies.

As *B. microplus* is a one-host tick, it must cope with the pressures of the immune response resulting from the feeding of all lifestages on a single host. The identification of proteins expressed at the different lifestages of the tick will provide a resource that could be mined for biologically important molecules for the development of novel control strategies. The work presented here describes the identification of 19 proteins that are abundantly expressed during the larval stage of *B. microplus* development. It is our intent that this information will be used in the assembly of a two-dimensional (2D) database of expressed larval proteins, which will extend our understanding of the repertoire of molecules produced by the tick and aid in the identification of proteins to which the host elicits an immune response at this stage.

2. Materials and methods

2.1. Tick strains

The *B. microplus* organophosphate (coumaphos) resistant strain, San Roman (SR), was collected in 1994 from a ranch located in Champoton, Campeche, Mexico. The strain was maintained at the Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX, as described by Davey et al. (1980). Unfed larvae between 12 and 16 days old were collected and were immediately frozen at -80°C until subsequent use.

2.2. Sample preparation

Soluble and insoluble proteins were isolated from larvae using the ReadyPrepTM Sequential Extraction Kit (BioRad, Hercules, CA). Tick larvae (0.6 g) were ground to a powder in BioRad Reagent 1 (40 mM Tris base) using a liquid nitrogen cooled mortar and pestle. The sample was further homogenized using three 10 s bursts with a Polytron, treated with FOCUS-NucleaseTM (Genotech, St. Louis, MO) and a protease inhibitor cocktail (FOCUS-Protease ArrestTM; Genotech) for 1 h at room temperature and subsequently ultracentrifuged at $210,000 \times g$ for 4 h at 4°C . Supernatant, consisting of soluble cytosolic proteins, were stored in 300 μl aliquots at -20°C . Urea-soluble, putative membrane or membrane-associated proteins were further extracted by resuspending the remaining pellet in BioRad Reagent 2, a buffer consisting of 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

(CHAPS), 2 mM tributylphosphine (TBP), 40 mM Tris, and 0.2% (w/v) Bio-LyteTM pH 3–10 ampholytes (BioRad). Highly insoluble proteins were removed from this mixture by ultracentrifugation at $210,000 \times g$ for 2 h at 25°C , and the membrane protein-enriched supernatants were collected in 300 μl aliquots and stored at -20°C .

Protein aliquots were extracted with an equal volume of water-saturated phenol, and proteins were precipitated from the phenol phase using 0.1 M ammonium acetate in methanol overnight at -20°C , as described by Hurkman and Tanaka (1986). The precipitate was washed three times with 0.1 M ammonium acetate in methanol and once with acetone prior to solubilization in BioRad Reagent 2.

The protein concentration of each sample was determined using the modified BioRad Protein Assay System, based on the Bradford dye-binding procedure (Bradford, 1976). The supernatants were analyzed by 2D gel electrophoresis.

2.3. Two-dimensional gel electrophoresis. First dimension: isoelectric focusing (IEF)

A 300 μl sample containing 400 μg of protein diluted in BioRad Reagent 2 was used to rehydrate ReadyStripTM Immobilized pH Gradient (IPG) strips (17 cm, linear pH 3–6, 5–8 and 7–10; BioRad) for at least 18 h at room temperature. Additionally, a 125 μl sample containing 50 μg of protein diluted in the same buffer was utilized for the rehydration of 7 cm IPG strips for each of the various pH ranges. IEF was performed using the PROTEAN[®] IEF Cell (BioRad) according to the manufacturer's protocol. Focused strips were stored at -20°C .

2.4. Two-dimensional gel electrophoresis. Second dimension: gradient gel electrophoresis

The focused IPG strips were equilibrated for 15 min at room temperature in a buffer comprised of 6 M urea (w/v), 2% SDS (w/v), 0.05 M Tris, pH 8.8, 20% glycerol (v/v), and 2% DTT (w/v). A second equilibration of 15 min was conducted in the same buffer with 2.5% iodoacetamide (w/v) instead of 2% DTT. Equilibrated strips (17 cm) were transferred to the IPG well of a PROTEAN[®] II ReadyGel[®], (8–16% acrylamide gradient; BioRad) and the second-dimensional electrophoresis was conducted in Tris/Glycine/SDS buffer (BioRad) at 24 mA/gel on a PROTEAN[®] II xi apparatus (BioRad). The shorter equilibrated strips (7 cm) were transferred to the IPG well of a ReadyGel[®], (8–16% acrylamide minigel; BioRad) and electrophoresed in the same buffer at 170 V for 75 min on a MiniProtean[®] II apparatus (BioRad) at 4°C .

2.5. Protein staining and image analysis

Mini-gels (7 cm) were used for analytical purposes and were stained using the SYPRO Ruby protein gel stain (BioRad) according to the manufacturer's protocol. Each of the small format gels was analyzed in triplicate. High-resolution gels (18 cm) were subsequently used to obtain more protein for spot excision and protein analysis by mass spectrometry. Proteins on the high-resolution gels were fixed and stained using a modified colloidal Coomassie Brilliant Blue (CBB)-G250 method (Neuhoff et al., 1988) as described in Haddow et al. (2002). Briefly, gels were fixed overnight [50% (v/v) ethanol, 3% (v/v) ortho-phosphoric acid] and subsequently washed for three intervals of 30 min in Milli-Q-processed water. The gels were equilibrated in Neuhoff's solution [16% (w/v) ammonium sulfate, 25% (v/v) methanol, 5% (v/v) ortho-phosphoric acid] for 1 h at room temperature, after which 1 g/L of CBB-G250 (BioRad) was sprinkled into the Neuhoff's solution. The staining was conducted with constant shaking at room temperature until distinct protein spots were visible (1–3 days).

Two-dimensional gel images were digitized from stained gels using a GS-800 calibrated densitometer (BioRad) coupled with the The Discovery SeriesTM Quantity One Quantitation Software (Version 4.4; BioRad). Data from 2D gels were analyzed using the Discovery SeriesTM PDQuest 2D Gel Analysis software (Version 7.1.0; BioRad). Overlapping pI ranges were identified across all of the gels by comparing spot patterns, and these regions were taken into consideration when conducting the PDQuest software analysis. Only those protein spots that were present on all three replicate gels were counted.

2.6. In-gel tryptic digestion of proteins

Protein spots were cored from at least 2 and up to four replicate (18 cm), colloidal-CBB stained 2D gels using a plastic straw (4 mm in diameter) and transferred to 1.5 ml reaction tubes that had been previously rinsed in 50% HPLC-grade methanol. For analysis by mass spectrometry, selected protein spots of interest were destained, reduced with dithiothreitol, alkylated with iodoacetamide and digested with modified porcine trypsin as described previously by Haddow et al. (2002).

2.7. MALDI-ToF mass spectrometry

Half of the tryptic digest for each protein spot (the remaining 10 μ l were reserved for Q-TOF mass spectrometry analysis) was desalted using a ZipTip[®] (C18 resin; P10, Millipore Corporation, Bedford, MA). The peptides were eluted from the ZipTip[®] with 1 μ l of matrix (α -cyano-4-hydroxycinnamic acid; Aldrich, Milwaukee, WI), directly onto a MALDI plate and analyzed using an Applied Biosystems Voyager DE-STR mass spectrometer

(Foster City, CA) in delayed extraction, reflectron mode. The masses of the observed tryptic peptides were submitted to MS-Fit (ProteinProspector software package; <http://www.prospector.ucsf.edu>; Clauser et al., 1999) and Mascot (Matrix Science; <http://www.matrixscience.com>) to perform the peptide mass fingerprint (PMF) searches.

2.8. Tandem mass spectrometry

Tryptic peptides were desalted using a microcolumn (glass capillary needles) of POROSTM R2 C18 resin (PerSeptive Biosystems, Foster City, CA) as described by Haddow et al. (2002) followed by extraction into sample needles using 1 μ l of 50% methanol (v/v)/1% formic acid (v/v). Selected peptides were fragmented and the fragmentation spectra were recorded on an Applied Biosystems QSTAR[®] Pulsar *i* mass spectrometer equipped with a nanospray ion source. Data were managed with BioanalystTM Software (Applied Biosystems). Peptide fragmentation data searching was performed using the Mascot MS/MS Ions Search algorithm (Matrix Science; London, UK; <http://www.matrixscience.com>) and peptide sequences were submitted to the MS-BLAST search algorithm at EMBL (<http://www.dove.embl-heidelberg.de/Blast2/msblast.html>; Shevchenko et al., 2001). Our group, in conjunction with The Institute for Genomic Research (Rockville, MD), developed a *B. microplus* EST database comprising approximately 8000 non-redundant EST sequences derived from a cDNA library synthesized from whole ticks and dissected organs of *B. microplus* (Guerrero, F.D. and Nene, V., personal communication). This database was searched by comparing the peptide sequences obtained by mass spectrometry to virtual translations of the EST sequences. Those ESTs to which peptides aligned were submitted to MS-Digest (ProteinProspector software package) to obtain predicted tryptic peptide mass maps. EST nomenclature reported here was designated as such in the database. Proteomic tools provided by the Expert Protein Analysis System (ExpASY; <http://www.us.expasy.org/tools>; Gasteiger et al., 2003) were utilized for evaluation of assigned protein identities, as was the Simple Modular Architecture Research Tool (SMART; <http://www.smart.embl-heidelberg.de>; Letunic et al., 2002) and the Protein Families (Pfam; <http://www.sanger.ac.uk/Software/Pfam/>) database of annotated protein domains (Bateman et al., 2004).

3. Results and discussion

3.1. Two-dimensional gel analysis of larval protein extracts

Representative 2D electropherograms of Tris-soluble and urea-soluble proteins are presented in Fig. 1.

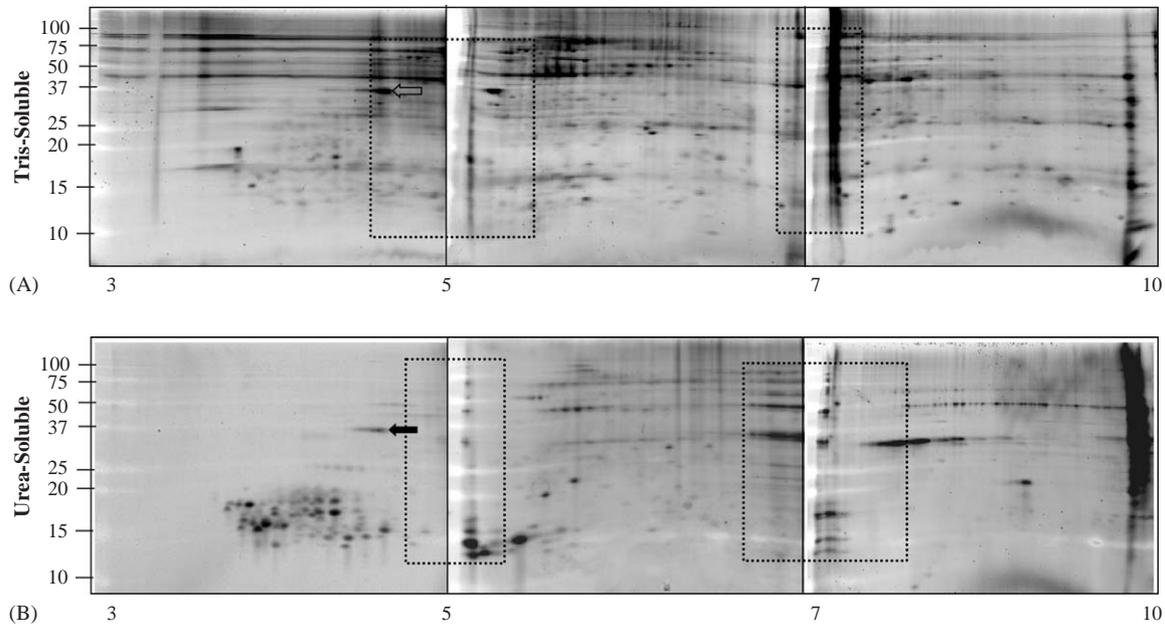


Fig. 1. Representative 2D gel electropherograms of Tris-soluble (Panel A) and urea-soluble (Panel B) protein fractions isolated from unfed larvae of *B. microplus*. Proteins (50 μ g) were separated by IEF (horizontal axis) using 7 cm IPG strips with a pI range of 3–6, 5–8, and 7–10 followed by resolution in the second dimension on 8–16% gradient polyacrylamide gels (vertical axis). Molecular masses in kiloDaltons (kDa) are indicated on the left of each panel. Regions of gel overlap between pI ranges are defined by a thatched box. The white arrow in Panel A highlights the source of Tris-soluble protein carryover into the urea-soluble fraction.

Samples were analyzed in triplicate to confirm overall reproducibility of the protein spot patterns observed and only minor differences were apparent between gel replicates. In total, 550 Tris-soluble and 250 urea-soluble proteins were resolved in the isoelectric point (pI) range of 3–10. The majority of Tris-soluble proteins was present in the pI range of 5–8 and in the molecular mass (M_r) range of 10–100 kDa, whereas abundantly expressed urea-soluble proteins were predominantly acidic and of low molecular mass. The Tris- and urea-soluble protein fractions were subsequently separated using high-resolution (18 cm) 2D gels and the proteins were stained with colloidal Coomassie Brilliant Blue G250 to identify abundant protein spots for subsequent analysis by mass spectrometry (Fig. 2). Colloidal Coomassie Blue is thought to be linearly bound by most proteins; thus, staining intensity is deemed proportional to the abundance of the protein, taking into consideration that proteins will differ in their staining characteristics.

3.2. Protein identification by mass spectrometry

Six Tris-soluble and 14 urea-soluble proteins, based on their abundance, were selected for identification by mass spectrometry. The results of the MALDI-ToF mass spectrometry and tandem MS (MS/MS) analyses are summarized in Tables 1 and 2. Carbamidomethylated tryptic peptides were initially assessed by MALDI-ToF mass spectrometry and peptide mass fingerprints (PMF) were obtained for all 20 proteins. However, only

1 protein (SP23; *Boophilus microplus* tropomyosin: GenBank accession no. AAD17324.1) was unambiguously identified by this approach. The eleven SP23 signature masses elucidated covered 38% of the complete protein sequence (Fig. 3) and the predicted M_r (33 kDa) and pI (4.7) of the *B. microplus* tropomyosin agree with the location of SP23 on the analytical 2D gel (Fig. 1B; black arrow).

The putative identities for 18 of the remaining 19 proteins were deduced using peptide sequence information obtained from tandem mass spectrometry (MS/MS) followed by an MS/MS ion search of several databases and an in-house search of a *B. microplus* EST database. Two peptide sequences from SP26 did not align to sequences in either database, thus this protein remains unidentified. The ESTs encoding protein sequences to which the tryptic peptides aligned were analyzed for tryptic peptides in order to generate hypothetical peptide mass fingerprints. When possible, the predicted tryptic peptide masses were compared to the tryptic peptide mass list generated from MALDI-ToF analysis to provide additional confirmation of the putative identities assigned. Overall, the calculated and observed molecular masses for all of the proteins identified from a search of the public database were consistent with each other (Table 1). However, the theoretical and experimental pI data were not always in agreement. These discrepancies could be attributed to post-translational modifications (especially phosphorylations and glycosylations), multiple protein isoforms, and different protein

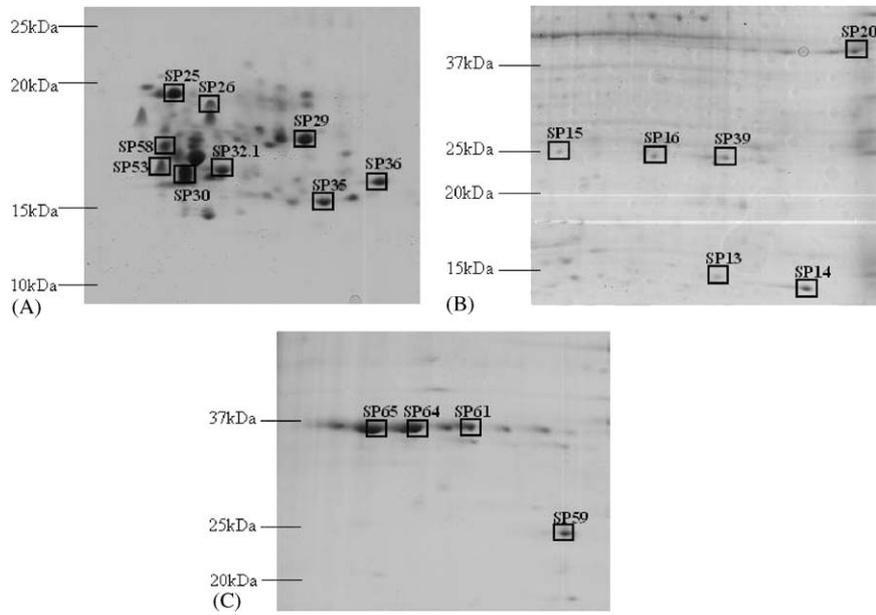


Fig. 2. High resolution 2D gel separation of urea- (Panel A and C) and Tris- (Panel B) soluble proteins for analysis by MS. Proteins isolated from larvae were separated in the first dimension by IEF using 17 cm IPG strips (Panel A: 3–6, Panel B: 5–8, and Panel C: 7–10) and in the second dimension by large format 8–16% gradient gels. Molecular masses in kilo Daltons (kDa) are indicated on the left of each panel. The proteins identified by a square and 2D spot number were selected for subsequent protein identification by MS.

compositions utilized by *B. microplus* homologs of the reported proteins.

3.3. Cytoskeletal proteins

Two proteins identified in this study, a troponin I-like protein (SP15) and tropomyosin (SP23), are involved in actin regulation. The putative identity of SP15 was assigned based on significant sequence similarity of an octamer peptide to a troponin I-like protein from the hard tick, *Haemaphysalis longicornis*. In addition, five theoretical masses, covering 27% of a 647 bp translated EST encoding troponin I in *B. microplus* (TC86), matched masses obtained from MALDI-ToF analysis of SP15.

Although the theoretical M_r and pI data for tropomyosin correspond well with the position of SP23 on the 2D gel, the protein was isolated from an experiment in which urea-soluble proteins were resolved. The *B. microplus* tropomyosin sequence does not contain any transmembrane domain regions characteristic of integral membrane proteins and although it is possible that the tropomyosin sequence is membrane associated via a post-translational modification, there does not appear to be any precedence for this in the tropomyosin literature. Examination of the 2D gels in which Tris-soluble proteins were separated reveals that there is an abundant protein present at the expected position for tropomyosin (Fig. 1A; white arrow). This suggests that SP23 may be a Tris-soluble protein contaminant carried over during the extraction proce-

dures, a common problem encountered when dealing with membrane protein preparations (Santoni et al., 2000).

3.4. Exoskeletal/chitin-binding proteins

Eight acidic, low molecular mass, urea-soluble proteins (SPs 58, 32.1, 25, 53, 35, 29, 30 and 36; Fig. 2A) were identified as putative cuticular proteins, as summarized in Table 2. This is based on sequence similarity of peptides from SPs 58, 32.1, 25, 53, and 29 to cuticular proteins from other arthropod species, namely *Cancer pagurus* (crab), *Araneus diadematus* (spider), and *D. melanogaster* as well as the alignment of numerous peptides from the eight proteins with *B. microplus* cuticle protein-encoding ESTs. Comparison of the peptide mass fingerprints and deduced peptide sequences from the eight proteins revealed considerable overlap in the masses and sequences of the tryptic peptides, respectively, i.e. SPs 32.1 and 25 shared two and three peptides with SP58, while SP29 and SP36 share a common peptide. In addition, peptides with similar, although not identical, sequences were shared between SP35/36, SP29/36, and SP53/30. Cuticle genes isolated from insects have been described as representative of small multigene families, accounting for similarities in the coding regions of these proteins (Horodyski and Riddiford, 1989; Charles et al., 1997; Rondot et al., 1998). This has been observed within cuticular proteins purified from the horseshoe crab, *Limulus polyphemus*, in which pairs of cuticular proteins differed by only 3/59

Table 1
Summary of proteins identified by MS in unfed larvae of *B. microplus*

2D spot number	Protein ID ^a (Genbank accession No.)	Peptide sequences ^{b,c}	% Sim. of peptide to protein	<i>M_r</i> (kDa) ^d pI ^e	EST ^f /Homol. protein to translated EST	% Sim. of peptide to EST
SP15	Troponin I-like <i>Haemaphysalis longicornis</i> (BAB55451)	¹¹¹ YDLEYEVR ¹¹⁸	100	23.4; 23 9.77; 5.5	TC86/Troponin I-like	100
SP23	Tropomyosin <i>Boophilus microplus</i> (AAD17324.1)	Identified using peptide mass fingerprint ^g	n/a	33;35 4.7;4.7		
SP20	Arginine kinase <i>Callinectes sapidus</i> (AAF43436)	³³¹ MGLTEYZAV ³³⁹ ²³⁰ IISMZVGG ²³⁷	78 63	40.3; 40 6.19; 7	TC260/Arginine Kinase	89 88
SP16	CG14207 gene product <i>Drosophila melanogaster</i> (AAN09508)	¹⁰⁸ FDVSQYAPEEIVVK ¹²¹ ¹⁷ SKKDNEFSSLR ²⁷	100 73	21.8; 23 6.14; 6	—	—
SP39	HMG-like protein <i>Dermacentor variabilis</i> (AAO92280)	¹¹ MSAYAFFVQTCR ²² ¹¹⁹ PDSSVGEVAK ¹²⁸ ¹³⁵ NEVGDDVK ¹⁴²	100 90 100	24.2; 25 5.88; 6.5	—	—
SP14	n.i. ^h	VTLEDGK VEGDDGESLK GDQEVTLVR	— — —	11.3; 13 5.56; 6.8	TC274/Fatty-acid binding protein	100 — 88
SP13	n.i.	TPVLAEEAYK	—	17.6; 15 6.09; 6.4	BEACZ37/Thioredoxin	100
SP59	20/24 kDa glycine-rich immunodominant saliva protein <i>Rhipicephalus appendiculatus</i> (AAO60049)	FP ⁷⁰ PPPGIGAAER ⁷⁸ VVYGGVLR	82 —	19.7; 23 9.41; 9	TC2896/Salivary gland-associated protein	100 100
SP65	n.i.	GPFINNR	—	n/a; 37	TC4923/Salivary gland protein	100
SP64	n.i.	HNTNVVPR GPFINNR	— —	n/a; 37	TC4923/Salivary gland protein	— 100
SP61	n.i.	HNTNVVPR	—	n/a; 37	TC4923/Salivary gland protein	—
SP26	n.i.	YGYTDAFVAYR VDYVADFGQR	— —	n/a	n.i.	— —

^aProtein in the NCBI database to which significant peptide mass matching or sequence similarity was observed. Genbank Accession numbers are provided.

^bSequence information obtained from tandem mass spectrometry. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.

^cThe isobaric amino acids isoleucine (I) and leucine (L) cannot be distinguished using Q-TOF mass spectrometry. Hence, I and L are interchangeable in the peptide sequences reported here. When possible, translated ESTs were used to assign either I or L in the peptide sequence.

^dPredicted values determined by database search algorithms (n/a, not applicable); observed mass estimated from position of protein spot on gel.

^ePredicted values determined by database search algorithms; observed pI estimated from position of protein spot on gel.

^fEST from a *B. microplus* database to which the peptide displayed sequence similarity.

^gAn unequivocal identity was determined for SP23 using peptide mass mapping. The 11 tryptic peptides covered 38% of the protein (see also Fig. 3).

^hn.i., not identified.

(LpCP7a and LpCP7b) and 2/130 (LpCP14a and LpCP14b) amino acid residues, respectively (Ditzel et al., 2003). The sequence information suggests that larval cuticular proteins from *B. microplus* may also be encoded by a multigene family, but to our knowledge this has not been investigated. Interestingly, an amino acid sequence alignment of the cuticle protein-encoding ESTs presented in Table 2 revealed that the sequences conform to a conserved arthropod chitin binding domain (Fig. 4; Rebers and Willis, 2001), within which a number of the sequenced peptides were observed.

3.5. Putative housekeeping proteins

Two peptide sequences deduced from SP20 aligned significantly (78% and 63%) with arginine kinase sequences from the blue crab, *Callinectes sapidus*, as well as the corresponding protein from other arthropods. In addition, both peptides were identified in a *B. microplus* EST encoding arginine kinase (TC260; with 89% and 88% sequence similarity) but only five peptide masses covering 13% of the 1178 bp EST matched the masses obtained from MALDI-ToF mass spectrometry.

Table 2
Summary of putative chitin-binding proteins identified by MS in unfed larvae of *B. microplus*

2D spot number	Protein ID ^a (Genbank Accession No.)	Peptide sequences ^{b,c}	%Sim. of peptide to protein	<i>M_r</i> (kDa) ^d pI ^e	EST ^f /Homol. protein to translated EST	% Sim. of peptide to EST
SP58	Exoskeletal cuticle protein <i>Cancer pagurus</i> (CPA2_CANPG)	YLSLVSGR	—	11.6; 15	Similar to TC22/cuticle protein	78
		TVT ⁶³ YVADEFGYR ⁷¹	90	4.43; 4		100
		VSLPTNEVRK	—	—		—
		NYVADDQTCR	—	—		—
		ADVVTNEPGTESR	—	—		100
SP32.1	Exoskeletal cuticle protein <i>Cancer pagurus</i> (CPA2_CANPG)	YLSLVSGR	—	11.6; 15	Similar to TC22/cuticle protein	78
		TVT ⁶³ YVADEFGYR ⁷¹	90	4.43; 4.3		100
SP25	Exoskeletal cuticle protein <i>Cancer pagurus</i> (CPA2_CANPG)	YLSLVSGR	—	11.6; 20	Similar to TC22/cuticle protein	78
		TVT ⁶³ YVADEFGYR ⁷¹	90	4.43; 4		100
		ADVVTNEPGTESR	—	—		100
SP53	Adult-specific rigid cuticular protein <i>Araneus diadematus</i> (P80519)	V ⁸⁰ SIDTNEPGT ⁸⁸ K	73	15.7; 15	Similar to TC48/cuticle protein	100
		VSLDTCNCPGK	—	9.01; 4		—
		TANYVADDQTCR	—	—		83
		TGSYSYQTPDRYR	—	—		92
		FENGELVDL	—	—		—
SP35	n.i. ^g	YTLSLADGR	—	n/a; 15	Similar to TC2976/cuticle protein	100
		TYTEFTYHK	—	n/a; 5		—
		LVTNEQATESK	—	—		91
SP29	CG2560-PA gene product <i>Drosophila melanogaster</i> (AAL49153.1)	⁹⁹ VSGSYTFTLADG ¹¹⁰ R	83	n/a; 15	Similar to TC109/cuticle protein	—
		VSGSYTMTLADGR	—	n/a; 4.9		100
SP36	n.i.	YTMTLADGR	—	n/a; 15	Similar to TC297/cuticle protein	78/100
		NVTNELGTESK	—	n/a; 5.3		(TC109)
		TVTYTADENGYR	—	—		82
		LVTNELGDESK	—	—		92
SP30	n.i.	VSLDTNEAT	—	n/a; 15	Similar to TC48/cuticle protein	78
		SYQTPDTFAR	—	n/a; 4.1		70

^aProtein in the NCBI database to which significant peptide mass matching or sequence similarity was observed. Genbank Accession numbers are provided.

^bSequence information obtained from tandem mass spectrometry. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.

^cThe isobaric amino acids isoleucine (I) and leucine (L) cannot be distinguished using Q-TOF mass spectrometry. Hence, I and L are interchangeable in the peptide sequences reported here. When possible, translated ESTs were used to assign either I or L in the peptide sequence.

^dPredicted values determined by database search algorithms (n/a, not applicable); observed mass estimated from position of protein spot on gel.

^ePredicted values determined by database search algorithms; observed pI estimated from position of protein spot on gel.

^fEST from a *B. microplus* database to which the peptide displayed sequence similarity.

^gn.i., not identified.

Arginine kinase (ArgK-EC 2.7.3.3) is a type of phosphagen kinase that catalyzes the transfer of a phosphoryl group from a guanidino phosphagen, e.g. arginine phosphate, to adenosine diphosphate (ADP), generating a molecule of adenosine triphosphate (ATP). ArgK is usually present in tissues that require continuous delivery of ATP (high energy demand) or that experience short bursts of energy demand (Lang et al., 1980; Kotlyar et al., 2000).

The CG14207 *D. melanogaster* gene product, to which SP16 had significant sequence similarity, is presently

uncharacterized, but has been described as having putative chaperone activity based on the presence of a conserved HSP20 domain in its sequence. Sequence similarity was also observed to ENSANGP00000023810 from *Anopheles gambiae* (GenBank accession no. XP_309128). One of the SP16 tryptic peptides (FDVSQYAPEEIVVK) aligns exactly to a region within the putative HSP20 domain of CG14207, while the other peptide is 73% similar to the sequence (Fig. 5A). The HSP20 family of small heat shock proteins (sHSPs) is one of six major families of molecular chaperones

MEAIKKKMQAMKLEKDNAVDR**AETAEQ**QSR**E**AALRAEKAE**EE**VRSLQKKIQQIENELDQVQEQLSQA
 NSKLEEKDK**ALQAAEA**EVA**AHNRR**IQLLEEDLERSEERLKIATQK**LEEASQA**ADESERMRKMLEHRS
ITDEERMDGLEQ**LKE**ARTMAEDAD**RKY**DEVARKLAMVEADLERAE**E**RAETGETK**IVELEEE**ELRVVG
 NNLKSLEVSEE**KALQKE**ETYEMQIRQMTNRLQEA**E**ARAEFAERSVQKLQ**KEVDR**LEDEL**VQEK**EKYK
 AISDELQDTFSELTGY

Fig. 3. Peptide mass fingerprint analysis of tryptic peptides obtained from protein SP23 by MALDI-ToF MS. The sequences shown in bold type indicate the tryptic peptides identified by MS overlaid on the complete sequence of tropomyosin from *B. microplus*. The peptides covered 38% of the tropomyosin sequence.

TC109	95	<u>GSYTM</u> <u>LADGR</u> QRTVRYTAD ETGFH PEIVTNEQGTESNSP	134
TC22	62	GRYSLSLADGRTRTVTYVADEF GYRAD VVTNEPGT ESR PS	101
TC48	73	<u>GSYSYQ</u> TPDG VYRT ANYVAD DQ GF RV SIDTNEPGTKAENP	112
TC2976	75	GRYTL SLADGR TRTVTYTADENGYRAEIVTNEQGT ES KNP	114
CPA2_CANPG	47	GTFRFL LPD GTTAEVRYVADEF GYR PESPL LPV GP EL PPH	86
P80519	54	GSY TIT DI DGR ARRVDYVADAAGFRASIKTNEPGTALSAP	93
		* : ** . *.** *:: . *.	
R-R consensus:		GxxxxxxxxGxxxxxxYxAxExGYxxxxxxxxPxxP	

Fig. 4. A CLUSTALW (Thompson et al., 1994) amino acid sequence alignment of regions from *B. microplus* ESTs encoding putative chitin-binding proteins (TC109, TC22, TC48, and TC2976) with regions of a *C. pagurus* exoskeletal cuticle protein (CPA2_CANPG) and an *A. diadematus* adult cuticular protein (P80519). Identical and similar residues are identified by (*) and (: , .), respectively. Peptide sequences deduced by MS/MS that aligned exactly to the *B. microplus* EST are indicated by a double underline (=), while those peptides similar to the EST are underlined. The Rebers-Riddiford (R-R) consensus sequence, conserved in arthropod chitin binding domains, is displayed below the alignment.

SP16		SLLDNEFSSLR	
Dm AAN09508	MAE-ANKRNIPIKLGDF	SVIDTEFSN IRERFDSEMRKMEEMAKFRHELMNREANFFEST	59
Ag XP_309128	MADNANKRNIPIKLGDF	SVIDTEFSS IRERFDSEMKKMEEMARFRSDLMHREPNFFETT	60
SP16			FDVSQYAP
Dm AAN09508	S--STKKT TTTTSS --TTNSALPSRIPKQONYVSDISSPLIQDEGDNKVLKLR	FDVSQYAP	115
Ag XP_309128	SSFSSK KVATSS SSSTTSNAATSMKLPQSQS-GSDIC SPLIQ DDGDKVLKLR	FDVSQYAP	119
SP16		EEIVVK	
Dm AAN09508	EEIVVK TVDQKLLVHAKHEEKSDTKSVYREYNREFLLPKGVNPESIRSSLSKDGVLTVDA		175
Ag XP_309128	EEIVVK TVDNKLLVHAEHAEKSDTKSVYREYNREFMLPKGCLPENIKSSLSKDGVLTVDA		179
Dm AAN09508	PLP--ALTAGETLIPIAHK		192
Ag XP_309128	PIQPEALLAGETMV-----		193
(A)			
SP39		MSAYAFFVQTCR	
Dv AAO92280	MGKGDKPRGR	MSAYAFFVQTCR EEHKKKHPNENNVFAEFSKKCAERWKT MSESEK KRFHQ	60
SP39			PD
Dv AAO92280	MADKDKRFDTEMADYKPPKGDKSKKRKRAKDPNAPKRPLSAFFWFCNDRPNVRQ ESPD		120
SP39		SSVGEVAK NEVGDDVK	
Dv AAO92280	ASVGEVAK ELGRR WNEVGDDV KSKYEGLAAKDKARYEKELKAYKGKPKAASPPKEKAKK		180
Dv AAO92280	KEEDDDEDDDEEDEVEDAEDDDDDDED		208
(B)			

Fig. 5. Alignment of peptide sequences, obtained using MS/MS, from proteins SP16 (Panel A) and SP39 (Panel B) to high scoring proteins from the public database. (A) Two peptides from SP16 align with sequences of uncharacterized gene products from *D. melanogaster* (Dm) and *A. gambiae* (Ag). (B) Three peptide sequences from SP39 align with the sequence of an HMG-like protein from *D. variabilis* (Dv).

found in prokaryotes and eukaryotes (Garcia-Ranea et al., 2002). The main function of sHSPs is to assist in the refolding and hence prevention of the aggregation of denatured proteins and has been postulated to play a

role in the protection of cells from stress (Murashov et al., 1998; Mearow et al., 2002).

Three peptide sequences, obtained from SP39, displayed significant similarity to a putative high mobility

group (HMG)-like protein from the American dog tick, *Dermacentor variabilis*. HMG proteins are chromatin-associated and contain a DNA binding domain (the HMG box) that binds in either a sequence-specific (HMG-A) or a non sequence-specific fashion (HMG-B) (Laudet et al., 1993; Agresti and Bianchi, 2003). Typically, HMG-B proteins contain multiple HMG boxes. The *D. variabilis* sequence contains two HMG-box domains and all three peptide sequences of SP39 align within these two domains (Fig. 5B).

Although sequence information for three peptides from SP14 did not align significantly to any sequences in the public database, two of the three peptides aligned (100% and 88%) to a *B. microplus* EST (TC274) encoding a fatty-acid binding protein that is most similar to that of the Antarctic eel pout, *Lycodichthys dearborni* (GenBank accession no. AAC60353). Fatty acid binding proteins typically bind to hydrophobic ligands (like fatty acids) and are believed to play a role in lipid metabolism (Maatman et al., 1994).

No proteins in the public database showed any sequence similarity to an octamer peptide of SP13. However, the peptide aligned exactly to a *B. microplus* EST (BEACZ37) encoding a thioredoxin (Thrx)-like protein with similarity to Thrx from *Caenorhabditis elegans* (GenBank accession no. NP_503954). Comparison of the SP13 tryptic peptide mass list to the predicted peptide mass fingerprint for the EST revealed four matched masses covering 27% of the 482 bp EST. Thrx is a molecule that plays a role in antioxidant defense and redox regulatory processes (Rahlfs et al., 2003) and, along with protein disulfide isomerase, is a substrate for thioredoxin reductase (ThrxR). Molecular chaperone-like properties have also been suggested for the Thrx system based on studies in *Escherichia coli* (Kern et al., 2003). Interestingly, ESTs encoding both protein disulfide isomerase and ThrxR were identified in the database, suggesting that the Thrx system is present in *B. microplus*, possibly as a means of managing toxic haem.

3.6. Salivary gland-associated proteins

One of two peptide sequences obtained from SP59, a basic, urea-soluble protein, shared significant sequence similarity with a glycine-rich 20/24 kDa salivary gland protein (annotated as immunodominant) from the African brown ear tick, *Rhipicephalus appendiculatus*, while both peptides aligned exactly to a *B. microplus* EST (TC2896) encoding a homolog of the salivary gland protein (42% sequence identity). Five tryptic peptide masses encompassing 28% of the 647 bp EST matched masses obtained via MALDI-ToF analysis. Peptide sequences obtained from three additional basic, urea-soluble proteins (SP65, 64, and 61) revealed that they

contained shared sequences, i.e. of 2 peptides from SP64, one was identical to a peptide from SP65 and the other was identical to a peptide from SP61. As these three proteins were present as a series of spots on the horizontal axis (Fig. 2C), it is possible that they represent different isoforms or post-translational modifications of the same protein resulting in a pI shift on the gel. Although the peptide sequences obtained for these three proteins did not align to sequences in the public database, one of the peptides (GPFINNR) aligned to a *B. microplus* EST (TC4923) that encodes a protein with a glycine-rich repeat region (data not shown). Such glycine-rich proteins have been found to be expressed in salivary glands of *R. appendiculatus* (Trimnell et al., 2002; Bishop et al., 2002; Nene et al., 2004) and the tropical bont tick, *Amblyomma variegatum* (Nene et al., 2002), and are believed to be components of tick cement, the proteins of which are rich in glycine, serine, leucine, tyrosine, and proline (Kemp et al., 1982). Tick cement is produced following the production of a lesion in the host skin and aids in strengthening attachment of the tick to the host (reviewed in Kemp et al., 1982). Analysis of the amino acid composition of the translated open reading frames of the *B. microplus* ESTs T2896 (full-length) and TC4923 (not full-length) revealed that they contain 15.7% glycine/17.2% proline and 30% glycine/12% tyrosine, respectively, suggesting that these proteins (SPs 59, 65, 64, and 61) may be components of *B. microplus* tick cement.

4. Conclusions

Proteome analysis of *B. microplus* larvae was initiated to establish a resource that could be used to identify potential targets for the development of novel control strategies. Here, we presented the 2D gel electrophoretic protein expression map of unfed larvae (12–16 d old) and obtained peptide mass maps and peptide sequence information that allowed us to identify abundant proteins at this life cycle stage. The limited number of acarine sequences available in the public database was an obvious hindrance to the identification of the tick proteins using peptide mass maps generated by MALDI-ToF analysis and was also a limitation when conducting sequence similarity searches. In fact, identification of six of the nineteen proteins would not have been possible without access to the *B. microplus* EST database (Guerrero, F.D. and Nene, V., personal communication).

The majority of the proteins identified were intriguing, as they had not previously been reported in *B. microplus*. Indeed, there are only a few examples of such proteins in the public database from the Subclass Acari, i.e. two troponin I-like sequences (*H. longicornis* and

Sarcoptes scabiei), two ArgK sequences (*Psoroptes ovis* and *S. scabiei*) and one each of the HSP20-like (*D. variabilis*) and HMG-like sequences (*D. variabilis*). Of particular interest is the cluster of proteins putatively identified as chitin-binding proteins since it appears that they may be encoded by a multigene family. Hackman (1974, 1975) described cuticular proteins from engorged, adult female *B. microplus* as being >25 kDa in size and of a pI between 6 and 9, which is in contrast to the much more acidic, low M_r proteins observed from larvae. This suggests that there may be different subsets of cuticular proteins expressed at the various life cycle stages of the tick. However, this requires further investigation. Nevertheless, our data represents the first, to our knowledge, amino acid sequences from cuticle proteins of *B. microplus*.

It is our intent that this database of proteins will be updated continuously and will serve as a growing reference for future studies on the biological role of proteins expressed by *B. microplus*.

Acknowledgements

We thank the Cattle Fever Tick Research Lab in Mission, TX for the constant supply of larval material and the staff of the University of Victoria -Genome BC Proteomics Centre (Victoria, B. C.) for excellent services. We are also grateful to Kevin Temeyer and John Pruett for stimulating discussions and encouragement, Barbara Drolet for critical review of early drafts of this manuscript, and Vish Nene of The Institute for Genomic Research (Rockville, MD) for aiding in searches of the *B. microplus* EST database. This work was supported in part by a Discovery Grant (to TWP) from the Natural Sciences and Engineering Research Council of Canada. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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