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Toxicon 51 (2008) 1017–1028

TOXICON

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Immunochemical and proteomic technologies as tools for unravelling toxins involved in envenoming by accidental contact with *Lonomia obliqua* caterpillars ☆

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Received 15 December 2006; received in revised form 22 January 2008; accepted 28 January 2008
Available online 3 February 2008

Abstract

The accidental contact with *Lonomia obliqua* caterpillar causes local and systemic symptoms (such as fibrinogen depletion), leading, in some cases, to serious clinical complications (acute renal failure and intracranial haemorrhage). Fortunately, a successful therapeutical approach using anti-Lonomic serum, produced in horses against *L. obliqua*'s bristle extract, has already been put in place. However, a global view of immunogenic toxins involved in the coagulation disorders could help to elucidate the envenoming process. In the present study, our aim was to identify bristle extract's immunogenic components, especially those related to the haemostasis, coupling proteomics and immunochemical approaches (bidimensional electrophoresis, mass spectrometry and immunoblotting). The bidimensional map of bristle extract showed a broad profile of 157 silver-stained spots, where at least 153 spots were immunochemically revealed. Twenty-four of these spots were submitted to sequencing by mass spectrometry and three different categories of proteins were identified: lipocalins, cuticle proteins and serpins. From these protein families, it was observed that the most abundant was the lipocalin family, specifically represented by different isoforms of Lopap (a prothrombin activator protein), reinforcing its relevance during envenoming. Peptide sequences of several other immunochemically revealed spots showed no correspondence to any known sequence and were classified as unknown proteins. These proteins could represent new immunogenic molecules and/or toxins. The sequences presented in this article can be used for oligonucleotide design aiming the amplification of cDNAs coding for new molecules using *L. obliqua* bristles' cDNA libraries or isolated RNAs as template.

Keywords: Caterpillar; *Lonomia obliqua*; Bristle extract; Bidimensional electrophoresis; Immunoblotting; Mass spectrometry

☆ *Ethical statement:* All experiments were done according to good laboratory practices and to current animal manipulation techniques. All authors agree with the content and publication of the present manuscript.

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doi:10.1016/j.toxicon.2008.01.013

1. Introduction

Coagulation disorders have been reported after contact with the Saturniidae caterpillars from the *Lonomia* genus. Specifically, accidents caused by *Lonomia achelous* have been described in Mexico, Venezuela, Guiana and north Brazil (Amazonian region) (Arocha-Pinango, 1967; Arocha-Pinango et al., 2000). Since 1989, accidents involving *Lonomia obliqua* species were reported in Argentina, Paraguay, Uruguay and in the south of Brazil (Kelen et al., 1995; Zannin et al., 2003). In the last years, accidents have also been reported at other geographical areas of the Brazilian territory, such as the southeast region (Garcia and Danni-Oliveira, 2007). This organism's biological cycle is composed of 4 phases with distinct durations: egg (17 days); caterpillar (6 instars in 90 days); pupa (1–3 months) and moth (8 days) (Lorini and Corseuil, 2001). The physical contact with the Lepidoptera larvae in its 5th instar induces a toxic secretion from bristle spicules, which promotes local and systemic symptoms in the victim between 6 and 72 h after contact, such as a burning sensation, intense haematuria, disseminated intravascular coagulation-like reactions (severe depletion of coagulation factors) and secondary fibrinolysis (Zannin et al., 2003). Serious clinical complications, such as acute renal failure (Duarte et al., 1990) and intracranial haemorrhage (Kelen et al., 1995), may also occur. The envenomation process is influenced by the amount of venom injected, the instar stage, the number of smashed larvae, the extension of the skin area affected and the deepness of the injury.

The *L. obliqua* bristle extract has a complex toxic composition, from which two procoagulant proteins were already described: prothrombin activator (Lopap, ***L****onomia* ***o****bliqua* **p**rothrombin **a**ctivator **p**rotease) (Reis et al., 2001a, b, 1999) and FX activator (Losac, ***L****onomia* ***o****bliqua* **S**tuart factor **ac**tivator) (Alvarez Flores et al., 2006). Native Lopap was characterized as a 69 kDa lipocalin (isoelectric point (p*I*) around 6.0) harbouring serine protease-like activity (Reis et al., 2001b). It converts prothrombin into thrombin, by a prothrombinase complex-independent pathway, activating the coagulation system and leading to fibrinogen depletion (Chudzinski-Tavassi and Alvarez Flores, 2005; Reis et al., 2001a, b). In human umbilical vein endothelial cells (HUVECs), Lopap induced a higher expression of ICAM-1 and E-selectin, but not of VCAM-1 (Chudzinski-Tavassi et al., 2001), and stimulated the release of nitric oxide (Fritzen et al., 2005). In contrast to native Lopap, its recombinant active form was expressed in *Escherichia coli* as a monomer of about 20 kDa (Reis et al., 2006).

Another procoagulant toxin, Losac, an FX activator (~43 kDa) induces a similar cleavage pattern when compared with RVV-X, a P-IV class metalloproteinase from the venomous snake *Vipera russelli* (Chudzinski-Tavassi and Alvarez Flores, 2005). Losac is a growth stimulation agent with anti-apoptotic activity on HUVECs (Alvarez Flores et al., 2006).

Some other haemostasis-acting proteins were also identified in a cDNA library constructed upon bristle extract, including hyaluronidases, bradykinin agonist, cathepsin and phospholipase A_2-like molecules (Gouveia et al., 2005; Seibert et al., 2006; Veiga et al., 2005).

From a therapeutic standpoint, the anti-Lonomic serum, produced against the crude bristle extract from *L. obliqua* (5th instar) in horses by Instituto Butantan (Rocha-Campos et al., 2001), has been successfully used to re-establish the physiological coagulation parameters in poisoned patients. No more deaths were reported since serum therapy has been applied, according to clinical data from the Toxicological Center at ''Universidade Federal de Santa Catarina'' (CIT-SC). However, a global view of immunogenic toxins involved in the coagulation disorders could help to elucidate their role in the envenoming process.

The purpose of the present study was to highlight the main immunogenic proteic components present in *L. obliqua* venom by the use of proteomics methodologies based on their separation (bidimensional electrophoresis) and identification (mass spectrometry (MS)) coupled with immunochemical characterization (immunoblotting).

2. Materials and methods

2.1. Sample preparation

L. obliqua caterpillars from Santa Catarina (south of Brazil) in the 5th instar were anaesthetized by freezing. Their bristles were cut and ground with a mortar and pestle, in the presence of liquid nitrogen, and stored at −80 °C until use.

2.2. Two-dimensional electrophoresis

L. obliqua's bristles were extracted in the rehydration solution, which was composed of 8 M urea,

1 M thiourea, 2% w/v CHAPS and 0.5% v/v immobilized pH gradient buffer (IPG buffer, pH 3–10) containing 65 mM dithiothreitol (DTT) (''reduced'' sample) or not (''non-reduced'' sample). Cell debris was removed by centrifugation (3000*g* for 3 min). Samples containing 100–150 μg of protein in rehydration solution (final volume of 350 μL) were applied to 18 cm 3–10 IPG strips and separated according to their p*I* using IPGphor® apparatus (GE Healthcare). Sample loading and strip rehydration were performed at 30 V for 10 h. Then, 5 focusing steps were carried out (200 V for 1 h; 500 V for 1 h; 1000 V for 1 h; gradient from 1000 to 8000 V for 30 min; constant 8000 V for 4 h), until about 33 kVh. After focusing, ''reduced'' sample strips were incubated for 15 min in 10 mL equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and 0.002% w/v bromophenol blue) containing 65 mM DTT, followed by a second incubation step in the same buffer solution, except for DTT, which was replaced by 135 mM iodoacetamide. ''Non-reduced'' sample strips were incubated for 30 min in 10 mL of equilibration buffer without DTT or iodoacetamide. Following the equilibration step, the strips were placed on top of 14% polyacrylamide gels and proteins were separated by their molecular masses on SDS-PAGE gels. Runs were performed at constant current (25 mA per gel, max. 200 mA) at 8 °C using a vertical system (Hoefer® DALT, GE Healthcare). Analytical gels (100 μg protein) were silver stained (Shevchenko et al., 1996), while gels containing 150 μg of protein were stained by colloidal Coomassie blue (Neuhoff et al., 1988). Image analysis was performed using the Image Master® software (GE Healthcare). The reproducibility of each set of 2D gels was evaluated by conducting three independent experiments.

2.3. Sera production

Anti-Lonomic serum, produced by hyperimmunization of horses with *L. obliqua* bristle extract, was obtained from ''Seção de Processamento de Plasmas Hiperimunes'', Instituto Butantan, SP, Brazil. Normal sera were obtained from non-immunized animals. Anti-Lopap serum was obtained by immunization of adult rabbits, by intradermal injection, using the recombinant purified protein (10 μg in 100 μL of PBS) absorbed with an equal volume of a 10% solution of $Al(OH)_3$. The injections were repeated 5 times at 15-day intervals. Blood samples were collected 10 days after the last injection and sera stored at −20 °C.

2.4. Immunoblot

''Non-reduced'' sample bidimensional gels were blotted onto PVDF membranes (Towbin et al., 1979). The membranes were blocked with PBS containing 5% BSA and incubated at 37 °C for 2 h. Subsequently, the membranes were incubated for 1 h at room temperature with the anti-Lonomic horse serum produced by Instituto Butantan, diluted 1:500 in PBS/0.1% BSA or with the rabbit serum against Lopap, diluted 1:250. The membranes were washed 3 times for 10 min with PBS/0.05% Tween 20 and then incubated with specific secondary antibodies labelled with alkaline phosphatase in PBS/0.1% BSA (anti-horse 1:4000; anti-rabbit 1:7500) and incubated for 1 h at room temperature. The membranes were washed 3 times (PBS/0.05% Tween 20) and, finally, the reactions developed by the addition of the substrates NBT and BCIP (Promega), following the manufacturer's instructions.

2.5. Spot selection

A set of experiments in non-reducing conditions was performed for spot selection based on their immunogenic property and also on their abundance on colloidal Coomassie blue-stained 2D gels. A silver-stained 2D gel was taken as reference; two other gels were stained by colloidal Coomassie blue after protein blotting and visually compared with their respective PVDF membranes (recognized by polyclonal antibodies). Spots corresponding to proteins that were recognized by the anti-Lonomic serum were excised from the Coomassie blue-stained gel for further MS analyses.

2.6. Spot processing for mass spectrometric analyses

Initially samples were destained, reduced with DTT and alkylated with iodoacetamide as previously described (Bastos et al., 2007). *In gel* tryptic digestion was then performed according to the literature (Leon et al., 2007). The extracted peptides were submitted to derivatization with 4-sulphophenyl isothiocyanate (SPITC) as described (Wang et al., 2004) with modifications. Briefly, a 10 μL aliquot of tryptic peptides sample was concentrated under vacuum centrifugation to approximately

0.5 μL followed by the addition of 8.5 μL of reagent solution (10 mg/mL of SPITC in 20 mM ammonium bicarbonate, pH 8.6) and incubated for 1 h at 56 °C. The reaction was stopped by addition of 1 μL of 5% (v/v) trifluoracetic acid in water and samples were desalted and concentrated using a C18 Zip tip® (Millipore), following the manufacturer's instructions and analysed by MALDI-TOF/TOF MS.

Furthermore, a second peptide extraction from the gel plugs was performed by the addition of 30 μL of a 1:1 10% (v/v) formic acid in water/acetonitrile solution with vigorous vortexing for 20 min and subjected to ultrasonication for 10 min. The extracts were transferred to new clean tubes and the extraction process was repeated once. The final 60 μL peptide-containing samples were concentrated by vacuum centrifugation to approximately 10 μL. An aliquot of each sample was submitted to LC-ESI-ION TRAP and the remaining material was desalted and concentrated using a C18 Zip tip® (Millipore), following the manufacturer's instructions, and analysed by MALDI-TOF/TOF MS. When not being analysed, peptide samples were kept at −20 °C.

2.7. MALDI-TOF/TOF MS

MALDI-MS was performed on a 4700 Proteomics Analyzer® using its version 3.0 software (Applied Biosystems). MS spectra were acquired in positive ion reflector mode with 1250 laser shots per spot, processed with default calibration and the 6 most intense ions subjected to fragmentation. PSD spectra were acquired with 3000 laser shots and 1 keV collision energy with CID off (1e−8 Torr). The MS/MS data were analysed both by running Mascot (Perkins et al., 1999) as well as through manual analysis in order to obtain a larger number of *de novo* sequences to be compared with the NCBI non-redundant (NCBInr) database using the BLAST software (Altschul et al., 1997) for short sequences. All SPITC-derived peptides were manually analysed.

2.8. Mascot search parameters

All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 1.9.05). Mascot was set up to search our *Lonomia* EST database (available upon request) assuming the digestion enzyme as trypsin and allowing for 2 missed cleavages, a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 0.3 Da. Carbamidomethylation of cysteine was set as a fixed modification. Methionine oxidation and serine and threonine phosphorylation were specified in Mascot as variable modifications. Positive identifications were accepted, considering (a) the appearance of at least 3 consecutive fragment ions in the spectrum; (b) consistent fragment ions error pattern and (c) Mascot individual ion scores indicating identity or extensive homology with $p < 0.05$.

2.9. LC-ESI-ION TRAP MS

Analyses were done following the procedure described elsewhere (Bastos et al., 2007). Briefly, digested samples were resolved on a 15 cm × 300 μm i.d. ProteCol® C18 column (SGE, Australia) and the eluting peptides in the column effluent were directly electrosprayed into a LCQ Deca XP Plus ion trap mass spectrometer (Thermo Finnigan, USA). Acquired data were either analysed by the SEQUEST algorithm or manually interpreted and obtained sequences searched through the NCBI non-redundant database using the BLASTp algorithm (Altschul et al., 1997).

2.10. SEQUEST search parameters

Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by BioWorks version 3.3. All MS/MS samples were analysed using SEQUEST (ThermoFinnigan, San Jose, CA; version 27, rev. 12). Searches were conducted using our *Lonomia* EST database (available upon request) assuming the digestion enzyme as trypsin. SEQUEST was searched with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 1.4 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine, tryptophan oxidation to formylkynurenin and acrylamide adduct of cysteine were specified as variable modifications. Accepted SEQUEST results followed the criteria established in the literature (Washburn et al., 2001). In brief, ΔCn score of at least 0.1 (regardless of charge state) and cross-correlation (Xcorr) values bigger than 1.8 ($z = +1$), 2.5 ($+2$) and 3.75 ($+3$) were necessary to establish a confident hit.

3. Results and discussion

L. obliqua bristle extract is used, with no protein reduction and/or alkylation, as an antigen for the

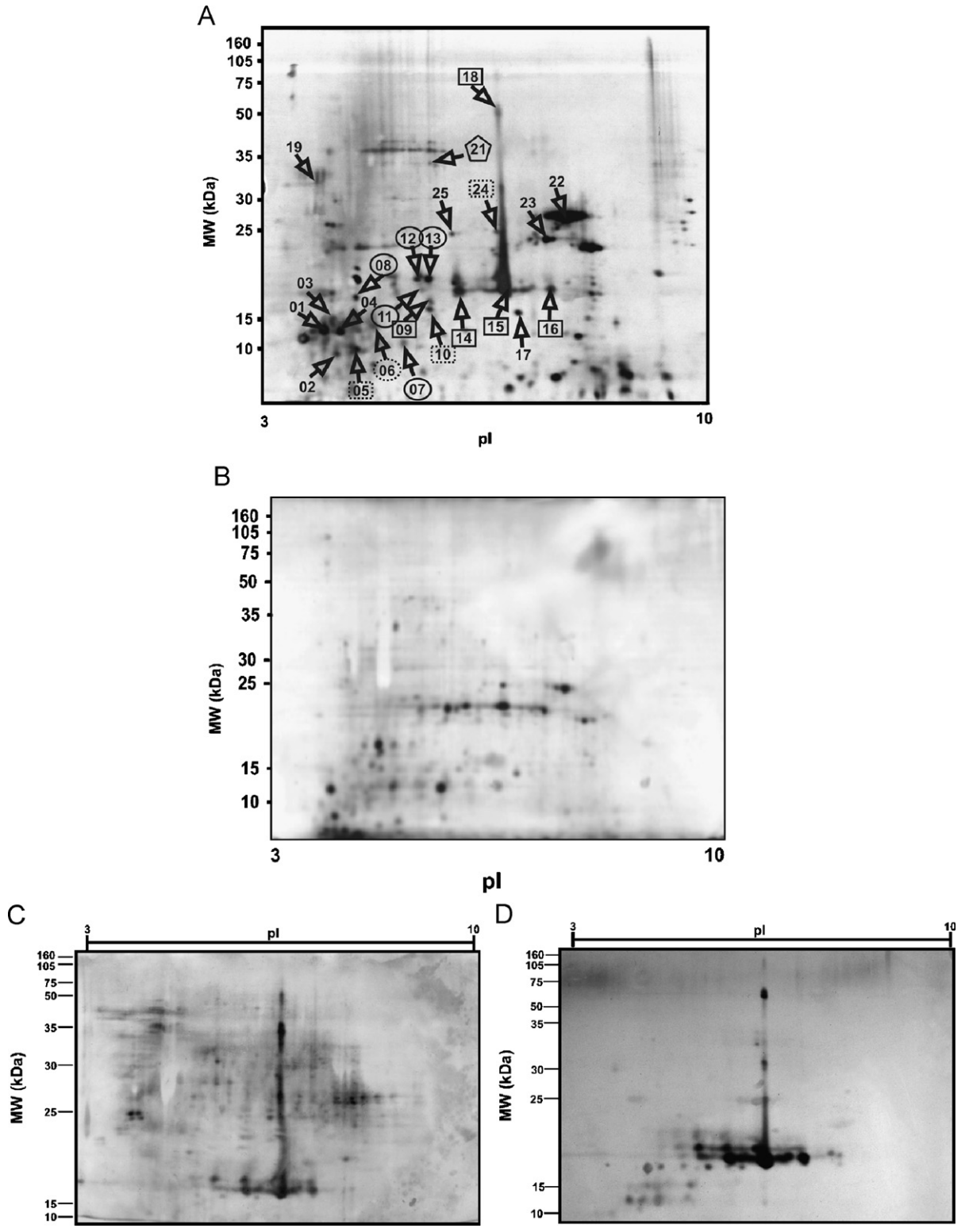

Fig. 1. . Bidimensional electrophoreses and immunoblotting from *Lonomia obliqua*'s bristle extract. Panels (A) and (B)—silver-stained gels (100 μg of protein applied) under non-reducing (A) or reducing conditions (B). Spot protein identifications are indicated by geometrical forms: Lopap (rectangle); cuticle protein (circle); serpin (pentagon). Dashed forms indicate more than one protein identification per spot. Panels (C) and (D)—PVDF immunoblotted 2D gels (non-reducing conditions) incubated with anti-Lonomic horse serum diluted 1:500 (C) or with anti-Lopap rabbit serum diluted 1:250 (D).

Table 1
Identified proteins from *Lonomia obliqua*'s bristle extract subjected to bidimensional electrophoresis under non-reducing conditions (Fig. 1A)

Spot	Protein	Accession (GenBank)	Protein, exp. p*I*	Protein, exp. M_r	*m*/*z*	*z*	Peptide sequence[a,b]
1	Unknown	–	4.4	13,000	1286.49	1+	NSCDCDJE[294][c,d]
		–			1964.80	1+	[283]WSSCSAYGFD[346]R[c]
		–			1612.64	1+	HDEDYGFNEDTDR[c,d]
		–			603.48	2+	[505]JJJNTK[d]
2	Unknown	–	4.5	9000	727.51	2+	[265]EEVQETVNJK[d]
		–			1453.58	1+	DE[230]YEYSVYR[c]
3	Unknown	–	4.5	15,000	1286.50	1+	NSCDCDJE[294][c,d]
		–			1964.81	1+	[283]WSSCSAYGFD[346]R[c]
		–			1612.57	1+	HDEDYGFNEDTDR[c]
		–			1187.63	1+	[432]AJJJPTK[d]
4	Unknown	–	4.6	13,000	1453.58	1+	DE[230]YEYSVYR[c,d]
5	Cuticle protein 1	EU106115	4.9	10,000	1508.71	1+	AVE[142]VQYNTDATR[c]
	Lopap	AY908986			1000.84	1+	VNJWJJSR[c,d]
					557.23	2+	AGHVEDDJEK[d]
					891.43	2+	FDMNAYQGTWYEJK[d]
					862.65	1+	FVVGVJTK[d]
6	Unknown	–	5.1	14,000	1453.59	1+	DE[230]YEYSVYR[c]
		–			1537.80	1+	E[243]EJJGSGHJJAR[c]
	Cuticle protein 1	EU106115			1508.70	1+	AVE[142]VQYNTDATR[c]
7	Cuticle protein 1	EU106115	5.4	11,000	1508.72	1+	A[228]AAVQYNTDATR[c,d]
					787.39	1+	QVAEANR[c]
					726.29	2+	[214]JDAAVAAQDHAR[d]
					633.63	2+	[279]ESGAYVT[260][d]
					947.45	2+	AGDAQAAAJDAAVAAQDHAR[d]
8	Cuticle protein 3	EU106114	4.9	18,000	1291.66	1+	GAJPGYVAPQYR[c]
					729.71	2+	YRPNVEGNAAVJR[c,d]
					620.33	2+	SDSEVSEQGFR[d]
					608.90	2+	YAYETEN[170]R[d]
					863.34	2+	[315]AGJFDDGS[647][d]
9	Lopap	AY908986	5.6	16,000	1000.55	1+	VNJWJJSR[c]
					557.03	2+	AGHVEDDJEK[d]
					1782.46	1+	FDMNAYQGTWYEJK[d]
					862.63	1+	FVVGVJTK[d]
					1101.61	1+	FVETDFSEK[d]
10	Unknown	–	5.6	15,000	1358.67	1+	NVV[147]DG[453]VR[c]
	Lopap	AY908986			1000.54	1+	VNJWJJSR[c]
11	Cuticle protein 2	EU106116	5.5	20,000	1297.72	2+	AEAAGVYNEGSYNVYNNQAAFANR[d]
					657.53	1+	NAAJJR[d]
					818.48	1+	SJEENAR[d]
12	Cuticle protein 2	EU106116	5.5	21,000	1297.78	2+	AEAAGVYNEGSYNVYNNQAAFANR[d]
					1389.98	2+	HQYPYQYQSNRPYNTLGY[504][d]
					2477.18	1+	[532]VA[225]YYYGGPGYY[234]GR[c]
					818.37	1+	SJEENAR[c,d]
					716.32	2+	[250]TGDDGQQYA[246][d]

Table 1 (*continued*)

Spot	Protein	Accession (GenBank)	Protein, exp. p*I*	Protein, exp. M_r	*m*/*z*	*z*	Peptide sequence[a,b]
13	Cuticle protein 2	EU106116	5.6	21,000	1297.19	2+	AEAAGVYNEGSYNVYNNQAAFANR[d]
					2477.14	1+	[532]VA[225]YYYGGPGYY[234]GR[c]
					818.38	1+	SJEENAR[c,d]
					716.33	2+	[250]TGDDGQQYA[246][d]
					657.54	1+	NAAJJR[d]
14	Lopap (Lipocalin 1/4)	Q5ECE3	5.8	20,000	500.39	2+	VNJWJJSR[c,d]
					891.22	2+	FDMNAYQGTWYEJK[c,d]
					1101.75	1+	FVETDFSEK[c,d]
					2066.01	1+	NAGTSDAEJTJSVVVGDYVR[c]
					2435.19	1+	VAPJWJVSTDYDNYAJGYSCK[c]
					668.45	2+	[214]VJDGACPD[294][d]
					556.39	2+	AGHVEDDJEK[d]
					862.67	1+	FVVGVJTK[d]
15	Lopap (Lipocalin 1/4)	Q5ECE3	6.0	20,000	1000.65	2+	VNJWJJSR[c,d]
					1765.73	1+	FDMNAYQGTWYEJK[c,d]
					2066.02	1+	NAGTSDAEJTJSVVVGDYVR[c]
					2435.22	1+	VAPJWJVSTDYDNYAJGYSCK[c]
					660.32	2+	DVVJDGACPDMK[d]
					1112.70	1+	AGHVEDDJEK[d]
					862.65	1+	FVVGVJTK[d]
					550.84	2+	FVETDFSEK[d]
16	Lopap	AY908986	6.5	20,000	1000.55	1+	VNJWJJSR[c]
					891.31	2+	FDMNAYQGTWYEJK[c,d]
					550.83	2+	FVETDFSEK[c,d]
					862.70	1+	FVVGVJTK[d]
					556.40	2+	AGHVEDDJEK[d]
17	Unknown	–	6.2	16,000	727.56	2+	[216]JEHNAEEJRK[c,d]
		–			735.40	1+	Q[147]TAJR[c]
		–			708.77	2+	[227]TAVQNTAQQTK[d]
18	Lopap	AY908986	6.1	69,000	1000.70	1+	VNJWJJSR[c,d]
					1765.75	1+	FDMNAYQGTWYEJK[c,d]
					1101.60	1+	FVETDFSEK[c,d]
					556.33	2+	AGHVEDDJEK[d]
					862.66	1+	FVVGVJTK[d]
19	Unknown	–	4.2	33,000	690.41	2+	VGEAFWSAEEQK[d]
21	Serpin 3	AY829816	5.7	34,000	719.56	2+	KSDAJYVSEAVQK[d]
22	Unknown	–	6.7	28,000	1780.92	1+	[198]MA[241]DQAAGVA[352]R[c,d]
		–			953.62	2+	AJJEJDNJFSESEVJSK[d]
23	Unknown	–	6.5	23,000	1780.93	1+	[198]MA[241]DQAAGVA[352]R[c,d]
24	Unknown	–	6.0	25,000	1471.68	1+	[375]J[260]QESG[147]R[c]
		–			968.57	1+	EVJJEJPR[c,d]
	Lopap	AY908986			1000.57	1+	VNJWJJSR[c]
25	Unknown	–	5.7	24,000	1471.68	1+	[375]J[260]QESG[147]R[c]
		–			968.57	1+	EVJJEJPR[c,d]

[a]J is L (leucine) or I (isoleucine).
[b]Numbers in brackets indicate non-assigned masses when the peptide sequence was determined by manual analysis.
[c]Data generated by MALDI-TOF/TOF MS and analysed by the MASCOT algorithm.
[d]Data generated by LC-ESI-ION TRAP MS and analysed by SEQUEST algorithm.

production of the anti-Lonomic horse hyperimmune serum. Hence, in order to identify immunogenic molecules, we decided to run 2D gels following two different treatments: (a) without protein reduction and alkylation (non-reducing conditions) and (b) with the traditional reduction (during first dimension and equilibration step) and alkylation (equilibration step).

A broad protein profile (157 spots) was obtained from *L. obliqua* bristle extract silver-stained bidimensional electrophoresis under non-reducing conditions. Molecular masses ranged from less than 10 to approximately 105 kDa and a complex distribution of p*I*'s was observed, with most of the proteins displaying an acidic–neutral composition ($4 < pI < 7$), while 9 spots had alkaline properties (Fig. 1A). On the other hand, when the sample was subjected to reducing conditions, a simpler profile was observed (129 spots), especially in the lower molecular mass range (Fig. 1B). This could indicate that many of these low mass spots, especially in the alkaline region, are 2 to *n*-polypeptide chain molecules that after disulphide bridge disruption cannot be detected any more under the electrophoretic conditions used in this work. Future investigations using the well-established Tricine–SDS-PAGE methodology (Schagger and von Jagow, 1987) should shed some light into the matter. Other abundant spots, like 14, 15 and 16, have had their molecular masses shifted upward upon reduction. This could be explained as a consequence of the use of DTT as a reducing agent since it disrupts the tertiary structure of polypeptide chains leading to a change in the molecule's hydrodynamic properties, which can eventually result in slower electrophoretic migration (Westermeier, 2001). Finally, when the gels shown in Fig. 1 were run again and stained with colloidal Coomassie blue, only 81 and 88 spots were detected for the reduced and non-reduced conditions, respectively (data not shown). This is expected due to the smaller sensitivity of this staining method, although the spots subjected for MS needed to be excised from a Coomassie stained gel due to technical limitations. Overall, image analysis revealed the same profiles seen in Fig. 1 (panels A and B).

Abundant spots from 2D gels (colloidal Coomassie blue-stained spots) were selected for MS analyses based on their potential immunogenicity and 25 spots were subjected to tryptic digestion followed by MS/MS sequencing. Using the described proteomic approach, we could identify 3 different categories of proteins (Table 1), e.g. 8 hits for lipocalins (spots 5, 9, 10, 14–16, 18 and 24); 7 for cuticle proteins (spots 5–8 and 11–13), 1 for serpin (spot 21) and, finally, 12 samples containing peptides that were no match to known proteins (spots 1–4, 6, 10, 17, 19 and 22–25). However, spots 5, 6, 10 and 24 were assigned to more than one protein. An analysis of the major ions generated by MS spectra for these spots indicates that spot 5 is mainly composed of Lopap-originated peptides, spot 6 by unknown protein(s)-originated peptides, and spots 10 and 24 by unknown protein(s) and Lopap. It should be stressed that spot 10 also presented major keratin contamination. For a better identification of the proteins, different spectrometers (MALDI-TOF-TOF and LC-ESI ion trap) and search tools (MASCOT, SEQUEST, BLASTp and manual interpretation) were used as well as a derivatization technique (SPITC). The identification searches were done against an *L. obliqua* bristles' EST database. The peptides for unknown proteins did not match confidently any protein in the NCBInr database as well. For the spots identified as Lopap or cuticle protein, a graphical view of the peptide coverage resulting from MS sequencing is displayed in Fig. 2.

Lopap was initially characterized as a 69 kDa molecule both by molecular exclusion chromatography and by SDS-PAGE under reducing conditions (Reis et al., 2001b). Hence, a fair assumption was that the molecule was composed of a single polypeptide chain. However, in a later work where the recombinant molecule's obtention and biochemical characterization was done, the authors demonstrated that mature Lopap is a 20.8 kDa protein and they suggested, based on molecular modelling, a tetrameric conformation held together by non-covalent bonds (Reis et al., 2006). Our data show that under denaturing (SDS only, no heating) and non-reducing conditions (Fig. 1A), only one of the identified spots correspond to Lopap's 69 kDa form (spot 18). Most of the spots are in the 25 (spot 24) to 20 kDa (spots 14–16) or less (spots 5, 9 and 10) molecular mass range. Upon reduction (Fig. 1B), no band is seen in the 69 kDa range and the central smear, probably due to the presence of different incomplete unfolded forms, in the middle of the non-reduced gel disappears leaving only the expected 20 kDa spots. This result also agrees with the abundance of lipocalins reported in *L. obliqua* bristles' cDNA libraries. Two independent cDNA libraries were constructed from *L. obliqua*'s bristles. The first one was deposited at GenBank in 2004

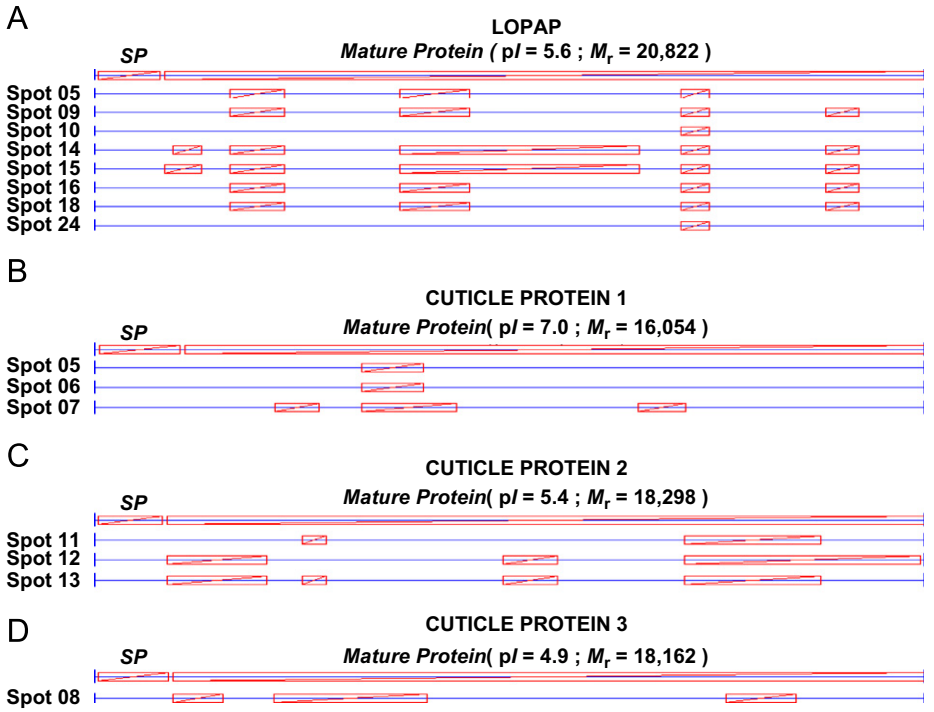

Fig. 2. Graphic representation of the sequence coverage for the identified Lopap (panel A) and different cuticle proteins (panels B–D). Signal peptide (SP) and mature protein sections of each protein are indicated. Signal peptide was determined using the SignalIP 3.0 Server (Bendtsen et al., 2004) and the individual graphical bars were created using CAITITU 1.0 and RAPADURA 1.0 software (http://www.buscario.com.br/caititu/). Peptide sequence coverage and distribution are graphically represented by boxes (sequenced region) and lines (no sequence data) relative to the molecule's sequence deposited in the database (full box—first line of each inset).

(1503 ESTs: CX815710-CX820336), reporting the presence of 24 clones containing the sequence of Lopap (accession number AY908986) (Reis et al., 2006). However, a second cDNA library revealed 3 different forms of lipocalins (accession numbers: AY829809; AY829833; AY829856) (Veiga et al., 2005), but 96.43% of the sequenced clones account for a protein 99% identical to Lopap (AY829833) (Reis et al., 2006).

Moreover, some spots from the bidimensional map were visualized with similar molecular weight and different p*I*, probably as a consequence of their amino acid residue composition and they could be initially considered as isoforms. In Table 1, an example of the isoforms identified in this study, corresponding to spots 14–16 (p*I* 5.8–6.5; M_r 20,000), can be observed. After that, their identities were confirmed as lipocalins by MS analyses even though changes in their amino acid composition were observed (AY908986—spots 5, 9, 10, 16 and 18, and Q5ECE3—spots 14 and 15).

For immunological identification of the components from bristle extract, an immunoblotting analysis was performed from proteins isolated by 2D gels, which were transferred to PVDF membrane. At least 153 spots were detected by the polyclonal horse anti-Lonomic hyperimmune serum (Fig. 1C), while only 30 of them could be recognized by the anti-Lopap-specific rabbit serum (Fig. 1D). The silver-stained 2D gel, used as reference, showed a total of 157 spots (Fig. 1A). It is important to point out that the most intense spots immunodetected corresponded to the more abundant proteins in this sample. Otherwise, the low abundant spots can be detected only by very sensitive methods, such as immunoblotting, and were not isolated from silver-stained 2D gels. These results indicate that the majority of the components present in *Lonomia* bristle extract is immunogenic and suggest that Lopap is present as multiple isoforms or that it shares epitopes with other components in the extract.

Considering the expression, the immunogenic properties and the diversity of forms of lipocalins in bristles from *L. obliqua*, it is reasonable to suppose that they should play an important role in

the envenomation process. Lopap, a prothrombin-activating lipocalin, was the first lipocalin identified having serine protease-like activity. It is known that lipocalins have a functional diversity; however, the abundance of functionally redundant lipocalins targeting the same molecule is essential (Andersen et al., 2005; Flower, 1996; Ribeiro et al., 2004). Lipocalins can play a role in homeostasis and inflammation, as a defence mechanism in haematophagous arthropods. The most abundant lipocalins from *Rhodnius prolixus* are nitrophorins, acting as a vasodilator and platelet aggregation inhibitor (Andersen et al., 2005). Prolixin S, a nitrophorin and potent anti-coagulant, was isolated from the saliva of *R. prolixus* (Ribeiro et al., 1995). In ticks, the histamine molecules bind to lipocalins, suppressing the host response to inflammation and allergenic processes. So, lipocalins can also be a scavenger for low molecular weight signalling or toxic components (Mans, 2005). A hydrophobic pocket that could harbour ligands of the same nature was predicted for Lopap (Reis et al., 2006).

An evolutionary relationship among lipocalins from arachnids and insects was described (Mans, 2005). The lipocalins identified by the transcriptome in *L. obliqua* bristle extract were correlated to biliverdin-binding protein from the saturniine eri-silkmoth *Samia cynthia ricini*, to lipopolyssaccharide-binding protein from the silkworm *Bombyx mori* and insecticyanin from the haemolymph of *Manduca sexta* (Saito, 1998; Veiga et al., 2005).

Some immunogenic components from bristle extract were identified as cuticle proteins (spots 5–8 and 11–13—Figs. 1A and 2), but they are probably not involved in the envenoming process or in the coagulation disorders, since they are present in the extracts as a result of the maceration of spicules containing the venom glands. On the other hand, one cannot rule out a possible inflammatory response to these proteins present in the spicule during the envenomation process.

Another protein identified was a serpin-like protein (spot 21—Fig. 1A), one of the minority proteins, which is supposed to act as a proteinase inhibitor. According to bristles' cDNA library, serpin represented about 2.8% of the venom composition and it had homology to serpins from *B. mori* and *Anopheles gambiae* (Veiga et al., 2005). These facts suggested that this protein could protect their host from infection by pathogens or regulate endogenous proteases involved in coagulation and cytokine activation (Veiga et al., 2005).

Spots 1–4, 6, 10, 17, 19 and 22–25 had some peptidic fragments sequenced but until now they were identified as unknown proteins because no correspondence was obtained with the translated sequences from *L. obliqua*'s bristle cDNA databank. Based on anti-Lonomic recognition properties, other toxic proteins in low abundance in bristle extract can be isolated by synthesizing oligonucleotides to find them in the ESTs databank or even using enrichment techniques, such as affinity columns. From these clues, new immunogenic molecules should be identified and characterized.

A proteomic analysis of the silk gland proteins from *B. mori* identified the proteins involved in silk production and transport as the major components in this tissue; proteins involved in distinct functions, such as metabolism and defence, could also be identified. In addition, 3 minor components identified in this proteome were 14 kDa apoptotic proteins (Zhang et al., 2006). In *L. obliqua*, anti-apoptotic proteins were already identified, in extracts from bristles and haemolymph (Alvarez Flores et al., 2006; Souza et al., 2005), and it is possible that proteins still defined as hypothetical proteins can be related to apoptosis.

4. Concluding remarks

This is a preliminary study concerning the identification of the immunogenic proteins involved in accidents, consisting in the contact of human skin with caterpillars, by a proteomic approach. From these results, we could conclude that lipocalins can have a relevant role in the envenoming process and it is sure that Lopap is one of the molecules that responds to the caterpillar's potent procoagulant activity. In spite of the fact that several proteins detected by the polyclonal anti-Lonomic serum could represent components not directly involved in envenoming, the employed methodologies proved useful for this kind of screening. Further efforts have to be made in order to identify other minority proteins that could have some influence on the haemostatic syndrome triggered by *L. obliqua* accidents.

Acknowledgements

We are grateful to Dr. Tetsuo Yamane, Nanci do Nascimento and Maria Aparecida Pires Camillo who allowed Maria Esther Ricci-Silva to work in the Biotechnology Laboratory at IPEN (São Paulo)

and also to Dra Marlene Zannin and Dra Danielle Bibas Legat Albino from CIT (Santa Catarina) who provided specimens of the caterpillars. For the financial support, we are thankful to CNPq, FAPERJ, FAPESP, FINEP and PDTIS-FIO-CRUZ.

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