Molecular characterization of an exceptionally acidic lysozyme-like protein from the protozoon *Entamoeba histolytica*

Rosa Nickel, Thomas Jacobs[1], Matthias Leippe*

Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany

Received 1 August 1998; received in revised form 8 September 1998

Abstract The protozoan parasite *Entamoeba histolytica* contains a second antibacterial protein with lysozyme-like properties. The newly recognized bacteriolytic protein was purified from extracts of amoebic trophozoites to allow amino-terminal sequencing. Subsequent molecular cloning revealed that it is an isoform of the amoeba lysozyme described previously but also demonstrated a substantial sequence divergence of the two forms. As lysozymes typically are basic proteins, the novel amoebic protein differs markedly in having a p*I* of 4.5. There is no significant similarity of both amoeba lysozymes with any bacteriolytic protein of other organisms reported so far; however, striking sequence identity is found with predicted gene products of unknown function derived from the bacteria-feeding nematode *Caenorhabditis elegans*.

Key words: Acidic lysozyme; Antibacterial protein; *N*-Acetylmuramidase; Protozoon; *Entamoeba histolytica*

1. Introduction

Antimicrobial systems have been characterized at the molecular level primarily for vertebrates and arthropods. Despite the fact that the protozoon *Entamoeba histolytica* is a human pathogen and a dramatically active killer of host cells, it may be viewed also as an insatiable phagocytic cell that inhabits the lower intestine of man and uses bacteria of the gut as a nutrient source. The enormous capacity of the amoebae to phagocytoze bacteria [1] implies that they possess a mechanism by which bacteria can be continually engulfed and then efficiently killed and degraded. The virtually anaerobic conditions in the lower intestine preclude the possibility that amoebae, like other phagocytes, bring antimicrobial mediators such as oxygen metabolites and NO into action. Accordingly, *E. histolytica* appears to rely on an oxygen-independent principle (e.g. antibacterial peptides) to combat intracellular microbial growth within their digestive vacuoles. We were interested to analyze the molecular antimicrobial armament of this protozoon as a prototype of a primitive phagocytic cell. Until now, we have isolated from lysosome-like granular vesicles of the amoebae membrane-active peptides termed amoebapores [2] as well as a lysozyme-like protein [3]. The latter is a 23-kDa protein, the molecular mass of which and some mild amino acid sequence identity (particularly at the N-terminus) placed it among the *Chalaropsis*-type rather than among other classes of lysozyme.

*Corresponding author. Fax: (49) (40) 31182-512.
E-mail: leippe@bni.uni-hamburg.de

[1]Present address: National Research Center for Biotechnology, Mascheroder Weg 1, 38124 Braunschweig, Germany.

We hypothesized that engulfed bacteria inside the phagosomes are exposed to an array of antimicrobial factors upon fusion with the cytoplasmic granules. In a survey for additional antibacterial peptides, we detected a lysozyme-like activity apart from that displayed by the enzyme previously described [3]. Here, we report the purification of a second lysozyme-like protein from trophozoite extracts which after its N-terminal sequencing and molecular cloning turned out to be an isoform of the already known amoeba lysozyme and which has an extraordinarily acidic p*I*. Since the similarity of the second isoform to *Chalaropsis*-type lysozymes is even less prominent, it is suggested that the amoebic enzymes constitute a lysozyme class of their own.

2. Materials and methods

2.1. Cultivation and harvesting of E. histolytica

Trophozoites of the *E. histolytica* isolate HM-1:IMSS were cultured axenically in medium TYI-S-33 in plastic tissue culture flasks [4]. These amoebae do not represent cloned cells. Trophozoites from cultures in late-logarithmic phase were harvested after being chilled on ice for 10 min, sedimented at $430\times g$ for 3 min at 4°C and washed three times in ice-cold 20 mM sodium phosphate, 145 mM NaCl, pH 7.4.

2.2. Purification of amoeba lysozyme II for amino-terminal sequence analysis

Freshly harvested and washed amoebae (5×10^8 cells) were extracted overnight with 5 vol. 10% acetic acid. The extract was centrifuged at $150\,000\times g$ at 4°C for 1 h, and the resulting supernatant was adjusted with 1 M sodium acetate to pH 4.0. The material was loaded onto a Resource S cation-exchange column (6 ml; Pharmacia LKB) equilibrated with 50 mM sodium acetate, pH 4.5. Adsorbed protein was eluted by washing the column with the same buffer (40 ml) and by use of a 0–500-mM NaCl gradient (120 ml) and a final wash of 1 M NaCl. After a second passage over the same column, the flow-through containing lysozyme II was dialyzed exhaustively against the starting buffer of the next column in tubing with a molecular mass cut-off of 6000 Da (Spectra/Por 6; Spectrum Industries) and was applied to a Mono Q HR 5/5 column (Pharmacia LKB) equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed with the same buffer (5 ml) and developed with a 25-ml gradient of 0–500 mM NaCl. Active fractions were pooled and dialyzed against 1 mM sodium phophate, pH 5.8. This material was subjected to a hydroxyapatite cartridge (1 ml Econo Pac CHT II; Bio-Rad) equilibrated with the same buffer. Elution was by washing with 1 ml equilibration buffer and by use of linear gradients of 1–10 mM $MgCl_2$ (10 ml) and 1–500 mM sodium phosphate, pH 5.8 (15 ml). The active 23-kDa protein eluted with 2 mM $MgCl_2$ was subjected to SDS-PAGE and subsequent semi-dry electroblotting using a poly(vinylidene difluoride) membrane (Problott; Applied Biosystems) and analyzed by protein sequencing using a gas-phase protein sequencer (model 437 A; Applied Biosystems).

2.3. Molecular cloning

The cDNA coding for lysozyme I was radiolabelled and used to screen the HM-1:IMSS cDNA library as described [5]. The method was modified by hybridizing the radioactive probe at low stringency

PII: S0014-5793(98)01220-4

temperature of 37°C overnight. The washings were performed in 2× SSC, 0.1% SDS at 42°C for 1 h. Cross-hybridizing clones were isolated and the plasmids were released according to the instructions of the manufacturer (Stratagene). The 5′-ends of all isolated cDNAs were sequenced [6]. Only cDNAs that differed in their partial nucleotide sequence from the coding sequence of lysozyme I were completely sequenced on both strands.

2.4. Enzyme assays

For monitoring lysozyme activity during purification, column fractions were analyzed using the lysoplate technique [7]. Five-μl samples were added to wells, 4 mm in diameter, that had been punched into a plate of 0.9% agarose containing lyophilized *M. luteus* (0.5 mg/ml; Sigma) and 50 mM sodium phosphate, pH 6.0. For determination of the pH for optimal activity, the plates contained 0.1 M citric acid, 0.2 M disodium phosphate in different ratios (Geigy scientific tables) to cover a pH range from 2.2 to 7.8. The ionic strength of all buffer mixtures were equalized by adding NaCl. After incubation at 37°C for 20 h, the diameters of the cleared zones were measured. Chitinolytic activity was assayed using chitin glycol (Sigma) as a substrate [8].

2.5. Miscellaneous methods

Southern and Northern blotting were performed according to the published procedures [9]. Blots were preincubated in 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA, pH 7.2 at 50°C for 1 h and subsequently hybridized with the radiolabelled cDNA in the same solution and at the same conditions overnight. The blots were washed twice with 2× SSC, 0.1% SDS at 20°C and again at 50°C for 1 h. For sequence homology searches, the BlastP 2.0.4 program [10] was used. SDS-PAGE was performed according to Laemmli [11] with a 13% separation gel and a 4% stacking gel.

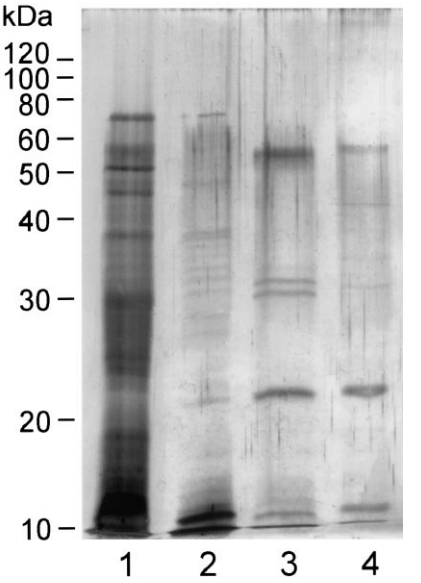

Fig. 1. Amoeba lysozyme II at different stages of purification. Aliquots (20 μl) were subjected to SDS-PAGE under reducing conditions and silver stained. Lane 1: Acidic trophozoite extract; lane 2: through-flow of the Resource S column; lane 3: Mono Q fraction; lane 4: hydroxyapatite fraction. Molecular mass standards are indicated on the left.

```
  1 CTT TTG GCT CTT GCT TCA GCC GAA TCA TAT GGT GTA GAT GTA TCT CAA CCA ACA
 -7  L   L   A   L   A   S   A   E   S   Y   G   V   D   V   S   Q   P   T

 55 TCT GCT AGT TCT ATT TCT TGC CTT AGA AAT AAT GGA TTC ACT GGA TTC TTC ATT
 12  S   A   S   S   I   S   C   L   R   N   N   G   F   T   G   F   F   I

109 TGC CGT GCT TGG TGT TCA CCA GGA TAT TTT GAT GAT AAT GCT CCA TAT ACT CTT
 30  C   R   A   W   C   S   P   G   Y   F   D   D   N   A   P   Y   T   L

163 GGA CAA GCT CAA TCT GTT GGA TTT GTT GGA GAT AAC TCA GAA GTC TAT TTC TAT
 48  G   Q   A   Q   S   V   G   F   V   G   D   N   S   E   V   Y   F   Y

217 CCA TGC CTC TCA TGT GGT AAT GCT GCT GGT CAA GTC CAA TCT TTC TGG TCA AGC
 66  P   C   L   S   C   G   N   A   A   G   Q   V   Q   S   F   W   S   S

271 GTT ATT GCA AAT CAA ATG AAA TTC AAG AGA GTT TGG TTC GAT ATT GAA GGT CAA
 84  V   I   A   N   Q   M   K   F   K   R   V   W   F   D   I   E   G   Q

325 TGG TAT TCA GAT GTT GGA TCA AAT AGA GCT TTC TTC CAA GAA CTT ATT GAT ACT
102  W   Y   S   D   V   G   S   N   R   A   F   F   Q   E   L   I   D   T

379 GCC CTT GGA ATG GGT ATT GTC TCT GGT GTT TAT TCA TCT CAA TAT TAT TGG GGA
120  A   L   G   M   G   I   V   S   G   V   Y   S   S   Q   Y   Y   W   G

433 TGC ATT TTT GGA TCT GGA TAC AGT TAT GGT CCA GCT GCT TCT CTC CCA TTA TGG
138  C   I   F   G   S   G   Y   S   Y   G   P   A   A   S   L   P   L   W

487 TAT GCT CAC TAT GAT AAT TGG GCT TCA TTC GGT GAT TTC TCT TCA TTC GGT GGA
156  Y   A   H   Y   D   N   W   A   S   F   G   D   F   S   S   F   G   G

541 TGG TCA TGG CCA ACT ATG AAA CAA TAT AGA GGA GAT GTT TCT ATT TGC TCT GCT
174  W   S   W   P   T   M   K   Q   Y   R   G   D   V   S   I   C   S   A

595 GGT GTT GAC TAT AAC TAC AGA GAA TAA GCT ACT CAT TTC TTT TT
192  G   V   D   Y   N   Y   R   E   ★
```

Fig. 2. Nucleotide and deduced amino acid sequences of lysozyme II. The N-terminal amino acid sequence obtained by protein sequencing is underlined. The asterisk represents a stop codon. These data have been deposited with the EMBL, GenBank and DDBJ sequence databanks and are available under accession number AF034843.

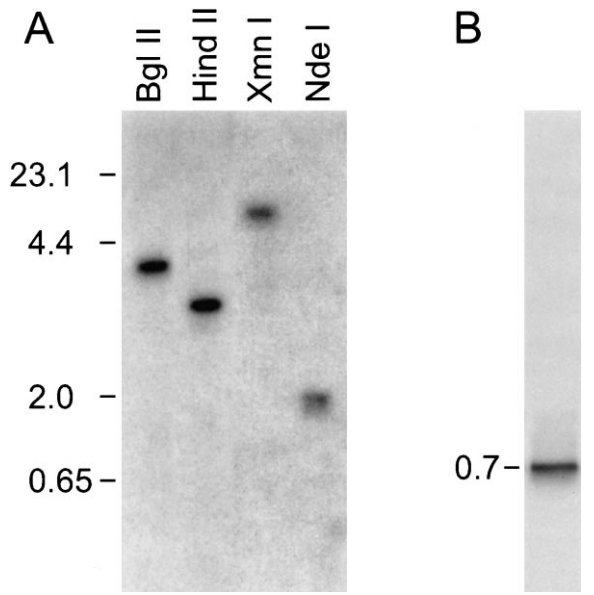

Fig. 3. Southern and Northern analysis of lysozyme II. A: Genomic DNA (10 μg) prepared from *E. histolytica* was digested with *Bgl*II, *Hin*dII, *Xmn*I and *Nde*I and hybridized with the cDNA of lysozyme II. B: Total RNA (10 μg) of *E. histolytica* was separated on a formaldehyde-agarose gel and hybridized with the cDNA of lysozyme II.

3. Results

During purification of amoeba lysozyme it became apparent that an additional lysozyme-like activity is present in amoebic extracts. A crucial step for separation of the two activities was Resource S cation-exchange chromatography. The majority of the active material bound to the resin, was eluted at 140 mM NaCl, and represented the amoeba lysozyme already known (in the following termed lysozyme I). However, active material still remained in the flow-through even after repeated subjection to the same resin indicating the existence of another lysozyme-like protein with a considerably less basic character. The active material of the flow-through was retained by a Mono Q anion-exchange column and was eluted at 120–200 mM NaCl. In activity assays, degradation of *M. luteus* cell walls was accompanied by the occurrence of a protein band of ~23 kDa. Purification to near homogeneity was achieved by adsorption to hydroxylapatite and elution with an $MgCl_2$ gradient and ascertained the molecular mass which is identical to that of lysozyme I (Fig. 1). Purification using the aforementioned scheme yielded only minute amounts of material which, however, were sufficient for N-terminal protein sequencing. The procedure gave sequence information up to residue 50 (see Fig. 2). A sequence identity of 58% with lysozyme I revealed that the purified protein is a second isoform, and consequently was named lysozyme II. In the lysoplate assay, the pH optimal for activity of lysozyme II was 3.5 in citrate/phosphate buffer at a ionic strength of 10 mS. In a parallel experiment, lysozyme I exerted optimal activity at pH 4.0. As was the case with lysozyme I [3], chitinase activity of lysozyme II could not be detected (data not shown).

We used the cDNA of lysozyme I to isolate cross-hybridizing cDNAs coding for lysozyme II. Sequencing of the longest cDNA insert of 638 bp revealed the complete coding region for lysozyme II which was preceded by a part of a putative signal peptide. The mature protein consists of 199 residues and has a calculated molecular mass of 22 136. The overall sequence identity between the isoforms is 61% and concerning amino acids with similar properties at the same position the similarity is 71%. All four cysteine residues of lysozyme I are conserved in lysozyme II suggesting two identical disulfide connectivities, but lysozyme II possesses three additional cysteine residues. The most remarkable difference between the two isoforms is their isoelectrical point explaining the different behavior on the cation exchange column. Whereas lysozyme I is – like most lysozymes – a highly basic protein (p*I* 9.5), the predicted isoelectrical point at pH 4.5 of lysozyme II is exceptionally acidic.

For Southern blot analysis, genomic DNA from trophozoites was digested with several enzymes, for which the lysozyme II gene does not possess restriction sites. Subsequent hybridization with the lysozyme II cDNA yielded single hybridization fragments in each digest varying in size between 2 and 9 kb (Fig. 3). As probing the blot with lysozyme I at the same conditions resulted in a markedly different hybridization pattern (not shown), cross-hybridization of the two lysozyme genes could be excluded. The data suggest that lysozyme II is, like lysozyme I [3], encoded by a single-copy gene. Northern blot analysis performed with total amoebic RNA revealed that the probe hybridized to an RNA of ≈650 bp, which is in agreement with the expected size of the message (Fig. 3).

A database search for homologue proteins showed no significant sequence identities to lysozyme sequences of other organisms. The highest degree of overall sequence identity was found to several functionally uncharacterized, putative gene products of *C. elegans* (Fig. 4). The putative coding sequences have been derived from the *C. elegans* genome project [12] and have been predicted by computer analysis using the program Genefinder (Green, P., unpublished) and other available information. The *C. elegans* sequences may be divided into two groups of related gene products. f58b3.1, f58b3.2, f58b3.3 and f17e9.11 are members of the first group with 67–81% sequence identities to each other. f17e9.11 is exceptional among them in that it contains an insertion of 16 amino acid residues. Identities and similarities between this group and the amoebic lysozymes are, according to Blastp 2.0.4 criteria, 30–38% and 44–58%, respectively. The second group of putative gene products are af016415, c17g10.5, y22f5a.a, y22f5a.4 and y22f5a.5 with 48–70% sequence identities to each other. The overall identities and similarities of the latter group to the amoebic lysozymes are 24–30% and 43–49%, respectively. The isoelectrical points of the aforementioned deduced amino acid sequences of *C. elegans* were predicted to be at pH 5.1–6.7, with the exception of f17e9.11, y22f5a.4 and y22f5a.5 which appear to represent basic proteins with isoelectrical points of 8.2–8.7.

4. Discussion

Lysozymes are bacteriolytic enzymes that have been found in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals [13]. In animals, they constitute key components of the antibacterial defense. Whereas many of the animals investigated appear to possess a single type of lysozyme only, multiple forms of lysozymes can be found when the protein has also been recruited as a digestive enzyme. The latter differ in some char-

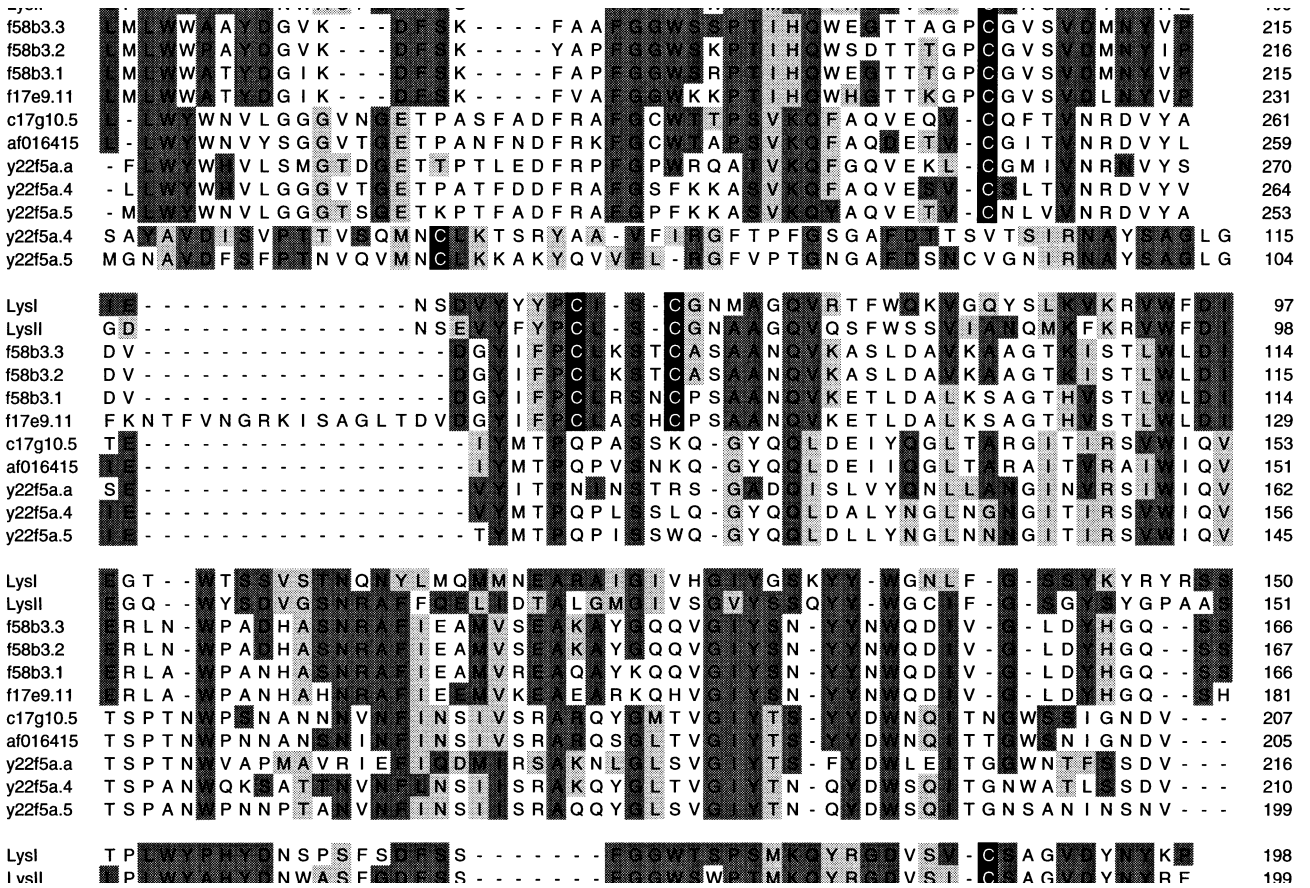

Fig. 4. Amino acid sequence homology of the amoeba lysozymes and functionally uncharacterized sequences of *C. elegans*. Residues identical in at least one amoebic lysozyme isoform and one *C. elegans* sequence are darkly shaded and conserved cysteine residues are in white letters on a black background; those with similar properties found in both species are lightly shaded. Similar amino acids were defined as follows: AG; ST; ED; RKH; QN; VILM; YF; W; P; C. Gaps introduced to maximize amino acid alignments are indicated by hyphens. Numbers refer to the positions of the residues at the end of each block in the respective protein. The EMBL/GenBank/DDBJ accession number for lysozyme I is X87610. The accession number for the *C. elegans* sequences f58b3.1, f58b3.2, f58b3.3 is Z73427. For c17g10.5, af016415 and f17e9.11, the accession numbers are U28739, AF016415 and AF047656, respectively. The amino acid sequences y22f5a.a, y22f5a.4 and y22f5a.5 share the accession number AL021479.

acteristics from conventional lysozymes of birds and mammals, e.g. they have their activity optima at low pH and are resistant to proteolytic degradation by pepsin [14]. In addition, these lysozymes are model proteins for adaptive evolution at the molecular level [14,15].

It is reasonable to assume that the amoebic lysozymes, like in other phagocytes, are constituents of an intracellular armament that prevent uncontrolled microbial growth within the digestive vacuoles and, in addition, fulfil a digestive function to exploit bacteria for nutrition. The acidic pH optima of both isoforms are in agreement with the acidic interior pH in amoebic intracellular vesicles [16]. The two isoforms of amoeba lysozyme, albeit of virtually identical molecular mass, show a substantial sequence divergence and, most strikingly, amoeba lysozyme II has an acidic p*I*. To date, the only lysozyme known to possess such an acidic p*I* (4.4) is one of eight isoforms from *Drosophila melanogaster* which is expressed exclusively in the digestive tract and hence presumably has been recruited as a digestive enzyme [17]. The second isoform described here appears to be of relatively low abundance compared to lysozyme I. Since at the moment we were capable of purifying only minute amounts of lysozyme II from the natural source, the biochemical comparison of the two isoforms has to be the subject of future studies using recombinantly expressed proteins. It is noteworthy that in older studies another amoeboid organism, *Hartmanella glebae*, was also found to contain two forms of bacteriolytic proteins, which were purified and biochemically classified as *N*-acetylmuramidases [18,19]. One of the two enzymes is produced by *Hartmanella* exclusively in response to a particular bacterium [18]. In *E. histolytica*, both lysozymes were purified from amoebae grown in the absence of bacteria and hence are continuously ex-

pressed, at least at a low level. However, in vivo, i.e. in the presence of the mixed bacterial flora of the gut, their amounts may differ.

When we reported previously the primary structure of amoeba lysozyme I, the first of protozoan origin, we suggested its membership to the lysozymes of the *Chalaropsis* type [3]. Now that we have in addition the primary structure of the second isoform, the similarity to this distinct class of lysozymes appears even weaker. Notably, only one of two acidic amino acids considered essential for the catalytic activity of *Chalaropsis* lysozyme [20], i.e. the most N-terminal aspartic acid residue, is found at the same position in both amoebic lysozymes. This residue is also conserved in the putative proteins of *C. elegans*. The second critical acidic residue involved in the catalytic action of most lysozymes may be positioned differently. Alternatively, one acidic amino acid residue inside the catalytic cleft might be sufficient for bacteriolytic activity as it is described for goose-type lysozymes [21]. If the *C. elegans* sequences are indeed coding for lysozymes, the nematode genome contains at least 9 of such genes. All these nematode products are much more similar to the amoeba lysozymes than to those of the *Chalaropsis* type. The evidence of their bacteriolytic activity would strengthen our notion that we are dealing with a novel class of lysozymes, which then would include the amoeba lysozymes and the putative proteins of the bacteria-feeding nematode.

The enzymes most likely are instrumental in rapid killing and degradation of engulfed bacteria and may act in synergy with antibacterial factors such as the amoebapores. The latter are a family of pore-forming 77-residue-peptides which are stored also in the cytoplasmic granules. These peptides are cytolytic and antibacterial by permeating the cytoplasmic membranes [22]. It is interesting to note that homologs have been found in lymphocytes of pig [23] and man [24]. Since, in addition to the lysozymes, numerous sequences of putative amoebapore-like peptides can be found within the genome of *C. elegans* upon a search in databases, it is tempting to suggest that a common bacteriolytic principle exists in such phylogenetically diverse organisms as amoebae and nematodes.

Acknowledgements: We thank Dr. Thomas Marti for protein sequencing and Ulrike Fröhlke for technical assistance. The studies were supported by the Deutsche Forschungsgemeinschaft by Grant LE 1075/2-1 and by a Heisenberg fellowship to M.L.

References

[1] Mirelman, D. (1987) Microbiol. Rev. 51, 272–284.
[2] Leippe, M., Andrä, J., Nickel, R., Tannich, E. and Müller-Eberhard, H.J. (1994) Mol. Microbiol. 14, 895–904.
[3] Jacobs, T. and Leippe, M. (1995) Eur. J. Biochem. 231, 831–838.
[4] Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) Trans. R. Soc. Trop. Med. Hyg. 72, 431–432.
[5] Tannich, E., Scholze, H., Nickel, R. and Horstmann, R.D. (1991) J. Biol. Chem. 266, 4798–4803.
[6] Sanger, F., Coulson, A.R., Barell, B.G., Smith, A.J. and Roe, B.A. (1980) J. Mol. Biol. 143, 161–178.
[7] Osserman, E.F. and Lawlor, D.P. (1966) J. Exp. Med. 124, 921–951.
[8] Yamada, H. and Imoto, T. (1981) Carbohydrate Res. 92, 160–162.
[9] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[10] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., Favello, A., Fulton, L., Gardner, A., Green, P., Hawkins, T., Hillier, L., Jier, M., Johnston, L., Jones, M., Kershaw, J., Kirtsen, J., Laister, N., Latreille, P., Lightning, J., Lloyd, C., McMurray, A., Mortimore, B., O'Callaghan, M., Parsons, J., Percy, C., Rifken, L., Roopra, A., Saunders, D., Showkeen, R., Smaldon, N., Smith, A., Soonhammer, E., Staden, R., Sulston, J., Thierry-Mieg, J., Thomas, K., Vaudin, M., Wilkinson-Sproat, J. and Wohldman, P. (1994) Nature 368, 32–38.
[13] Jollès, P. and Jollès, J. (1984) Mol. Cell. Biochem. 63, 165–189.
[14] Prager, E.M. (1996) EXS 75, 323–345.
[15] Irwin, D.M. (1996) EXS 75, 347–361.
[16] Ravdin, J.I., Schlesinger, P.H., Murphy, C.F., Gluzman, I.Y. and Krogstad, D.J. (1986) J. Protozool. 33, 478–486.
[17] Daffre, S., Kylsten, P., Samakovlis, C. and Hultmark, D. (1994) Mol. Gen. Genet. 242, 152–162.
[18] Upadhyay, J.M. and Difulco, T.J. (1972) Arch. Biochem. Biophys. 149, 470–475.
[19] Hemelt, D.M., Mares, B. and Upadhyay, J.M. (1979) Appl. Environ. Microbiol. 38, 373–378.
[20] Fouche, P.B. and Hash, J.H. (1978) J. Biol. Chem. 253, (19) 6787–6793.
[21] Weaver, L.H., Grütter, M.G. and Matthews, B.W. (1995) J. Mol. Biol. 245, 54–68.
[22] Leippe, M. (1997) Parasitol. Today 13, 178–183.
[23] Andersson, M., Gunne, H., Agerberth, B., Boman, A., Bergman, T., Sillard, R., Jörnvall, H., Mutt, V., Olsson, B., Wigzell, H., Dagerlind, A., Boman, H.G. and Gudmundsson, G.H. (1995) EMBO J. 14, 1615–1625.
[24] Peña, S.V., Hanson, D.A., Carr, B.A., Goralski, T.J. and Krensky, A.M. (1997) J. Immunol. 158, 2680–2688.