

Isolation and characterisation of a 13.8-kDa bacteriolytic enzyme from house dust mite extracts: homology with prokaryotic proteins suggests that the enzyme could be bacterially derived

Leslie T. Mathaba^a, Catherine H. Pope^a, Jason Lenzo^a, Maria Hartofilis^a,
Helen Peake^a, Robert L. Moritz^b, Richard J. Simpson^b, Andreas Bubert^c,
Philip J. Thompson^d, Geoffrey A. Stewart^{a,*}

^a Division of Inflammation and Infectious Diseases, Department of Microbiology, The University of Western Australia, Crawley 6009, Australia

^b Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research and The Walter and Eliza Hall Institute for Medical Research, Royal Melbourne Hospital, Melbourne, Vic., Australia

^c Microbiological Analytics, Frankfurter Strasse 250, Darmstadt, Germany

^d Asthma and Allergy Research Institute and Department of Medicine, The University of Western Australia, Crawley 6009, Australia

Received 5 September 2001; received in revised form 21 January 2002; accepted 23 January 2002

First published online 17 March 2002

Abstract

Bacteriolytic activity was detected in extracts of whole mite and spent growth medium (SGM) from the clinically important *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* mites and was most abundant in whole mite extract. Gram-positive organisms *Micrococcus lysodeikticus*, *Bacillus megaterium* and *Listeria monocytogenes* were preferentially lysed and the lytic activity was enhanced by thiols, destroyed by mite proteases, inhibited by HgCl₂ and high concentrations of NaCl but was resistant to heat and acid treatment. Substrate SDS–PAGE analysis indicated the presence of several lytic enzymes, two of which were isolated from *D. pteronyssinus* spent growth medium extract by hydroxyapatite chromatography. The N-terminal amino acid sequence of one of them was then used in PCR-based cloning studies. The complete amino acid sequence of this protein was determined and cDNA found to encode a 130-amino acid residue mature protein with a 20-amino acid leader sequence. The deduced protein demonstrated sequence similarity with the C-terminal regions of a group of bacterial proteins belonging to the P60 superfamily. These data suggest that the enzyme is derived from bacteria within the mites rather than from mites per se. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mite; Bacteriolysin; *Listeria monocytogenes*; *Mycobacterial* invasin; P60 superfamily; Der p 2

1. Introduction

Mites are economically and medically important arachnids, which cause food spoilage in agricultural industries, and disease in both humans and animals. With regard to spoilage, storage mites such as *Lepidoglyphus destructor*, *Glycyphagus domesticus*, *Acarus siro* and *Tyrophagus longior* are particularly significant whereas species such as the house dust mites *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Euroglyphus maynei* [1], the para-

sitic mite *Sarcoptes scabiei* [2] and the ectoparasitic mange mites *Psoroptes cuniculi* and *Psoroptes ovis* [3] are important in both human and animal disease. Because of the above associations, mites have been extensively studied and a large body of data has emerged, particularly with regard to their biochemistry and molecular biology (reviewed in [4,5]). These studies showed that mite components comprising faecal particles that are either inhaled (e.g. house dust and storage mites) or deposited in tissue (e.g. mange and scabies mites) play important roles in disease.

Others and we have shown that many proteins present in mite faecal pellets are enzymes associated with digestion, which become entrapped by a peritrophic membrane during the passage of food through the alimentary canal

* Corresponding author. Present address: Department of Microbiology, The University of Western Australia, Nedlands, WA 6907, Australia. Tel.: +61 (8) 9346 3915; Fax: +61 (8) 9346 2912.

E-mail address: geoffrey@cyllene.uwa.edu.au (G.A. Stewart).

[6]. A number of enzymes have been isolated, and include a cysteine protease [7], trypsin [8], chymotrypsin [9], a collagenase-like protease [10] and amylase [11], from *Dermatophagoides* and *Euroglyphus* species. During the course of investigations on mite proteins, bacteriolytic enzymes were also shown to be present in mite extracts [12–14]. Although the responsible enzyme(s) was not isolated, it was assumed that bacteriolytic activity was due to a type c lysozyme-like enzyme. However, in studies designed to determine the allergenicity of the lytic enzyme from dust mites, it became clear that the mite enzyme(s) possessed some properties distinct from those of previously described lysozymes. In the study described herein, we report on physicochemical properties of some of the mite lytic enzymes present in whole mite and faecal extracts and the isolation of cDNA encoding one of these bacteriolytic enzymes. The data obtained suggest that mites possess several lytic enzymes, one of which shows marked similarity to the C-terminal regions of a variety of bacterial proteins associated with invasion.

2. Materials and methods

2.1. Bacterial strains and mites and cDNA libraries

The *Listeria monocytogenes* rough mutant RIII was derived from a smooth strain of serovar 1/2a [15,16] and was kindly provided by Dr J. Potel, Institute for Medical Academy, Hannover, Germany. Lyophilised *Micrococcus lysodeikticus* was purchased from Sigma (St. Louis, MO, USA). *Bacillus megaterium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus sanguis*, *Enterococcus faecalis*, *Lactobacillus casei*, *Propionibacterium acnes*, *Corynebacterium xerosis* and *Escherichia coli* were obtained from the Department of Microbiology, The University of Western Australia (Perth, Australia) culture collection. Competent *E. coli* TOP10F' and plasmids pCR2.1-TOPO and pUC18 were purchased from Invitrogen (Carlsbad, CA, USA). A *D. pteronyssinus* cDNA library was kindly provided by Prof. Wayne Thomas, TVW Telethon Institute for Child Health Research (Perth, Australia). *D. pteronyssinus* and *D. farinae* whole mites, spent growth media and unused growth medium were kindly provided by the Commonwealth Serum Laboratories (CSL) (Parkville, Australia).

2.2. Preparation of mite extracts and general reagents

Extracts (E) of *D. pteronyssinus* and *D. farinae* were prepared from both whole mites (WM) and faecally enriched spent growth medium (SGM). Except where stated, whole mite extracts were prepared by grinding the mites in 0.01 M phosphate buffer, pH 6.2, containing 50 mM EDTA, 2 mM dithiothreitol (DTT) and 50 U ml⁻¹ aprotinin (Bayer Australia, Sydney, Australia), whereas

SGMEs were prepared by stirring the dry powder upon which the mites were raised with the above buffer. An extract of fresh mite-naïve medium (UGM) was also prepared and used as an enzyme control. General chemicals were purchased from British Drug Houses (Sydney, Australia) whereas synthetic protease substrates, DTT and hen egg white lysozyme were purchased from Sigma. Other reagents were obtained as indicated.

2.3. Spectrophotometric determination of mite bacteriolytic activity

Lytic enzyme activity was determined using a modification of the lysis of *M. lysodeikticus* assay described previously [13]. In brief, 50 µl of test sample were added to 200 µl of bacterial suspension (200 µg ml⁻¹ in 0.01 M phosphate buffer, pH 6.2, containing 500 µg ml⁻¹ bovine serum albumin (BSA; CSL) and 2 mM DTT in plastic 96-well microtitre plates. The optical density (OD) at 450 nm was determined at the beginning of the assay and the plate incubated at room temperature or 37°C. The decrease in OD was then determined and the data expressed in units, where 1 U represented the decrease in OD × 1000 min⁻¹ mg⁻¹ protein or ml⁻¹ of extract [14]. The extracts were also assessed for bacteriolytic activity in a similar fashion using the organisms described above.

2.4. Determination of mite serine protease activity

Mite trypsin, chymotrypsin and the collagenase-like enzyme activities were determined using *N*-benzoyl-L-arginine-*p*-nitroanilide (BA_pNA), succinyl-alanyl-alanyl-prolyl-phenyl-alanyl-*p*-nitroanilide (SA₂PF_pNA) and succinyl-alanyl-alanyl-prolyl-leucyl-*p*-nitroanilide (SA₂PL_pNA) respectively, using a microtitre plate assay as described previously [10,17]. Data were expressed in nmol of *p*-nitroaniline released min⁻¹ mg⁻¹ protein.

2.5. Effect of acid and heat treatment on the bacteriolytic activity in SGM

Aliquots of *D. pteronyssinus* SGMEs were titrated to pH 2.7 with concentrated hydrochloric acid and incubated in a water bath at 67°C for 15 min. The pHs of the aliquots were then adjusted to 6.2 with 1 M NaOH and any resulting precipitate removed by centrifugation. The samples were then assayed for bacteriolytic and protease activities as described above.

2.6. Effect of mite proteases on bacteriolytic activity in SGM

Aliquots of *D. pteronyssinus* SGMEs were incubated at 4°C, 20°C, 37°C or 65°C for 24 h in the presence or absence of the general serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, 13 mM final concentration) or

aprotinin (Trasylol, Bayer, Leverkusen, Germany; 50 U mg⁻¹ SGM) as described previously [18]. The bacteriolytic and protease activities in the extracts were monitored throughout the incubation period using appropriate substrates as described above.

2.7. Determination of isoelectric point of the mite bacteriolytic enzyme(s)

The isoelectric point(s) (pI) of the mite bacteriolytic enzyme(s) was determined by chromatofocusing of mite SGM at 4°C as described [11]. Appropriate fractions were collected and analysed for enzyme activity after adjusting the pH, as described above.

2.8. Determination of the protein content in mite and faecally enriched extracts

The protein concentrations of individual samples were determined using the Lowry modification of the Folin assay as described previously [19], using BSA as the standard.

2.9. Gel filtration of the mite bacteriolytic enzyme(s)

The molecular weight (mol wt) of the mite bacteriolytic enzyme(s) was determined by gel filtration as described previously [20]. Gel filtration was performed on a 1 × 52 cm column of Sephadex G-75 (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M acetate buffer, pH 4.5, and apparent mol wt determined using the standards ovalbumin (apparent mol wt 45 K), chymotrypsinogen (25.5 K) and cytochrome *c* (11.7 K) obtained from Sigma. The elution of the mite bacteriolytic enzyme(s) was monitored using the *M. lysodeikticus* assay after neutralisation.

2.10. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography

SDS-PAGE was performed as described previously [21] and gels stained with Coomassie blue R-250. Bacteriolytic activity was determined using substrate SDS-PAGE as described [22]. Samples were boiled in SDS sample buffer devoid of DTT and electrophoresed on 13% homogeneous polyacrylamide gels containing 2% (v/v) autoclaved *M. lysodeikticus* at 20 mA for 3.5 h. Following electrophoresis, the gels were incubated in deionised water for 30 min, followed by either 25 mM Tris-HCl buffer, pH 7.2, containing 1% (v/v) Triton X-100 or this buffer containing 3.2 mM DTT for 18 h at 37°C, with gentle agitation. Gels were then rinsed with deionised water and photographed by dark field or stained with 0.01% (w/v) methylene blue in 0.01% (w/v) KOH for 5 min, destained in deionised water and then photographed [23]. The apparent mol wt of the lytic bands were then determined by comparison to hen egg white lysozyme (HEWL; Sigma) or prestained

standards containing myosin (apparent mol wt 220 kDa), phosphorylase b (97.4 K), BSA (66 K), ovalbumin (46 K), carbonic anhydrase (30 K), trypsin inhibitor (21.5 K), and lysozyme (14.3 K) (Amersham Pharmacia Biotech, Sydney, Australia).

2.11. Purification of mite proteins

2.11.1. Hydroxyapatite chromatography

Heat- and acid-treated *D. pteronyssinus* SGME was titrated to pH 6.2 with acetic acid and applied to a CM-Sepharose cation ion exchange column (Pharmacia) equilibrated with 0.01 M phosphate buffer, pH 6.2. Bound protein was eluted with water adjusted to pH 11 with ammonia and subsequently adjusted to pH 6.2 for hydroxyapatite chromatography. The partially purified protein was then applied to a column of hydroxyapatite (Bio-Gel HT, Bio-Rad Laboratories, Richmond, CA, USA), equilibrated in 0.001 M NaCl at room temperature, essentially as described previously [24]. The unbound material was eluted with the same solvent and the bound material was eluted step-wise with 0.005 M MgCl₂, 1 M MgCl₂ and 0.3 M phosphate buffer, pH 6.2. Individual fractions were pooled on the basis of bacteriolytic activity and concentrated by cation ion exchange chromatography (Econo-Pac S cartridges, Bio-Rad Laboratories).

2.11.2. Affinity chromatography

The group 2 allergens from *D. pteronyssinus* and *D. farinae* were isolated from SGME by affinity chromatography as described previously [25]. The appropriate monoclonal antibodies were kindly provided by Professor M.D. Chapman, Division of Allergy and Clinical Immunology, Department of Medicine, University of Virginia (Charlottesville, VA, USA).

2.11.3. N-terminal sequence analyses and molecular mass determination of mite bacteriolytic enzymes

The N-terminal sequences of various fractions were determined after SDS-PAGE and electrotransfer from a 12.5% (w/v) polyacrylamide gel to polyvinylidene difluoride membrane as described previously [11,26]. The derived N-terminal sequences were compared with sequences in the PIR data bank of the National Biomedical Research Foundation and the Swiss Protein data bank using the Basic Local Alignment Search Tool (BLAST) [27]. The molecular mass of the 0.001 M NaCl fraction from hydroxyapatite chromatography was determined by electrospray mass spectroscopy as described previously [10].

2.12. Isolation of a cDNA coding for the 0.001 M NaCl fraction from hydroxyapatite chromatography

PCR primers were designed based upon the preferred mite codon usage data [28] and the N-terminal amino

acid sequence of the hydroxyapatite fraction eluting with 0.001 M NaCl (see Table 5). The primers GS1 (5'-AATGGTGCTGCTATTGTTTCAGCT-3') and GS2 (5'-CAAATTGGTGTTCCATATTCATGG-3') were then used in conjunction with λ gt10 forward (5'-CTTTTGAGCAAGTTCAGCCTGGTTAAG-3') or reverse (5'-GAGGTGGCTTATGAGTATTTCTTCCAGGGTA-3') primers in a PCR-based screening of a *D. pteronyssinus* cDNA library to isolate the cDNA encoding the enzyme in this fraction. The PCR products obtained were cloned into vector pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions and sequenced either at the Australian Neuromuscular Research Institute or at the Department of Clinical Immunology, Royal Perth Hospital (Perth, Australia) using universal M13 (vector) primers. Based on the sequence data obtained, three sequence-specific primers were designed: a forward primer GSUTR1 (5'-CTATTATGAAATTCTTCTTCACT-3') and two reverse primers GS2R (5'-CCATGAATATGGAACACAATTTG-3') and GSR3 (5'-TTACCAACAACGAGCAACATTAGC-3'). These new primers were then used in conjunction with each other (e.g. GSUTR1 with GS2R, and GSUTR1 with GSR3) or with λ gt10 forward and reverse primers (e.g. GSUTR1 with either λ gt10 forward or reverse primers and GS2R and GSR3 with λ gt10 forward primer). The products obtained were cloned into vector pCR2.1-TOPO and sequenced. The final, complete nucleotide sequence was translated into an amino acid sequence using the Expert Protein Analysis System (ExPaSy) Proteomics Server of the Swiss Institute of Bioinformatics (SIB) and then compared as before.

3. Results

3.1. Optimisation of bacteriolysis

Experiments were performed to determine the optimum conditions for the extraction of mite lytic activity using *D. pteronyssinus* SGM, with particular emphasis on the effects of DTT, EDTA and protease inhibitors, since preliminary experiments indicated that addition of each increased bacteriolytic activity. The data in Table 1 show that an approximately two-fold increase in activity was achieved when either DTT (2 mM) or EDTA (50 mM) was added to the extraction buffer, and an approximately six-fold increase when extractions were performed in the presence of both reagents. Lytic activity was not detected in UGM. Bacteriolytic activity in SGME was found to decline on prolonged incubation at 37°C but this loss was inhibited by both PMSF and aprotinin (Fig. 1). In the absence of inhibitor, the times taken for 50% of the activity to be lost at 37°C and 65°C (data not shown) were 62 min and 4 min, respectively. Subsequent studies showed that heat and acid treatment (65°C for 15 min at pH 2.7 followed by neutralisation) was sufficient to destroy the activities of the mite serine proteases trypsin, chymotrypsin and the collagenase-like enzyme contained therein [8–10] but not the bacteriolytic activity (Table 1), although a slight reduction in total lytic activity was observed when SGME was both acid- and heat-treated in the presence of DTT but not EDTA. Further study showed that 80% of the bacteriolytic activity was destroyed by heating for 6 min at 100°C.

Table 1
Effect of EDTA and DTT on the bacteriolytic and proteolytic activities of *D. pteronyssinus* SGME

Specific enzyme activity and treatment	Enzyme activities obtained after extraction in ^{a,b}			
	PB	PB+DTT	PB+EDTA	PB+EDTA+DTT
<i>Bacteriolytic activity</i>				
No prior treatment	418	835	1137	2644
Acid treatment	449	846	1348	2512
Heat and acid treatment	357	447	1027	2256
<i>Protease</i>				
<i>BA_pNA</i>				
No prior treatment	0.8	0.8	0.6	0.6
Acid treatment	0.0	0.1	0.1	0.1
Heat and acid treatment	0.1	0.1	0.0	0.0
<i>SA₂PF_pNA</i>				
No prior treatment	9.0	8.5	8.5	8.5
Acid treatment	1.7	2.2	2.6	0.5
Heat and acid treatment	0.0	0.0	0.0	0.0
<i>SA₂PL_pNA</i>				
No prior treatment	5.3	5.4	5.4	5.5
Acid treatment	0.4	1.4	1.4	0.2
Heat and acid treatment	0.1	0.1	0.0	0.0

^aSGM extracted in 0.01 M phosphate buffer, pH 6.2 (PB) alone or containing either 50 mM EDTA or 2 mM DTT or both.

^bEnzyme activities were determined without prior treatment of SGME or after treating SGME with heat (65°C for 15 min) and/or acid (pH 2.7 for 15 min, then neutralised). Protease activities were determined using BA_pNA, SA₂PF_pNA and SA₂PL_pNA, and data are expressed as nmol min⁻¹ mg⁻¹ protein. Lytic activity was determined using the *M. lysodeikticus* assay, and data are expressed as units of enzyme activity where 1 U = ΔOD × 1000 min⁻¹ mg⁻¹ protein.

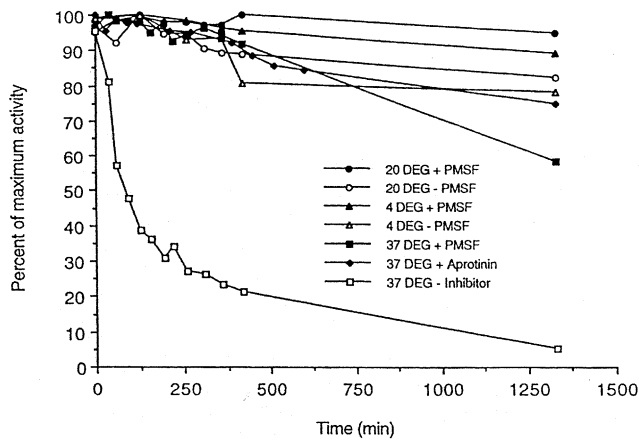


Fig. 1. The effect of mite serine proteases on bacteriolytic activity present in mite SGME. Aliquots of SGME were incubated at 4°C (▲, △), 20°C (●, ○) and 37°C (■, ◆, □) for 23.5 h in the presence (▲, ●, ■, ◆) or absence (△, ○, □) of the protease inhibitors, PMSF and aprotinin (◆). Bacteriolytic activity was determined at timed intervals.

3.2. Bacteriolytic activity against various bacterial species

Extracts of *D. pteronyssinus* and *D. farinae* were assessed for lytic activity using a range of environmental bacteria. The most susceptible species were *M. lysodeikticus*, *L. monocytogenes* and *B. megaterium* (Table 2). For each of the bacterial species, the most lytically potent extracts were obtained with WME. For example, *D. pteronyssinus* and *D. farinae* WME contained about nine- and 16-fold greater activity, respectively, than the corresponding SGME when assessed using *M. lysodeikticus*; 12- and three-fold greater activity when using *L. monocytogenes* RIII and 53- and 35-fold greater activity when using *B. megaterium*. *B. megaterium* was most susceptible to *D. farinae* extracts whereas *M. lysodeikticus* and *L. monocytogenes* RIII were most susceptible to *D. pteronyssinus* extracts. The ability of the lytic activity in the *D. pteronyssinus* SGME to cleave the long chains of cells of the *L. monocytogenes* p60-deficient RIII mutant [29] to single cells is shown in Fig. 2A,B.

Table 2
Sensitivity of various bacterial species to lysis by mite extracts

Bacterial species	Lytic activity units (U) in:			
	<i>D. pteronyssinus</i>		<i>D. farinae</i>	
	WME ^a	SGME	WME	SGME
<i>Micrococcus lysodeikticus</i>	234	26	105	7
<i>Bacillus megaterium</i>	123	4	351	7
<i>Listeria monocytogenes</i> RIII ^b	63	19	38	3
<i>Lactobacillus casei</i>	33	2	8	1
<i>Streptococcus sanguis</i>	14	4	8	1
<i>Enterococcus faecalis</i>	14	1	3	0
<i>Staphylococcus aureus</i>	44	8	24	1
<i>Staphylococcus epidermidis</i>	17	3	10	1
<i>Escherichia coli</i>	14	2	6	1

^aWME, whole mite extract.

^bp60-deficient mutant.

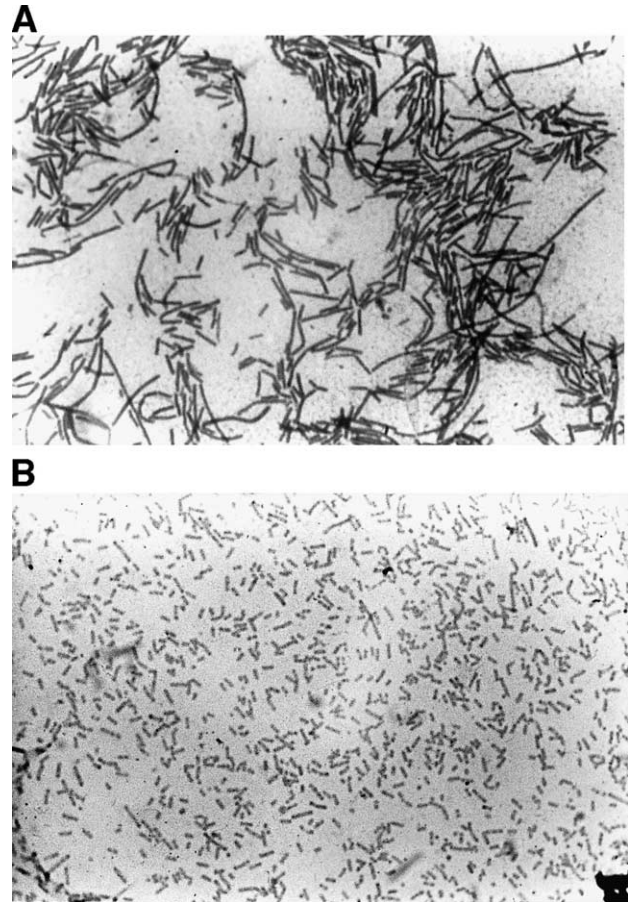


Fig. 2. Disruption of chains of cells of a deficient *L. monocytogenes* mutant by bacteriolytic activity present in mite SGME. Mutant cells were incubated at 37°C in the absence (A) and presence (B) of SGME.

3.3. Physicochemical characterisation of lytic enzymes in whole mite (WME) and SGME

3.3.1. Substrate SDS-PAGE analyses

WME and SGME from *D. pteronyssinus* and *D. farinae* were analysed by substrate SDS-PAGE to determine the spectrum of lytic enzymes present. When gels were renatured in buffer alone (Fig. 3A), lytic activity was observed within 1–2 h of incubation at 37°C. One major band (17 kDa), and 9–10 minor lytic bands (15–220 kDa) were detected in both WME and SGME of *D. pteronyssinus* and *D. farinae* after 2–8 h incubation (Fig. 3A; Table 3). When gels were renatured in buffer containing 3.2 mM DTT (Fig. 3B), at least three lytic bands were detected with apparent mol wt of 15, 17 and 20 kDa, respectively (Table 3) after 7 h incubation at 37°C. In *D. pteronyssinus* WME, the most prominent band was observed at 17 kDa whereas in the *D. farinae* WME, it was observed at 20 kDa. In both *D. pteronyssinus* and *D. farinae* SGME, the 20-kDa band was prominent, and the 17-kDa band was absent in SGME from both mite species. Further studies showed that renaturation in 100 mM KCl, >100 mM NaCl and 100 mM MgCl₂ markedly reduced lytic activity and that 10 mM HgCl₂ completely inhibited lysis (data not shown).

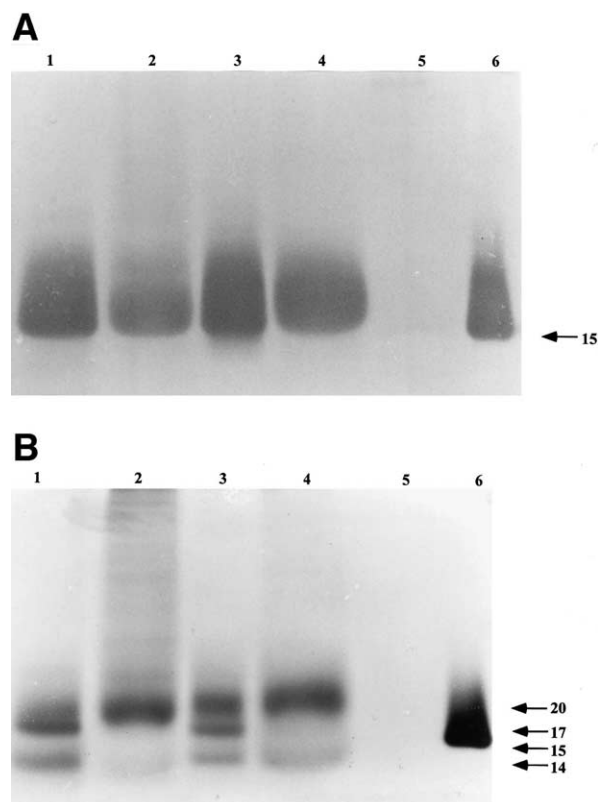


Fig. 3. Zymographic analysis of mite bacteriolytic enzymes. The mite bacteriolytic enzymes were separated by SDS-PAGE incorporating *M. lysodeikticus* as substrate. Following electrophoresis, gels were incubated in renaturation buffer without (A) and with DTT (B) overnight at 37°C. Lytic enzymes appeared as zones of clearing (indicated by arrows) within the opaque gels. The samples examined were WME of *D. pteronyssinus*, *D. farinae* (lanes 1 and 3 respectively), and SGME of *D. pteronyssinus* and *D. farinae* (lanes 2 and 4, respectively), and HEWL (lane 6). Numbers at right represent mol wts ($\times 1000$) of the lytic enzymes.

Lytic activity was not observed if samples were boiled in SDS sample buffer containing DTT.

3.3.2. Gel filtration and isoelectric focusing analyses of bacteriolytic activity

Only one major lytic peak was detected after extraction of *D. pteronyssinus* and *D. farinae* heat- or heat- and acid-

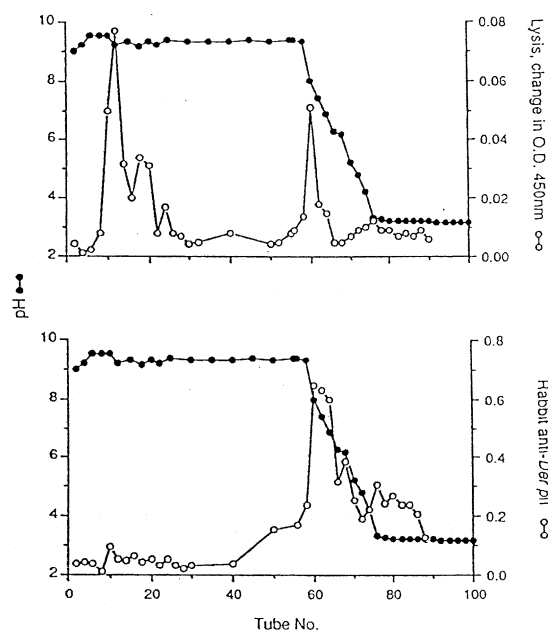


Fig. 4. Chromatofocusing analysis of mite bacteriolytic enzymes in partially purified heat- and acid-treated *D. pteronyssinus* SGMEs over the pH range 3–9 on columns (1×17 cm) of DE 53 diethylaminoethyl cellulose. ●, the elution profile of the enzymes at 280 nm; ○, lytic activity of the individual fractions.

treated SGME with EDTA and DTT as judged by G-75 gel filtration (data not shown). The apparent mol wt of the lytic peaks in each of these extracts were 12 K and 15 K, respectively. Chromatofocusing showed that several peaks of bacteriolytic activity were present in SGME from both mite species, but the pI of the majority of the bacteriolytic activity in both (approximately 80%) were in the range 6.2–>8.5 (Fig. 4), consistent with previous observations [13].

3.4. Isolation of bacteriolytic enzyme(s) by hydroxyapatite chromatography

Heat- and acid-treated SGME was partially purified by CM-Sepharose cation ion exchange chromatography and further fractionated on hydroxyapatite. Four fractions

Table 3
Presence of bacteriolytic bands in substrate SDS-PAGE gels

Mite species and apparent mol wt of lytic enzyme ($\times 1000$)	Extraction and treatment with buffer alone		Extraction and treatment with buffer+3.2 mM DTT	
	WM	SGM	WM	SGM
<i>D. pteronyssinus</i>				
15	—	—	++	+
17	++++	+++	+++	—
20	?	?	+	+++
<i>D. farinae</i>				
15	—	—	+	+
17	++++	++++	++	—
20	?	?	+++	+++

—, No visible zone of clearing; +, ++, +++, +++++, intensity of lysis, from less intense (+) to very intense (+++++); ?, not possible to determine whether a band at this position was present due to broad size of zone of clearing.

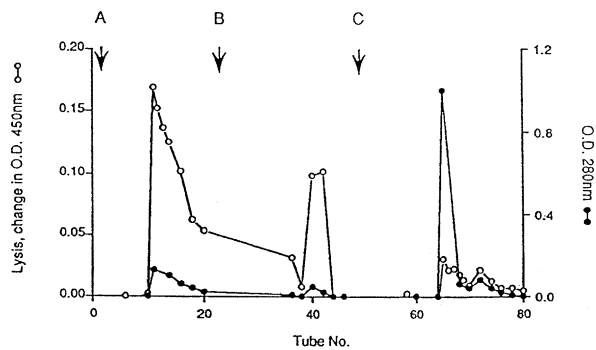


Fig. 5. Isolation of mite bacteriolytic activity from partially purified heat- and acid-treated SGME on hydroxyapatite. The column was equilibrated with 0.001 M sodium chloride and the unbound material was eluted with the same buffer (not shown). The bound material was eluted sequentially with 0.005 M (A) and 1 M (B) magnesium chloride and 0.3 M phosphate buffer, pH 6.2 (C) as indicated by the arrows.

were eluted with 0.001 M NaCl (unbound), 0.005 M MgCl_2 , 1 M MgCl_2 and 0.3 M phosphate buffer, pH 6.2 (Fig. 5). Each demonstrated lytic activity but the most potent with regard to specific and total activities were the two MgCl_2 fractions (Table 4). The bacteriolytic activity of the hydroxyapatite fractions increased after treatment with DTT, and a 13–14-fold increase was observed with the 0.001 M NaCl and 0.005 M MgCl_2 fractions as judged by the concentration required to achieve 50% maximum lytic activity (Fig. 6). The affinity-purified Der p 2 allergen did not possess lytic activity.

The mean apparent mol wt of the major bands in the 0.001 M NaCl, 0.005 M MgCl_2 , 1 M MgCl_2 and 0.3 M phosphate buffer fractions ($n=6$) were 15, 17, 16 and 17 K, respectively. The N-terminal sequences of the 0.001 M NaCl, 0.005 M MgCl_2 and 0.3 M phosphate buffer fractions were determined after pooling material from several runs and concentrating by cation exchange chromatography (Table 5). The sequence of the 0.001 M NaCl fraction was similar to that previously reported by us for a protein designated Dpt 36 [30], whereas the sequence of the material eluting with 0.005 M MgCl_2 was identical with that previously determined for the mite allergen Der p 2 [31]. The sequence of the phosphate-eluting material did not correspond to any proteins currently in the data banks.

Table 5

N-terminal sequences of proteins from hydroxyapatite fractionation of heat- and acid-treated *D. pteronyssinus* SGME

Fraction/protein	Amino acid sequence			
	1	10	20	30
0.001 M NaCl eluate	NGAAIVSAA	RSQIGVPYSW	GGGGIHGKSR	GI
Dpt 36 ^a	NGAAIVSAA	RSQIGVPY		
0.005 M MgCl_2 eluate	DQVDVKDXA	NHEIKKV		
Der p 2 ^b	DQVDVKDCA	NHEIKKVLVP	GCHGSEPCII	HRGK
0.3 M phosphate eluate	FVPXTNPSK	HVGHYIXXNQ	ERAALVQTNX	XRQPG

^aData from [30].

^bData from [31].

Table 4

Bacteriolytic activity in *D. pteronyssinus* mite fractions isolated by hydroxyapatite chromatography^a

Fraction	Total units recovered	Lytic activity (percent of total)	Specific activity (U mg^{-1})
Heat- and acid-treated SGME	ND ^b	—	20
0.001 M NaCl	17 568	25.2	36
0.005 M MgCl_2	31 824	45.6	1 896
1 M MgCl_2	10 440	15.0	22 667
0.3 M PO_4	9 888	14.2	961

^aAssay performed without reducing reagent.

^bND, not done.

3.5. Cloning and sequencing of the cDNA encoding the bacteriolytic 0.001 M NaCl-eluting enzyme from a *D. pteronyssinus* cDNA library

Using the degenerate primers (GS1 and GS2) designed from the N-terminal amino acid sequence data (Table 6) together with the universal $\lambda\text{gt}10$ reverse primer, two 450-bp PCR products were obtained and cloned into vector pCR2.1-TOPO. Sequencing of the two clones, termed pCR2.1-TOPO-A and pCR2.1-TOPO-B, produced identical partial cDNA sequences which ended at a stop codon at the 3'-end. Clone pCR2.1-TOPO-B had additional sequence data at its 5'-end. A new reverse primer (GSR3) was then designed based on the 3'-end of the new sequence data and used in a PCR with the universal $\lambda\text{gt}10$ forward primer. A 600-bp product was obtained, cloned and termed pCR2.1-TOPO-C and its DNA sequence is presented in Fig. 7 alongside the deduced amino acid sequence. Compared to pCR2.1-TOPO-B, the sequence of pCR2.1-TOPO-C was extended further upstream through the N-terminal amino acid sequence (Table 6). Sequencing of clone pCR2.1-TOPO-D, obtained using primers GSUTR1 and GS2R, confirmed the sequence upstream of the N-terminus (Table 6).

3.6. Analysis of the cDNA encoding the 0.001 M NaCl-eluting enzyme

The deduced amino acid sequence of the cDNA obtained (Fig. 7) showed that it encoded a mature protein comprising 130 amino acid residues and a 20-amino acid

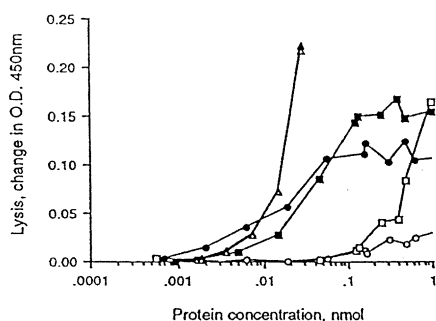


Fig. 6. The bacteriolytic activity of the hydroxyapatite fractions following treatment with DTT (■, ●). A 13–14-fold increase was observed with the 0.001 M NaCl (○) and 0.005 M MgCl₂ (□) fractions as judged by the concentration required to achieve 50% maximum lytic activity. Increases in lysozyme lytic activity due to DTT were not observed (▲, △).

leader sequence with no putative glycosylation sites. Three cysteines were found to be located at residue positions 42, 71 and 129. The calculated molecular mass of the mature protein was 13.7 kDa, which was similar to that determined using the native protein and electrospray mass spectroscopy, namely 13.8 kDa. A comparison of the deduced amino acid sequence with those available [32,33] demonstrated marked sequence similarity with the C-termini of members of the P60 family of bacterial proteins (Fig. 8).

Analysis of the deduced amino acid sequence between residues –20 and +31, to determine potential leader sequence cleavage sites, was undertaken using the SignalP program [34]. The highest scoring parameters were obtained using the eukaryote data base and two possible cleavage sites were revealed, namely between positions –5 and –4 and between positions –1 and +1. The latter

-20	GSUTR1	-10	-7	
ATG AAA TTC TTC TTC ACT	TTA GCT TTA TTC TGT ACA TTG GCT			42
M K F F F T	L A L F C T L A			
-6	-1	1	GS1	
ATC AGT CAA GTT TAT TGT AAT GGT GCC GCT ATT GTA TCG GCT				84
I S Q V Y C	N G A A I V S A			8
	GS2/GS2R			
GCA CGA TCA CAA ATT GGT GTT CCA TAT TCA TGG GGT GGT GGT				126
A R S Q I G V P Y S W G G G				22
GGT ATT CAC GGT AAA TCA CGT GGA ATA GGT GAA GGT GCC AAT				168
G I H G K S R G I				36
ACA GTT GGT TTT GAT TGT TCT GGC TTG GCA CAA TAT TCT GTT				210
T V G G F D	S G L A Q Y S V			50
TAT CAA GGT ACA CAT AAA GTA TTG GCA CGT GTT GCT TCT GGA				252
Y Q G T H K V L A R V A S G				64
CAA TAT TCC GAT CCA AAA TGT CAT CAT GTT GCA TAT GGT AGT				294
Q Y S D P K	H H V A Y G S			78
CAT CAA CCA GGT GAT TTA GTA TTT TTC GGT AAT CCT ATT CAT				336
H Q P G D L V F F G N P I H				92
CAT GTT GGT ATT GTT TCG GCA CAT GGT GGT ATG ATT AAT GCA				378
H V G I V S A H G R M I N A				106
CCA CAT ACC GGT ACA AAT GTA CGA GAA GAA AAT ATA TGG AGT				420
P H T G T N V R E E N I W S				120
	GSR3			
GAT CAT ATT GCT AAT GTT GCA CGA TGT TGG TAA				450
D H I A N V A R	W *			130

Fig. 7. Nucleotide and deduced amino acid sequences (top and bottom lines, respectively) of the 13.8-kDa bacteriolytic mite enzyme determined from cDNA. The N-terminus is underlined. The positions of the PCR primers are partially shaded in while the cysteines are fully shaded. The asterisk denotes the stop codon TAA.

corresponded to the observed N-terminus of the native protein (Fig. 7). In contrast, the Gram-positive and Gram-negative prokaryote data bases produced lower scores with possible cleavage sites between positions +8 and +9 and –5 and –4, respectively.

Table 6

Sequencing results from cloned cDNA products encoding the 13.8-kDa mite enzyme

	λgt10Forward	GS1	GS2	
N:		NC	ATVSAARS	QIGVPYSWGGGGIHGKSRGI
A:				
B:				---G-GG---KSRGI
C:				TIMKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGI
D:				TIMKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSW
	GSUTR1		GS2R	
A:	-----DCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDLVFFGNP			
B:	GEGA-TVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDLVGFNFP			
C:	GEGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDLVFFGNP			
A:	IHHVGIVSAHGRMINA			
B:	IHHVGIVSAHGRMINAPHTGTNVREENIWSDHIANVARCW			
C:	IHHVGIVSAHGRMINAPHTGTNVREENIGGDHIANVARCW			
		GSR3	λgt10Reverse	

Alignment of the N-terminus (N) of the mite protein in the 0.001 M NaCl hydroxyapatite fraction with the deduced amino acid sequences for clones pCR2.1-TOPO-A (A), pCR2.1-TOPO-B (B), pCR2.1-TOPO-C (C) and pCR2.1-TOPO-D (D) obtained using the primer combinations GS2+λgt10Reverse, λgt10Forward+GSR3 and GSUTR1+GS2R, respectively. The positions of the PCR primers relative to the amino acid sequence are partially shaded in while their direction is indicated by arrows.

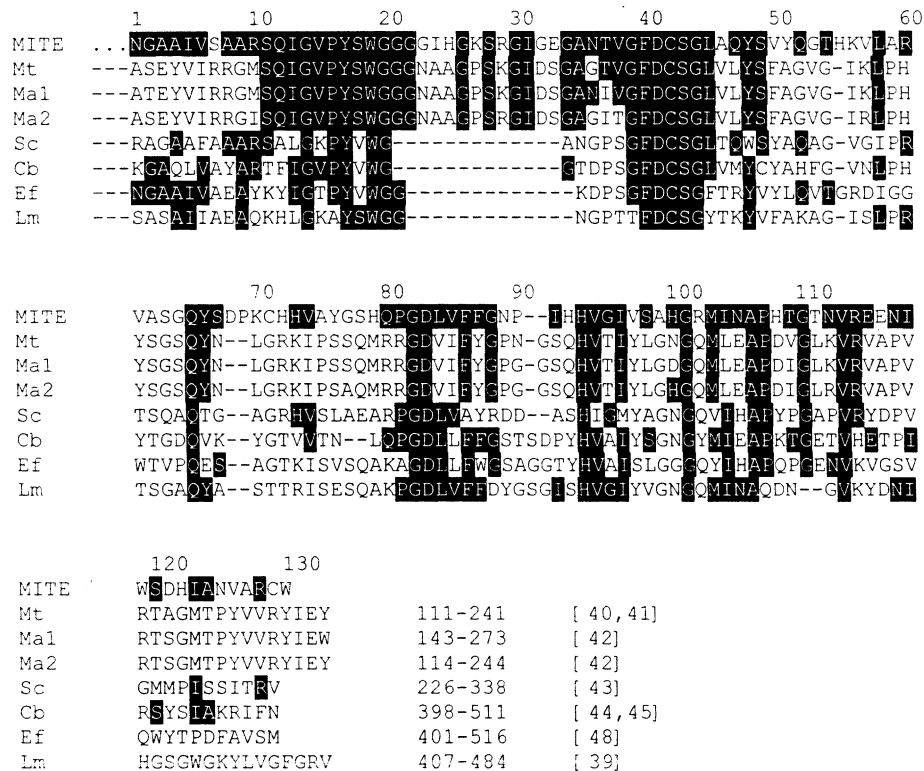


Fig. 8. Alignment of the full-length deduced amino acid sequence of the 13.8-kDa bacteriolytic mite enzyme with a sample of bacterial proteins belonging to the P60 family of proteins. Mi refers to mite lytic protein; Mt, *M. tuberculosis* hypothetical protein; Ma1, *M. avium* invasin 1; Ma2, *M. avium* invasin 2; Sc, *Streptomyces coelicolor* protein; Cb, *Clostridium beijerinckii* amylase; Ef, *Enterococcus faecium* P54 precursor protein; Lm, *L. monocytogenes* invasion-associated (p60) protein. Identical residues are fully shaded in. Upper numbers refer to mite lytic protein sequence. Numbers at right indicate the residues in the protein sharing identity or similarity with the mite protein and numbers in square brackets indicate the appropriate references. ‘–’ refer to gaps introduced to maximise homology.

4. Discussion

Data from these studies confirmed the presence of bacteriolytic activity in WME as well as SGME, suggesting that the enzyme(s) may be gut-derived. We also showed that the lytic activity was not restricted to *M. lysodeikticus* since a number of other Gram-positive bacterial species were susceptible, particularly *B. megaterium* and *L. monocytogenes*. Interestingly, *D. farinae* extracts were found to be more lytic when *B. megaterium* was used as the substrate, in contrast to extracts of *D. pteronyssinus*, which were more active using *M. lysodeikticus*. Whilst we have demonstrated lytic activity in mite extracts, the true function of these enzymes in the mite gut is unclear but they may be involved in both defence and digestion as reported for lysozymes from the gut of other eukaryotes including insects [35,36].

The bacteriolytic activity associated with mites was enhanced by reducing agents which, together with the inhibitory effects observed using HgCl_2 in zymography, suggested that a sulfhydryl group was necessary for catalysis. Interestingly, mites also possess a gut-derived cysteine protease [7], which has a similar pH optimum to that determined for the mite lytic enzyme(s), suggesting co-location within the digestive tract of the mite. In addition,

the mite lytic activity was shown to be susceptible to proteolysis, an observation which may account for the high lytic activity observed in WME compared to SGME which are relatively protease-rich compared to the former [17].

Zymography studies revealed the presence of 15-, 17- and 20-K lytic bands, in both *D. pteronyssinus* and *D. farinae*, although the prominence of the bands varied with the post-SDS–PAGE treatment. For example, with both mite species, the 17-K band was the most prominent under non-reducing conditions and appeared within 2 h following renaturation. However, the 15- and 20-K bands were the most prominent after renaturation in reducing conditions, although they became apparent only with extended incubation. These observations contrasted with those obtained in the microtitre tray assay where the bacteriolytic activities of WME, SGME and isolated fractions increased markedly when DTT was included. The reasons for this are unclear but may reflect the presence of SDS in zymography which would have the potential to facilitate protein unfolding and, therefore, enhanced susceptibility to reduction of conformationally important intrachain disulfide bonds. In support of this, we observed complete inhibition of lytic activity when DTT was included in the SDS sample buffer, and cDNA sequence data (see

below) suggest the presence of a disulfide bond in one of the lytic enzymes reported here. We also showed that EDTA enhanced, and divalent cations inhibited, lytic activity. Again the reasons for these observations are unclear but may reflect chelation of heavy metal ions or other interfering substances present in mite extracts that could inhibit sulfhydryl-dependent catalysis.

Four bacteriolytic fractions with apparent mol wt of 15, 17, 16 and 17 K were obtained by hydroxyapatite chromatography, three of which were subsequently sequenced. The N-terminal amino acid sequence obtained for the hydroxyapatite-unbound protein was found to be identical to Dpt 36, a lytic protein previously isolated from both WME and SGME of *D. pteronyssinus* and possessing allergenic properties [30]. In contrast, the N-terminal sequence of the major protein in the 0.005 M MgCl₂-eluting fraction was found to correspond to the group 2 mite allergen previously reported [31]. At present, it is not clear whether this protein was responsible for the low specific lytic activity determined. We (this study) and others [37] failed to demonstrate activity with affinity-purified Der p 2. The N-terminal amino acid sequence of the protein in the phosphate fraction did not correspond to any protein in the data banks, and the sequence of the 1.0 M MgCl₂-eluting fraction was not determined. More interestingly, the N-terminal sequence of the 0.001 M NaCl-eluting protein was found to be similar to a number of proteins belonging to the P60 superfamily. This family essentially comprises a range of bacterial proteins with diverse biological properties and include Listerial P60 proteins [38,39], invasion-associated proteins from *Mycobacteria* [40–42], a protein of unknown function from *Streptomyces coelicolor* [43], putative amylase from Clostridial species [44,45], D-glutamyl-L-diamino acid endopeptidase from *Bacillus sphaericus* [46,47], *Enterococcus faecium* P54 precursor protein [48] and the LytF hydrolase from *Bacillus subtilis* [49,50].

Several of these proteins possess bacteriolytic activity which is enhanced by thiols, for example, the Listerial P60 proteins [16], the D-glutamyl-L-diamino acid endopeptidase from *B. sphaericus* [46,47] and the LytF hydrolase from *B. subtilis* [49,50]. Each has been shown to be involved either in sporulation or in vegetative bacterial cell growth and, in this regard, an inverse link between the level of lysin synthesis and the grade of cell chain formation has been demonstrated for both *L. monocytogenes* and *S. faecalis* [16,51,52]. In this study, we showed that the 0.001 M NaCl-eluting enzyme converted the characteristic chains of the *L. monocytogenes* p60-deficient mutant into single bacterial cells with ultimate lysis, suggesting a similar mechanism of action and, as a result of the above associations, it was selected for further study using molecular techniques.

cDNA encoding this enzyme was obtained from a *D. pteronyssinus* cDNA library by PCR using oligonucleotide primers designed on the basis of the N-terminal amino

acid sequence data and the preferred mite codon usage [28]. The deduced amino acid sequence of the resulting full-length clone (pCR2.1-TOPO-C, GenBank accession number AF409109) showed that the cDNA encoded a protein comprising a mature 130-amino acid protein and a 20-amino acid leader sequence, indicating that it was a secreted protein. Three cysteine residues were present, namely one at position 42 and two at positions 71 and 129. The C₄₂ corresponded to a highly conserved cysteine residue in the C-terminal regions of most members of the P60 superfamily [32,33] (Fig. 8) and, on this basis, is likely to form part of the catalytic domain. C₇₁ and C₁₂₉ are, therefore, likely to be involved in the folding of the protein. These C-termini of members of the P60 superfamily share a domain which is often found in association with peptidoglycan binding domains in enzymes involved in bacterial cell wall degradation, whereas the N-terminal domain is responsible for anchoring the enzyme to the peptidoglycan [16,49,50,53–56]. In this regard, SignalP analysis indicated the presence of a leader sequence indicating that the protein isolated herein was the native one rather than a proteolytic cleavage product of a high mol wt protein comprising a catalytic domain and a peptidoglycan binding domain. The deduced amino acid sequence data confirmed the homology observed with the N-terminus of the native enzyme and the C-terminal regions of members of the P60 superfamily and indicate that the mite protein is the smallest member of the P60 superfamily thus far described.

The sequence data described above raise the issue of whether the lytic enzymes isolated from mite extracts were derived from bacteria cohabiting within the mites or from the mites per se since only prokaryotic sequence similarities were detected. Endosymbiotic bacteria, which play a variety of roles including the supply of essential amino acids and modulation of reproduction and digestion, have been demonstrated in some insects (e.g. *Buchnera* spp. and *Wolbachia* spp.) [57], but our knowledge of endosymbiotic bacteria in mites is limited. However, rod-shaped and coccoid bacteria have been demonstrated in the gut lumen and gut wall cells of *Histiogaster carpio* [58], in the guts of wild and laboratory-reared *D. pteronyssinus* and *D. farinae* [59], in *Psoroptes* mites [60] and in parenchymal tissue resembling insect mycetomes in soil mites [61]. However, the SignalP analysis suggested a eukaryotic leader sequence rather than a bacterial one indicating that, if the protein is shown to be mite-derived, then it is possible that it results from horizontal gene transfer from prokaryotes to eukaryotes [62].

In conclusion, we have demonstrated the presence of several bacteriolytic enzymes in both WME and SGME capable of causing the lysis of a number of environmental, Gram-positive bacteria which may form part of the mite diet or may be mite pathogens. The mite-associated enzymes were susceptible to proteolysis by endogenous proteases and were activated by reducing reagents indicating

the importance of sulphhydryl groups in catalysis. One of these enzymes showed similarities with bacterially derived proteins rather than with eukaryotic proteins. Our current investigations are, therefore, aimed at determining whether this enzyme is derived from mites per se or from endosymbiotic bacteria within the mites. Given the position of the observed homology, our data are consistent with previous studies suggesting that the known lytic activity of some of these proteins is associated with the C-terminal 110–130 amino acid residues [16]. The role of these bacteriolytic enzymes in mite biology and their mechanism of action are yet to be determined but they are likely to be involved in defence and digestion since bacteria form an important part of the mite diet. The availability of the cDNA will enable us to perform detailed analyses of the expressed recombinant protein, including a more accurate determination of its allergenicity and its precise origin.

Acknowledgements

This study was supported by the Australian Research Council Small Grants Scheme, the Australian National Health and Medical Research Council and the Asthma Foundation of WA Inc. We thank the Commonwealth Serum Laboratories for providing whole *D. pteronyssinus* and *D. farinae* mites, spent growth medium from both mite cultures and unused growth medium. We also thank Dr J. Potel, Institute for Medical Academy, Hannover, Germany, for providing *L. monocytogenes* rough mutant RIII and Professor Wayne Thomas, TVW Telethon Institute for Child Health Research, Perth, Australia, for providing the *D. pteronyssinus* cDNA library. In addition, we thank Dr Peter Yen and Mrs Jacquie Adams (Department of Microbiology, The University of Western Australia) for providing the bacterial cultures used in this study.

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