Trends in Entomology
Vol. 12, 2016

Original Communication

A new lysozyme found in the haemolymph from pupae of *Lonomia obliqua* (Lepidoptera: Saturniidae)

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ABSTRACT

Lysozymes are hydrolytic enzymes characterized by their capacity to cleave the 1,4-β linkages between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, causing cellular lysis and, subsequently, the death of microorganisms. Hence, lysozymes represent an important component of defence in most insects. In this work, one lysozyme was purified from uninfected and uninjured *Lonomia obliqua* pupae, which indicates that it is produced constitutively. However, the expression of genes that produce the lysozyme in *L. obliqua* was seen to increase threefold after infection. The increase in the production of antimicrobial molecules after injuries and infections is a common feature in insects. By transcriptomic analysis, the complete amino acid sequence of this enzyme was determined, and it consisted of 139 amino acids, with molecular mass of 15.9 kDa and pI value of 8.82. Alignment and comparison with other lysozymes indicate that it has 83.7% sequence identity with the lysozyme from the moth *Hyalophora cecropia* and higher identity with C-type than I-type lysozymes. The possible structure was predicted and indicates the presence of α helix, 3_{10} helix, β strand, (β) turn and other secondary structures. The description of a new lysozyme contributes to the overall knowledge of these important antimicrobial molecules.

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KEYWORDS: lysozyme types, C-type, *Lonomia obliqua*, transcriptomic analysis and innate immunity

INTRODUCTION

Insects represent 55% of all biodiversity in the world and 85% of all known animals. They can be found in almost all regions of the planet, and this wide distribution has stimulated the search for bioactive molecules among them [1]. Several studies indicate the presence of pharmacologically active substances in the haemolymph of insects. These substances can have, for example, enzymatic actions [2] and antibacterial and antifungal effects [3].

Many species of the order Lepidoptera, which includes moths and butterflies, can in their larval phase induce poisoning, whose symptoms vary according to species, degree of contact and the individual response of the victim. The most common symptoms are skin reactions, hives, pain and a local burning sensation, but can also include nausea, headache, fever, allergic reactions, conjunctivitis and, in rare cases, arthritis, coagulation disorders, bleeding and acute renal failure [4, 5].

Lonomia obliqua is the caterpillar whose venom has been most studied. Many of its principal components have been isolated and characterized, including two proteins: Losac, activator of the factor X coagulation cascade, and Lopap, activator of prothrombin [6].

Some bioactive molecules from the haemolymph of *Lonomia obliqua* were previously isolated and characterized. Lonofibrase, for example, is a molecule with a mass of 35 kDa that has fibrinogenolytic activity, cleaving the bond of chains αβ of the fibrinogen molecule [7, 8, 5]. Other examples include a molecule with a mass of 20 kDa that exhibits antiviral activity against poliomyelitis, measles and influenza virus [9, 10] and a protein with a mass of 51 kDa, with antiapoptotic action that can protect cultured *Spodoptera frugiperda* Sf-9 cells against DNA fragmentation and retard cell death [11]. Mendonça *et al.* [12] identified a protein with high molecular mass that stimulates the production of recombinant proteins. The production of G glycoprotein from the rabies virus, expressed in *Drosophila melanogaster* S2 cells, increased by approximately 59% through the action of this protein isolated from the haemolymph of *Lonomia obliqua*.

Lysozymes are hydrolytic enzymes characterized by their capacity to cleave the 1,4-β linkages between N-acetylmuramic acid and N-acetyl-glucosamine in peptidoglycan, the principal polymer of the bacterial cell wall. This polymer plays a structural role, giving strength, as well as counteracts the osmotic pressure of the cytoplasm in prokaryotic cells. Thus, the loss of integrity of peptidoglycan causes cellular lysis, and subsequently, death of the microorganism [13].

A wide variety of lysozymes have been described since the first report by Fleming [14]. These enzymes are widely distributed in nature and are mainly known for their role in defending against infections. In some animals, they also play an important role in digestion [15, 16].

The lysozymes were the first antimicrobial molecules purified from the haemolymph of insects [17, 18]. In the moth *Hyalophora cecropia,* the lysozyme is usually found in the haemolymph of larvae but is absent in the pupae. However, the production of this enzyme by the pupae can be induced after inoculation with bacteria [19, 18], in contrast to the behaviour of the lysozyme found in *Lonomia obliqua*.

MATERIALS AND METHODS

Obtaining the pupae

Pupae of *Lonomia obliqua* were ceded by Dr. Ronaldo Zucatelli Mendonça, from the Parasitology and Entomology Laboratory at Butantan Institute, São Paulo, Brazil.

Haemolymph extraction

Extraction of haemolymph was carried out by three different approaches: collection with apyrogenic syringe, centrifugation of pupae at 2,000 G for 10 minutes at room temperature and washing of fat body with PBS buffer (phosphate buffered saline) (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 10 mM; KH_2PO_4 1.76 mM; pH 7.4). Haemolymph was stored in vials containing phenylthiourea/phenylthiocarbamide crystals, in order to avoid phenoloxidase cascade activation.

Cell content extraction and purification

To perform cell content extraction, 30 mL of acetic acid 2 M was added to the haemolymph, agitated on ice for 30 minutes and centrifuged at 16,000 G at 4 °C for 30 minutes. The soluble part was pre-purified with classic Sep–Pak C18 cartridges with elution by acetonitrile (ACN) 40% in acidified water (trifluoroacetic acid - TFA - 0.05%). The obtained sample was concentrated in vacuum centrifuge, reconstituted with acidified water (TFA 0.05%) and applied to reverse phase high performance liquid chromatography (RP-HPLC) on a semi preparative Jupiter C18 column. Elution was performed with linear gradient of 2% to 60% of ACN in acidified water (TFA 0.05%) over 60 minutes at a flow rate of 1.5 mL/min and the ultraviolet absorbance was monitored at 225 nm. Eluted peak fractions were collected, vacuum dried, resuspended in 500 µL of ultrapure water and evaluated for the presence of antibacterial activity by a liquid growth inhibition assay against *Micrococcus luteus* A270 (Pasteur Institute, Paris).

Antimicrobial activity assays

Antimicrobial activities of samples obtained by RP-HPLC were monitored by liquid growth inhibition

assay. The assay was performed using 5-fold microliter broth dilution in 96-well sterile plates at a final volume of 100 µL, where each well containing 20 µL of the sample was filled up with 80 µL of bacterial dilution. The culture used was a mid-log phase of *Micrococcus luteus* (strain A270) in a final concentration of 1 x 10^5 colony forming units/mL, cultured in poor broth nutrient medium (1.0 g peptone in 100 mL of water containing 86 mM NaCl at pH 7.4). Plates were incubated for 18 hours at 30 °C. The growth inhibition was determined by measuring absorbance at 595 nm using the equipment Victor[3] (Perkin Elmer Inc.).

Protein digestion in solution (reduction, alkylation and trypsinization)

In-solution digestion of protein was carried out as described by Stone *et al.* [20]. Approximately 30 µg of the sample was resuspended in 20 µL of a solution of 8 M urea and 0.4 M ammonium bicarbonate, to which 5 µL of 45 mM dithiothreitol was added and incubated at 50 °C for 15 minutes. After cooling at room temperature, 5 µL of 100 mM iodoacetamide was added and incubated at room temperature for 15 minutes, and protected from light. 130 µL of ultrapure water and trypsin (Sigma-Aldrich) was added in a 1:25 (enzyme/protein) ratio. The sample was incubated at 37 °C for 24 hours and digestion was stopped by acidifying with TFA 0.1%. After protein digestion, the sample was desalinated by ZipTip® Pipette Tips (Merck Millipore) with a unique elution step using ACN 80%. Purified sample was concentrated in vacuum centrifuge and then analyzed by mass spectrometry.

Mass spectrometry and database searches

To determine amino acid sequence, the sample was resuspended in formic acid 0.1% and applied in Q-TOF Ultima API Mass Spectrometer (Waters Corporation), with electrospray ion source and operating in positive ionization mode. Spectra was analyzed by the software MassLynx, in which their doubly charged ions were subjected to "*de novo*" sequencing and the "y" and "b" fragments were used to elucidate the primary structure of the molecule. With these results, searches were performed using Mascot®.

Immunodot blot

The immunodot blot technique was employed based on the protocol described by Salazar-Anton *et al*. [21]. The sample was applied directly on nitrocellulose membrane and incubated overnight at room temperature. The membrane was blocked for 1 hour with 5% skim milk in PBS containing 0.05% Tween 20. Thereafter, the dots were incubated for 1 hour with anti-lysozyme chicken egg antibody (1:100) diluted in 5% skim milk–PBS and washed three times with PBS containing 0.05% Tween 20. This was followed by addition of anti-mouse IgG antibody conjugated with alkaline phosphatase (1:10000) diluted in 5% skim milk–PBS; the membrane was incubated for 1 hour in this step and, thereafter, was washed three times with PBS 0.05% Tween 20. The revelation was made using a solution with revelation buffer (Tris/HCl 100 mM, pH 9.5; NaCl 100 mM, $MgCl_2$ 5 mM), 6 mM NBT (nitro blue tetrazolium) and 10 mM BCIP (5-Bromo 4-chloro 3-indolyl phosphate p-toluidine salt). When colored dots appeared, the reaction was stopped with deionized water.

Sequence analysis and comparison to other lysozymes

The complete transcriptome of *Lonomia obliqua* was made by the Genomics and Transcriptomics Laboratory, CeTICS - Butantan Institute. The total RNA was extracted using TRIzol® reagent (Ambion, Life Technologies). The RNA integrity was assessed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano assay. mRNA was extracted with an oligo (dT) using Dynabeads® mRNA DIRECT kit (Ambion, Life Technologies). mRNA was quantified by Quant-iT™ RiboGreen® RNA reagent and Kit (Invitrogen, Life Technologies Corp.) and the integrity of mRNA was evaluated in a 2100 Bioanalyzer, picochip series (Agilent Technologies). cDNA library was prepared following the standard TruSeq RNA Sample Prep Kit protocol (Illumina, San Diego, CA). The cDNA libraries were sequenced on the Illumina HiSeq 1500 System, into a rapid paired end flowcell in a 300 cycles of 2*151 bp paired end technique, according to the standard manufacturer's protocol (Illumina). The reads were filtered by quality control and assembled by Trinity [22]. Results of

this transcriptomic analysis are not available for public, but access to the database was authorized by Dr. Ronaldo Zucatelli Mendonça, author of the study. The complete sequence of different types of lysozymes were obtained on UniProt database and employed to identify similar sequences on transcriptome of *L. obliqua* using the BLAST algorithm tblastn [23] and the results were checked manually with the e-value threshold $<10^{-7}$. The lysozyme identified in *L. obliqua* and other lysozymes were aligned by ClustalW [24] using default parameters and the sequences were manually edited using Jalview [25]. The identity and similarity percentages were calculated by SIAS server using default parameters (http://imed.med.ucm.es/Tools/sias.html).

Structure modeling

Structure modeling of lysozyme was made by CPHmodels 3.2 [26], a protein homology modeling server. In this server, a preliminary three-dimensional structural model was obtained with coordinates in pdb format, which were visualized by the Jmol software (http://jmol.sourceforge.net/).

RESULTS

Purification and identification of antimicrobial molecules in the haemolymph

In the first step of purification by RP-HPLC, 68 fractions were obtained, which were tested by liquid growth inhibition assay against the Gram-positive bacteria *Micrococcus luteus* (A270). Seven fractions showed antimicrobial activity (Fig. 1).

The fraction 61 was selected for thorough analysis and was repurified by RP-HPLC on a semi preparative Jupiter C18 column, with a linear gradient of 33% to 60% of ACN in acidified water (TFA 0.05%) over 60 minutes, at a flow rate of 1.5 mL/min, with ultraviolet absorbance monitored at 225 nm. The fractions obtained in this second step of purification were again tested by liquid growth inhibition assay against *Micrococcus luteus* (A270), and one fraction showed antimicrobial activity (Fig. 2).

Mass spectrometry and database searches

The analysis indicated that the fraction with antimicrobial activity obtained in the second step

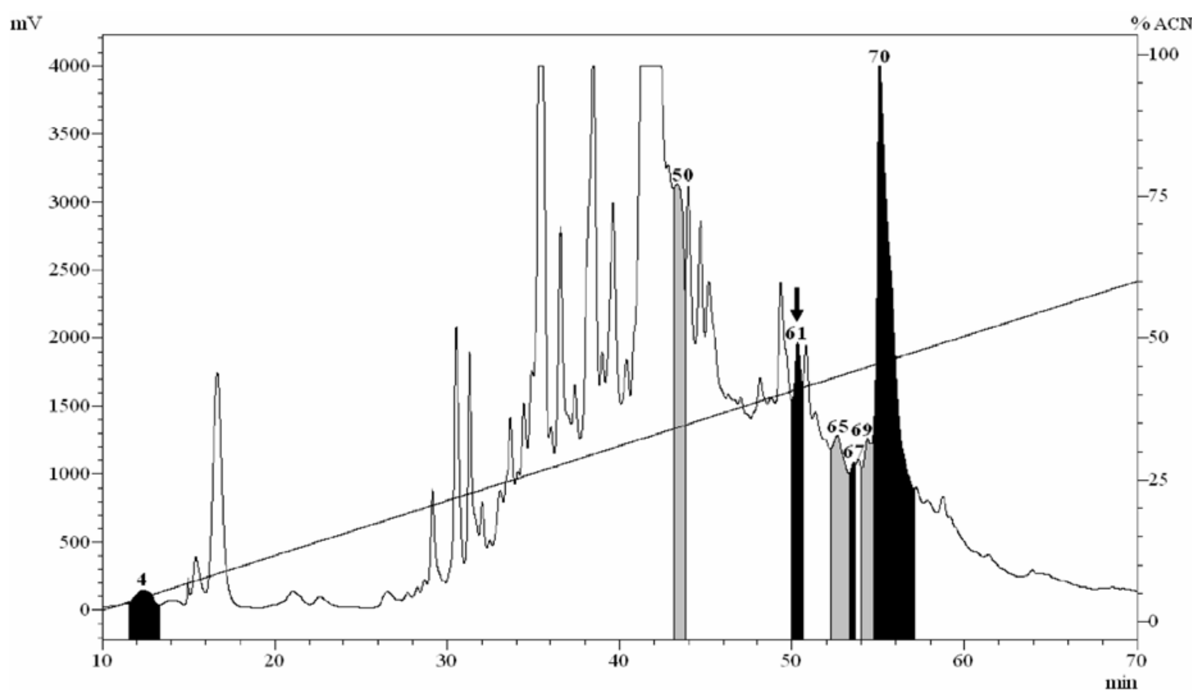

Fig. 1. Chromatographic profile of the first purification step of *Lonomia obliqua* pupae haemolymph. The fractions indicated (black and gray) showed antimicrobial activity against Gram-positive bacteria *Micrococcus luteus* (A270).

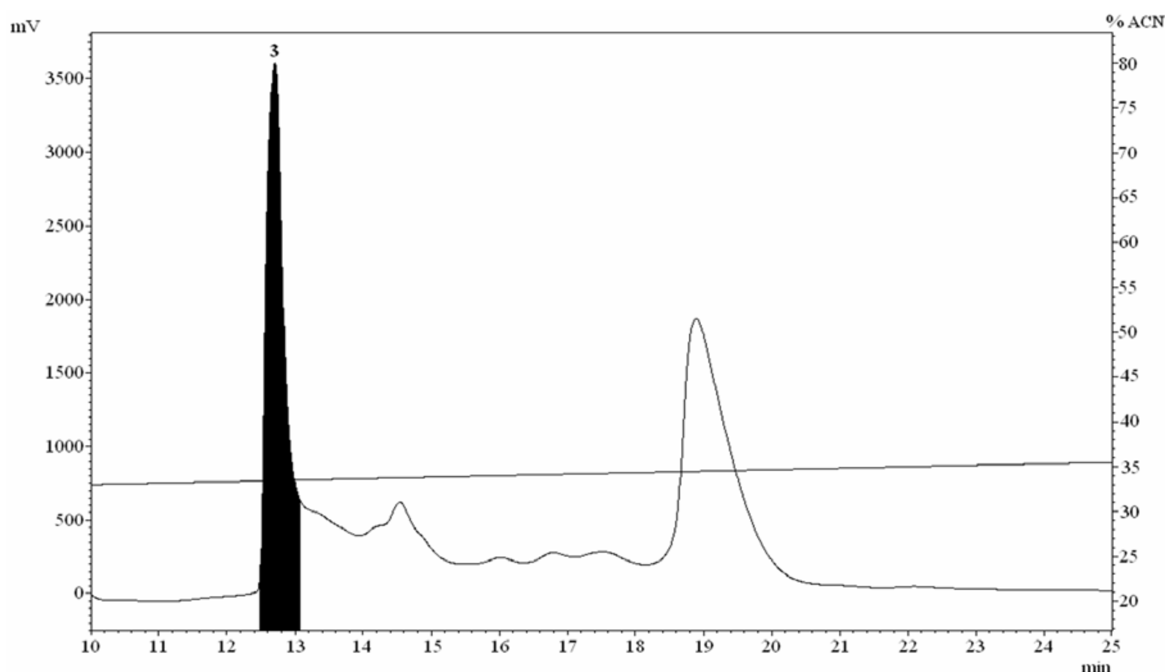

Fig. 2. Chromatographic profile of the second purification step of fraction 61. The fraction indicated (black) showed antimicrobial activity against Gram-positive bacteria *Micrococcus luteus* (A270).

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        10          20          30          40          50
MTKYVILLAV  LAFALHCDAK  RFTRCGLVQE  LRRLGFDETL  MSNWVCLVEN
        60          70          80          90         100
ESGRFTDKIG  KVNKNGSRDY  GLFQINDKYW  CSKGTTPGKD  CNVTCNQLLT
       110         120         130
DDISVAATCA  KKIYKRHKFD  AWYGWKNHCQ  HGLPDISDC
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Fig. 3. Amino acid sequence of *Hyalophora cecropia* lysozyme (obtained by Uniprot database, ID: P05105). The lysozyme identified in *Lonomia obliqua* showed identity with the two sequences indicated in the boxes.

of purification exhibited similarity with the lysozyme of the moth *Hyalophora cecropia,* having two fragments with identical amino acid sequences: **CGLVQELR** and **DYGLFQINDK** (Fig. 3). By "*de novo*" sequencing, the presence of these two fragments in the lysozyme isolated from the pupae of *L. obliqua* was confirmed (Figs. 4 and 5).

Immunodot blot

To perform the immunodot blot experiment, 16 µg of the lysozyme from pupae of *Lonomia obliqua* and 0.1 µg of the lysozyme from chicken egg were applied to the same nitrocellulose membrane at different points. The results indicated that the lysozyme from *L. obliqua* is not recognized by anti-lysozyme chicken egg antibody (Fig. 6).

Sequence analysis and comparison to other lysozymes

Transcriptomic analysis revealed three possible amino acid sequences for lysozyme identified in the haemolymph from pupae of *Lonomia obliqua*. Considering the results obtained by mass spectrometry analysis and "*de novo*" sequencing, only one of these amino acid sequences corresponds to lysozyme from *L. obliqua:* c17617_g1_i1 (Fig. 7).

This enzyme has 139 amino acids. Analysis by ExPASy server [27] indicates values of approximately

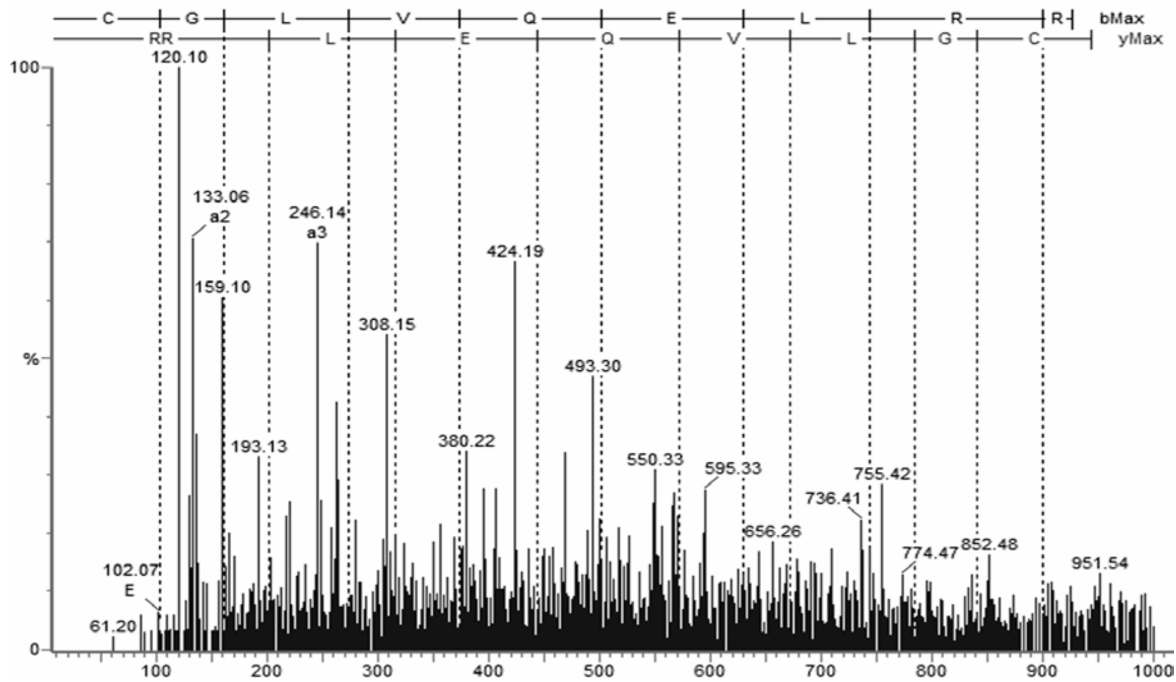

Fig. 4. Representative "*de novo*" sequencing of the first fragment (CGLVQELR), identical to amino acid sequence from *Hyalophora cecropia* lysozyme. The peptide sequence following "b" and "y" series orientation is shown on the top of the graph.

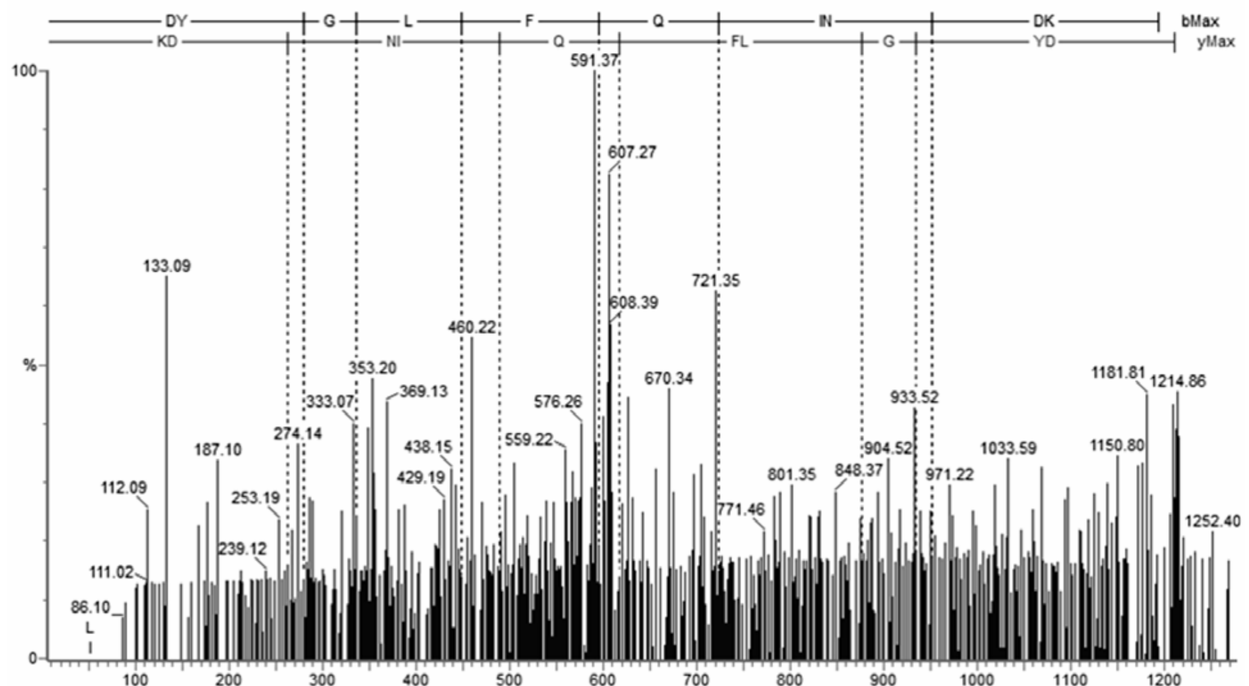

Fig. 5. Representative "*de novo*" sequencing of the second fragment (DYGLFQINDK), identical to amino acid sequence from *Hyalophora cecropia* lysozyme. The peptide sequence following "b" and "y" series orientation is shown on the top of the graph.

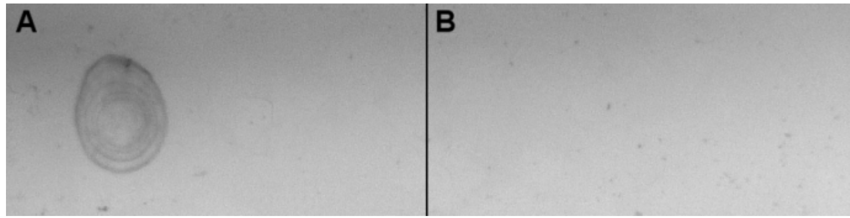

Fig. 6. Nitrocellulose membrane used in immunodot blot experiment. The gray dot (6A) indicates that chicken egg lysozyme was recognized by antibody, in contrast to *Lonomia obliqua* lysozyme. A) Lysozyme from the egg of *Gallus gallus*; B) lysozyme from *Lonomia obliqua* pupae haemolymph.

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                     10        20        30        40        50        60        70        80
c2646_g1_i1   1 MSGISAVLLLATTFAVHVVVIEAKIYDRCALASELRWKYNFPKDQIADWVCIAQHESSFNTAALGPPNSDGSRDHGLFQINDRYWC
c15693_g1_i1  1 MTSAVIKFSAILLVIGVCVADVSYMPQANPTPVTEACLGCICQAVSGCQYGKQCEGDHCGLFHITWAYWADAGKPTLLGHSADAPD
c17617_g1_i1  1 MKCVILFAVLALTLHCDAKHFTRCGLVQELRRQGFEESKLRDWVCLVENESSRYTDKVGKVNKNGSRDYGLFQINDKYWCSASATP

                   90        100       110       120       130       140       150
c2646_g1_i1  87 SPPGPHNDCGIDCSALRNDDIADDAKCIRKIYGRHGFSAW----------------------------------
c15693_g1_i1 87 AYPSCANDPRCAATAVQNYMARFGQDCNKDGVVNCYDYMAIHKLGGYGCTGDLPFNYVNVFNQCVAAVASHYG*
c17617_g1_i1 87 GKDCNTTCSQLLTDDISVAATCAKKIYRRHKFQAWYGWRNHCDGKTLPDISSC*--------------------
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Fig. 7. Three possible amino acid sequences for lysozyme identified on *Lonomia obliqua* pupae haemolymph. The sequences indicated in the boxes (contig c17617_g1_i1) are identical to those found in *Hyalophora cecropia* lysozyme.

15.9 kDa and pI 8.82. Amino acid residues 1 to 18 correspond to their signal peptide, determined by using the server SignalP 3.0 [28].

The alignment and comparison with other lysozymes from different animal species indicate that c17617_g1_i1 exhibits greater identity with C-type than I-type lysozymes (Fig. 8).

As expected, the alignment with the lysozyme from *Hyalophora cecropia* showed a higher identity percentage (83.7%). The comparison between amino acid residues located in the active site region from P05105, indicated in Fig. 8 (A), presents 84.21% identity and 94.73% similarity. This result indicates that the active site of lysozyme from *L. obliqua* might be in the same region of the lysozyme as in *H. cecropia* amino acid residues 51 to 69.

The identity percentage with the C-type lysozyme from the egg of *Gallus gallus* is 48.4%. Comparison between amino acid residues located in the region of the epitope from P00698 [29], indicated in Fig. 8 (B), shows 25% identity and similarity. This low identity percentage might explain why the antibody utilized in the immunodot blot experiment did not recognize the lysozyme from *L. obliqua*.

Comparing the lysozyme c17617_g1_i1 with an I-type lysozyme from *Periplaneta americana*, the identity percentage is rather low: 16.3%.

Using Jalview [25], a phylogenetic tree of lysozymes used in the alignment was calculated based on the average distance using the percentage identity parameter (Fig. 9). The result confirms that the lysozyme isolated from pupae of *L. obliqua* (c17617_g1_i1) is more closely related to C-type (P05105, I1XB29 and P00698) than I-type (I1XB28) lysozymes.

Structure modeling

To model the lysozyme (c17617_g1_i1), the signal peptide was removed; thus, only residues 19 to 139 were considered for the construction of the structure. The CPHmodels 3.2 server [26] identified homology with an antibacterial protein from *Antheraea mylitta* (PDB ID: 1IIZ), and based on this structure, the server provided a structure model for lysozyme from *L. obliqua.* This model indicated the presence of five types of protein secondary structures: α helix, 3_{10} helix, β strand, (β) turn and others (Fig. 10).

DISCUSSION

The haemolymph from uninfected and uninjured pupae of *Lonomia obliqua* was found to contain

Fig. 8. Alignment of amino acid sequences of different lysozymes and c17617_g1_i1. Variations in gray scale indicate levels of percent identity of the residues in each column with consensus sequence. The identity percentages, compared to first sequence, are indicated in the end of amino acid sequences. The symbol (-) represents gaps to improve the alignment. A) Alignment with C-type *Hyalophora cecropia* lysozyme (Uniprot ID: P05105), the signal peptide and active site are indicated; B) alignment with C-type lysozyme from the egg of *Gallus gallus* (Uniprot ID: P00698), the signal peptide, active site and epitope are indicated; C) alignment with I-type (Uniprot ID: I1XB28) and C-type (Uniprot ID: I1XB29) from *Periplaneta americana* lysozyme.

Fig. 9. Phylogenetic tree constructed by Jalview with I-type lysozyme from *Periplaneta Americana* (Uniprot ID: I1XB28), *Lonomia obliqua* pupae lysozyme (c17617_g1_i1) and C-type lysozyme from *Hyalophora cecropia* (Uniprot ID: P05105), *Periplaneta americana* (Uniprot ID: I1XB29) and from the egg of *Gallus gallus* (Uniprot ID: P00698).

7 molecules with antimicrobial activity against Gram-positive bacteria *Micrococcus luteus* (A270), including the new lysozyme. Data reported in the literature indicate that in insects, an increase in the production of molecules with antimicrobial properties occurs a few hours after injuries or infections [30, 31, 32]. The attacins and cecropins, for example, are antimicrobial molecules whose production is

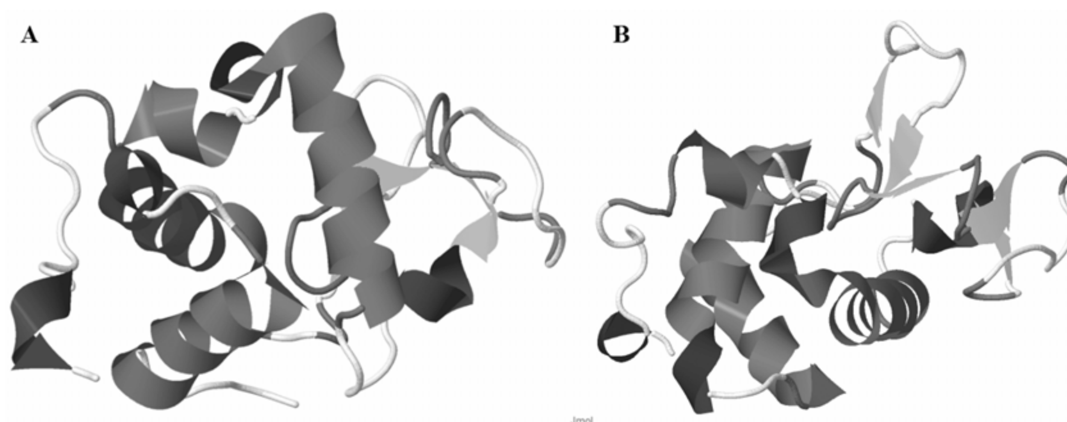

Fig. 10. Structure of lysozyme from the pupae of *Lonomia obliqua* (c17617_g1_i1), constructed by CPHmodels 3.2 based on homology with an antibacterial protein from *Antheraea mylitta.* A) Frontal view structure; B) top view structure.

induced in the pupae of *Hyalophora cecropia* after bacterial infection [18]. However, Lamberty *et al*. [33] observed that in the termite *Pseudacanthotermes spiniger*, the production of the antifungal peptide termicin is constitutive. As in this termite specie, in the pupae of *Lonomia obliqua,* the antimicrobial molecules identified are produced constitutively, as shown by their presence in uninfected and uninjured pupae.

Among these constitutive molecules, one lysozyme was identified, and the amino acid sequence was determined by a combination of proteomic and transcriptomic approaches. Transcriptomic data also revealed that the expression of the genes that produce this lysozyme in *L. obliqua* increased threefold after an infection/injury. As previously mentioned, increased production of antimicrobial molecules after injuries or infections is common in insects. Lysozymes represent an important component of defence in most insects and they show a clear increase in the haemolymph after an infection/injury [34].

Lysozymes are present in all main taxons of living organisms [13]. Based on differences in amino acid sequences and in biochemical and enzymatic properties, they can be classified into different types [35]. There are three principal types of lysozymes: C-type (chicken or conventional-type), G-type (goose-type) and I-type (invertebrate-type) [13]. Insects are the only metazoans known to have both C-type and I-type proteins. I-type lysozymes are similar in size to C-type lysozymes but differ both in primary sequence and in charge (acidic/neutral vs. basic for C-type). The C-type lysozymes exhibit muramidase activity, while the I-type lysozymes have diverged to fill other functional roles [36].

The lysozyme identified in this study has muramidase activity and higher identity with C-type than I-type lysozymes and thus it can be considered a C-type. The alignment and comparison between amino acid residues located in the region of the epitope from chicken egg lysozyme indicate low identity and similarity percentage. This result explains why the anti-lysozyme chicken egg antibody does not recognize the lysozyme identified in *L. obliqua*, although the two belong to the same group.

The structure of this new lysozyme was predicted by homology with an antibacterial protein from *Antheraea mylitta* [37]. The sequence identity between these two proteins is 77%. Considering this identity, the CPHmodels 3.2 server constructed the possible structure of lysozyme from *L. obliqua,* which includes α helix, 3_{10} helix, β strand, (β) turn and other secondary structures. According to Hegyi & Gerstein [38], proteins with alpha/beta folds, as in the structure obtained for the new lysozyme, tend to be enzymes.

Furthermore, the lysozyme from *Lonomia obliqua* is constituively expressed in pupae, in constrast to the moth *Hyalophora cecropia*. These results suggest that different strategies evolved in the innate defense of pupae from two closely related leptidopterans.

CONCLUSION

The complete amino acid sequence of lysozyme from the pupae of *Lonomia obliqua* was determined for the first time in this work. This enzyme acts in defence against infections, and the description of a new lysozyme contributes to increased knowledge about these important antimicrobial molecules.

ACKNOWLEDGEMENTS

This work was supported by FAPESP, Fundap and CNPq.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interest.

REFERENCES

1. Chernysh, S., Kim, S. I., Bekker, G., Pleskach, V. A., Filatova, N. A., Anikin, V. B., Platonov, V. G. and Bulet, P. 2002, Proc. Natl. Acad. Sci. USA, 99, 12628-12632.
2. Shiotsuki, T., Bonning, B. C., Hirai, M., Kikuchi, K. and Hammock, B. D. 2000, Biosci. Biotechnol. Biochem., 64, 1681-1687.
3. Lowenberger, C., Charlet, M., Vizioli, J., Kamal, S., Richman, A., Christensen, B. M. and Bulet, P. 1999, J. Biol. Chem., 274, 2092-2097.
4. Diaz, J. H. 2005, Am. J. Trop. Med. Hyg., 72, 347-357.
5. Veiga, A. B., Pinto, A. F. and Guimarães, J. A. 2003, Thromb. Res., 111, 95-101.
6. Chudzinski-Tavassi, A. M., Alvarez-Flores, M. P., Carrijo-Carvalho, L. C. and Ricci-Silva, M. E. 2013, Toxins from *Lonomia obliqua* - recombinant production and molecular approach, In An Integrated View of the Molecular Recognition and Toxinology - From Analytical Procedures to Biomedical Applications, G. R. Baptista (Ed.), InTech, 175-206.
7. Fritzen, M., Schattner, M., Ribeiro, A. L., Batista, I. F., Ventura, J., Prezoto, B. and Chudzinski-Tavassi, A. M. 2003, Thromb. Res., 112, 105-110.
8. Pinto, A. F., Dobrovolski, R., Veiga, A. B. and Guimarães, J. A. 2004, Thromb. Res., 113, 147-154.
9. Carmo, A. C., Giovanni, D. N., Corrêa, T. P., Martins, L. M., Stocco, R. C., Suazo, C. A., Moraes, R. H., Veiga, A. B. and Mendonça, R. Z. 2012, Antiviral Res., 94, 126-130.
10. Greco, K. N., Mendonça, R. M., Moraes, R. H., Mancini, D. A. and Mendonça, R. Z. 2009, Antiviral Res., 84, 84-90.
11. Souza, A. P., Peixoto, C. C., Maranga, L., Carvalhal, A. V., Moraes, R. H., Mendonça, R. M., Pereira, C. A., Carrondo, M. J. and Mendonça, R. Z. 2005, Biotechnol. Prog., 21, 99-105.
12. Mendonça, R. Z., Greco, K. N., Sousa, A. P. B., Moraes, R. H. P., Astray, R. M. and Pereira, C. A. 2008, Cytotechnology, 57, 83-91.
13. Callewaert, L. and Michiels, C. W. 2010, J. Biosci., 35, 127-160.
14. Fleming, A. 1922, Proc. R. Soc. London, 93, 306-317.
15. Cançado, F. C., Valério, A. A., Marana, S. R. and Barbosa, J. A. R. G. 2007, J. Struct. Biol., 160, 83-92.
16. Marana, S. R., Cançado, F. C., Valério, A. A., Ferreira, C., Terra, W. R. and Barbosa, J. A. R. G. 2006, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun., 62, 750-752.
17. Powning, R. F. and Davison, W. J. 1976, Comp. Biochem. Physiol. B, 55, 221-228.
18. Vargas-Albores, F. and Ortega-Rubio, A. 1994, Tópicos de Investigación y Posgrado, 4, 21-28.
19. Sun, S. C., Asling, B. and Faye, I. 1991, J. Biol. Chem., 266, 6644-6649.
20. Stone, K. L, Gulcicek, E. E. and Willians, K. R. 1996, Enzymatic digestion of proteins in solution and in SDS polyacrylamide gels, In The Protein Protocols Handbook, J. M. Walter (Ed.), Totowa, New Jersey, 415-421.
21. Salazar-Anton, F., Tellez, A. and Lindh, J. 2012, Parasitol. Res., 110, 2187-2191.
22. Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N. and Regev, A. 2011, Nat. Biotechnol., 29, 644-52.

23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990, J. Mol. Biol., 215, 403-410.
24. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. 2007, Bioinformatics, 23, 2947-2948.
25. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. and Barton, G. J. 2009, Bioinformatics, 25, 1189-1191.
26. Nielsen, M., Lundegaard, C., Lund, O. and Petersen, T. N. 2010, Nucleic Acids Res., 38, W576-W581.
27. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. and Bairoch, A. 2005, Identification and Analysis Tools on the ExPASy Server, In The Proteomics Protocols Handbook, J. M. Walker (Ed.), Totowa, New Jersey, 571-607.
28. Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. 2004, J. Mol. Biol., 340, 783-795.
29. Abbas, A. K., Lichtman, A. H. and Pillai, S. 2008, Imunologia Celular e Molecular. Elsevier Brasil, Rio de Janeiro, Brazil.
30. Engström, Y. 1998, Insect immune gene regulation, In Molecular mechanisms of immune responses in insects, P. T. Brey and D. Hultmark (Eds.), London, United Kingdom, 211-244.
31. Hoffmann, J. A., Reichhart, J. M. and Hetru, C. 1996, Curr. Opin. Immunol., 8, 8-13.
32. Manetti, A. G. O., Rosetto, M. and Marchini, M. 1998, Antibacterial peptides of the insect reprodutive tract, In Molecular mechanisms of immune responses in insects**,** P. T. Brey and D. Hultmark (Eds.), London, United Kingdom, 67-91.
33. Lamberty, M., Zachary, D., Lanot, R., Bordereau, C., Robert, A., Hoffmann, J. A. and Bulet, P. 2001, J. Biol. Chem., 276, 4085-4092.
34. Hultmark, D. 1996, Insect lysozymes, In Lysozymes: Model Enzymes Biochemistry and Biology, P. Jolles (Ed.), Birkhaüser Verlag, Basel, 87-102.
35. Herreweghe, J. M. V. and Michiels, C. W. 2012, J. Biosci., 37, 327-348.
36. Paskewitz, S. M., Li, B. and Kajla, M. K. 2008, Insect Mol. Biol., 17, 217-225.
37. Jain, D., Nair, D. T., Swaminathan, G. J., Abraham, E. G., Nagaraju, J. and Salunke, D. M. 2001, J. Biol. Chem., 276, 41377-41382.
38. Hegyi, H. and Gerstein, M. 1999, J. Mol. Biol., 288, 147-164.