Review

Lysozymes in the animal kingdom

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Lysozymes (EC 3.2.1.17) are hydrolytic enzymes, characterized by their ability to cleave the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan, the major bacterial cell wall polymer. In the animal kingdom, three major distinct lysozyme types have been identified – the c-type (*c*hicken or *c*onventional type), the g-type (*g*oose-type) and the i-type (*i*nvertebrate type) lysozyme. Examination of the phylogenetic distribution of these lysozymes reveals that c-type lysozymes are predominantly present in the phylum of the Chordata and in different classes of the Arthropoda. Moreover, g-type lysozymes (or at least their corresponding genes) are found in members of the Chordata, as well as in some bivalve mollusks belonging to the invertebrates. In general, the latter animals are known to produce i-type lysozymes. Although the homology in primary structure for representatives of these three lysozyme types is limited, their three-dimensional structures show striking similarities. Nevertheless, some variation exists in their catalytic mechanisms and the genomic organization of their genes. Regarding their biological role, the widely recognized function of lysozymes is their contribution to antibacterial defence but, additionally, some lysozymes (belonging to different types) are known to function as digestive enzymes.

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1. Introduction

The Scottish bacteriologist Sir Alexander Fleming (1881–1955) is usually associated with the discovery of penicillin in 1928. However, already in 1921, he observed that a drop of nasal mucus, which accidently fell onto an agar plate as Fleming was suffering from a cold, caused lysis of the bacteria present on this plate. This led him to the detection of a 'remarkable bacteriolytic element', which he later called *lysozyme* (Fleming 1922). Nevertheless, lysozyme proved useless as a direct bactericidal tool against many harmful human diseases and, therefore, was initially not considered to be of great importance as an antibacterial substance. More than 80 years later, lysozyme not only serves as a model system in protein chemistry, enzymology, crystallography and molecular biology, but its contribution to antibacterial defence in animals is also widely recognized and it is used as a preservative in foods and pharmaceuticals. Additionally, many aspects concerning the biological role of lysozymes, like the impact of peptidoglycan fragments released by the lytic action of this enzyme in bacteria–host interactions, or the biological relevance of the presence of different types of lysozymes in one host, are not yet completely understood. Judging by the high number of cited publications on lysozymes nowadays, Fleming's prophecy 'We shall hear more about lysozyme,' has certainly been fulfilled.

In this review, the different types of lysozymes that occur in the animal kingdom will be presented, followed by a comparison of their catalytic mechanism, the genomic organization of their genes and, finally, a discussion on their biological role.

Keywords. Animal kingdom; biological role; catalytic mechanism;; lysozyme; phylogenetic distribution

Abbreviations used: c-type, chicken or conventional type; EPEC, enteropathogenic *E. coli*; g-type, goose type; GEWL, goose egg white lysozyme; HEWL, hen egg white lysozyme; i-type, invertebrate type; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; NOD, nucleotide-binding oligomerization domain; PCR, polymerase chain reaction; PGRP, peptidoglycan recognition protein; pI, isoelectric point; proPO, prophenoloxidase; TjL, *Tapes japonica* lysozyme; TLR, toll-like receptor

2. Different types of lysozymes and their distribution

The common feature of all lysozymes (EC 3.2.17) is their ability to hydrolyse the β-(1,4)-glycosidic bond between the alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues of peptidoglycan, a unique bacterial cell wall polymer (figure 1). Lysozymes occur in all major taxa of living organisms. In this review, we will focus on the animal kingdom, where three major distinct lysozyme types have been identified, commonly designated as the c-type (*c*hicken or *c*onventional type), the g-type (*g*oose-type) and, more recently, also the i-type (*i*nvertebrate type) lysozyme. Lysozymes belonging to these different types differ in amino acid sequences, biochemical and enzymatic properties.

The phylogenetic distribution of lysozymes, reconstructed from studies reporting on the isolation and characterization, including sequence analysis, of lysozymes from different animals, and also from an increasing number of available DNA sequences (*www.ncbi.nlm.nih.gov/*) is presented in figure 2. Where the only evidence is sequence homology, only hits with an alignment score >40 bits were included. Further, an overview of published studies providing evidence for the presence of c-, g- and i-type lysozymes in the major taxa of animals is given in tables 1, 2 and 3, respectively.

2.1 *C-type lysozymes*

The archetype lysozyme, which has served as a model for studies on enzyme structure and function, is the c-type lysozyme from hen egg white (HEWL). C-type lysozymes are the major lysozymes produced by most vertebrates, including mammals. A BLAST search reveals that all available completely sequenced mammalian genomes

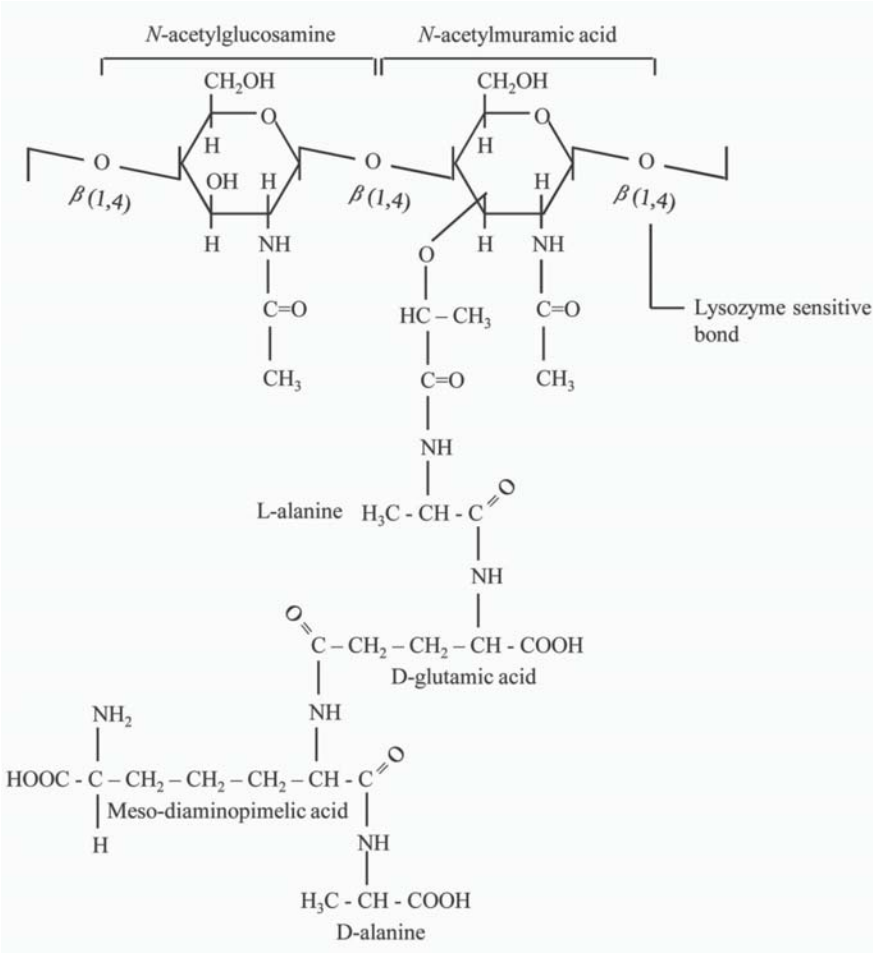

Figure 1. Structure of a repeating unit of the peptidoglycan cell wall structure and the glycan tetrapeptide. The structure given is that found in *Escherichia coli* and most other Gram-negative bacteria. Greatest variation occurs at the third amino acid of the peptide chain (mostly diaminopimelic acid in Gram-negative bacteria in contrast to L-lysine in Gram-positive bacteria) and at the interbridge, which cross-links the free amino group of diaminopimelic acid (or L-lysine) to the carboxyl group to the terminal D-alanine.

(representing five of twenty-one mammalian orders) contain at least one putative c-type lysozyme gene. Additionally, lysozyme gene and/or amino acid sequences from species belonging to these and four other orders of mammals are known and available (*www.ncbi.nlm.nih.gov/*). For seven of these orders of mammals, studies on lysozymes have been published (table 1). Human lysozyme was the first mammalian lysozyme to be sequenced and, along with HEWL, served as a model protein for a wide variety of studies (Peters *et al.* 1989; Prager and Jollès 1996). Besides mammals, other vertebrates such as birds, fish, reptiles and amphibians produce c-type lysozymes or at least harbour c-type lysozyme genes, but the information for the latter two groups is scanty (table 1). Apart from these reports, a BLAST search with HEWL (Genbank, December 2009) revealed the presence of c-type lysozyme homologues in some frogs and many fish species. Besides the subphylum Vertebrata, the chordates also comprise the subphyla Cephalochordata (the lancelets) and Urochordata (the tunicates). Organisms within these branches are considered to be intermediate between invertebrates and vertebrates. While Nilsen *et al.* (2003) reported that the urochordates *Ciona intestinalis* and *Oikopleura dioica* have no c-type lysozyme genes in their genome, Liu *et al.* (2006) demonstrated the existence of two copies of c-type lysozyme genes in the cephalochordate amphioxus (*Branchiostoma belcheri tsingtauense*) genome.

C-type lysozymes have also been reported in different classes of the Arthropoda phylum, namely in several species of lepidopteran, dipteran, isopteran and hemipteran insects, in arachnids and the crustaceans (table 1). Moreover, additional c-type lysozyme homologues can be retrieved in both the Coleoptera and Hymenoptera orders. This brings the spread of c-type lysozymes among insects to six out of twenty-seven known insect orders. Whether all insects have c-type lysozyme is difficult to say, but all available completely sequenced insect genomes contain at least one c-type lysozyme homologue (*www.ncbi.nlm.nih.gov/*). We

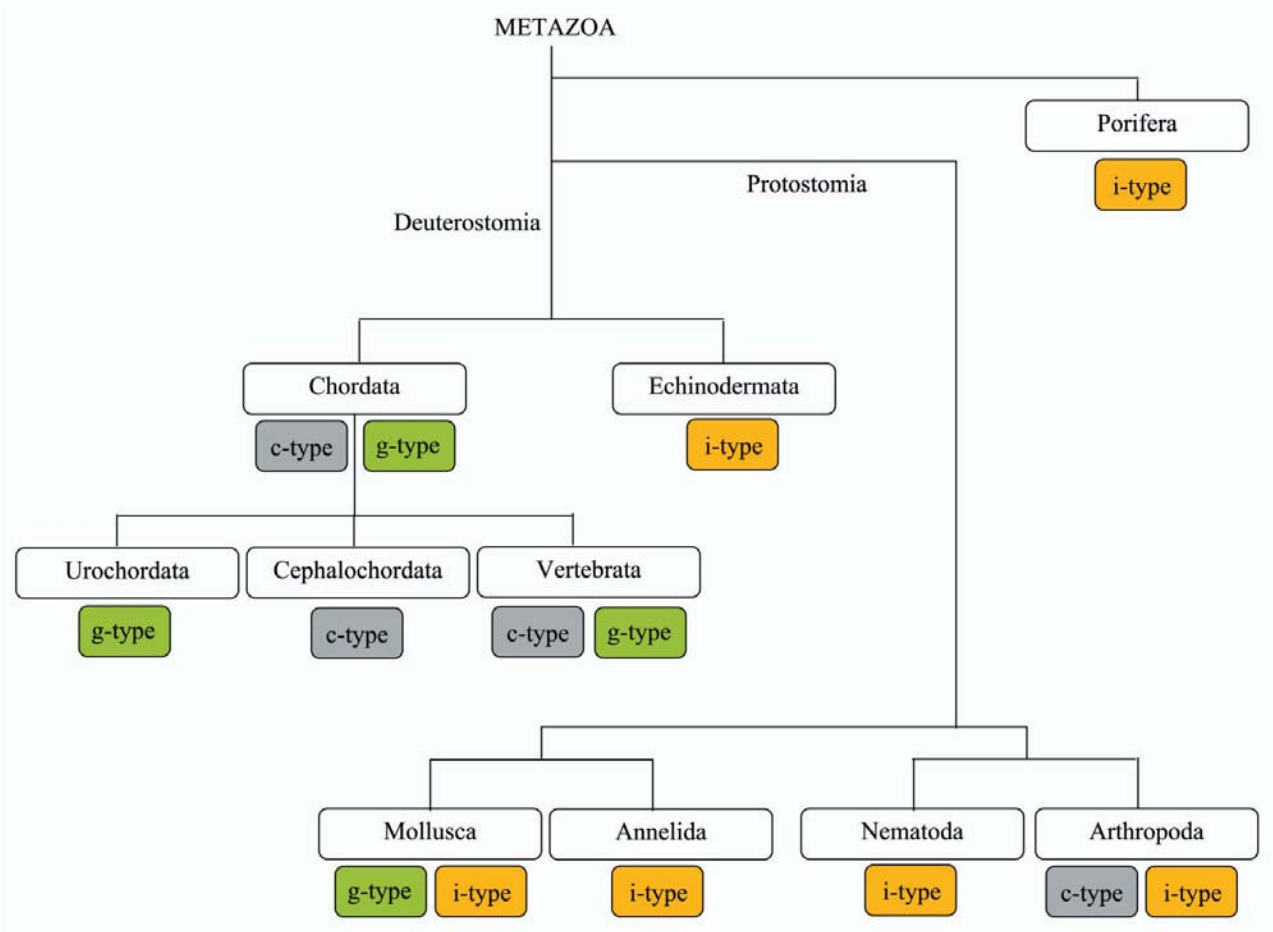

Figure 2. Distribution of different types of lysozyme in the animal kingdom. Simplified cladogram structure (based on Dunn *et al.* [2008]) only shows branches containing species where lysozyme was detected, either by available DNA sequences, or by functional studies from the literature.

Table 1. Reports of c-type lysozymes in the animal kingdom (AA sequence = amino acid sequence determination)

Class	Order	Organism	Type of identification	Reference
Phylum of Chordates Subphylum Vertebrates				
Mammals	Primates	Human	AA sequence	Peters *et al.* 1989
		Rhesus	cDNA isolation	Swanson *et al.* 1991
		African green monkey	cDNA isolation	Swanson *et al.* 1991
		Langur	cDNA isolation	Swanson *et al.* 1991
		New World monkeys	Partial DNA sequences	Singer *et al.* 2003
		Baboon	AA sequence	Hermann *et al.* 1973
	Artiodactylia	Cow	AA and/or DNA sequence	Irwin and Wilson 1989; Ito *et al.* 1993; Irwin 2004
		Sheep	cDNA isolation	Irwin and Wilson 1990
		Goat	AA sequence	Jollès *et al.* 1989; Jollès *et al.* 1990
		Deer	AA sequence	Jollès *et al.* 1989; Jollès *et al.* 1990
		Pig	DNA sequence	Yu and Irwin 1996
	Rodents	Mouse	Partial AA sequence	Hammer *et al.* 1987
		Rat	DNA sequence	Yeh *et al.* 1993
	Lagomorphs	Rabbit	AA sequence	Ito *et al.* 1990, 1994
	Carnivora	Southern elephant seal	DNA sequence	Slade *et al.* 1998
		Dog	AA sequence	Grobler *et al.* 1994
	Diprotodontia	Brush-tailed possum	cDNA isolation	Piotte *et al.* 1997
	Perissodactyla	Horse	AA sequence	McKenzie and Shaw 1985
		Ass	AA sequence	Godovac-Zimmermann *et al.* 1988
Birds		Chicken	AA sequence	Canfield 1963
		Turkey	AA sequence	Larue and Speck 1970
		Quail	AA sequence	Ibrahimi *et al.* 1979
		Pheasant	AA sequence	Araki *et al.* 1998a; Araki *et al.* 2003
		Peafowl	AA sequence	Araki *et al.* 1989
		Bobwhite	AA sequence	Prager *et al.* 1972
		Pigeon	AA sequence	Rodriguez *et al.* 1985
		Curassow	AA sequence	Araki *et al.* 2004
		Duck	AA sequence	Araki and Torikata 1999
		Hoatzin	AA sequence	Kornegay *et al.* 1994
		Lovebird	No sequence determination	Saravanan *et al.* 2009
		Chachalaca	AA sequence	Jollès *et al.* 1976
Reptiles		Turtle	AA sequence	Araki *et al.* 1998b; Thammasirirak *et al.* 2006; Siritapetawee *et al.* 2009
Amphibia		Toad	AA sequence	Zhao *et al.* 2006
Fish		Japanese flounder	DNA sequence	Hikima *et al.* 2000
		Rainbow trout	AA sequence and cDNA isolation	Dautigny *et al.* 1991

Table 1. *Continued*

		Common carp	cDNA isolation	Fujiki *et al.* 2000
		Brill	cDNA isolation	Jiménez-Cantizano *et al.* 2008
		Senegalese sole	cDNA isolation	Fernández-Trujillo *et al.* 2008
		Zebrafish	cDNA isolation	Liu and Wen 2002
Phylum of Chordates Subphylum Cephalochordata				
		Amphioxus	cDNA isolation	Liu *et al.* (2006)
Phylum of the Arthropoda				
Insect	Lepidoptera	e.g. Convolvulus hawk moth	cDNA isolation	Kim and Yoe 2003
	Diptera	e.g. *Drosophila melanogaster*	cDNA isolation	Kylsten *et al.* 1992; Daffre *et al.* 1994
	Isoptera	e.g. Wood-feeding termite	cDNA isolation	Fujita *et al.* 2002
	Hemiptera	e.g. Bug	cDNA isolation	Kollien *et al.* 2003; Araújo *et al.* 2006
Arachnids		Tick	cDNA isolation	Grunclová *et al.* 2003; Simser *et al.* 2004
		Scorpion	DNA sequence	Gantenbein and Keightley 2004
Crustaceans	Decapoda	Shrimp	cDNA isolation	Hikima *et al.* 2003; Sotelo-Mundo *et al.* 2003

also retrieved c-type lysozyme homologues in prawns in the crustacean class of Arthropoda (*www.ncbi.nlm.nih.gov/*, December 2009).

Based on the currently available genome sequences, c-type lysozymes do not seem to occur in invertebrates other than the arthropods and cephalochordates.

2.2 *G-type lysozymes*

The goose-type lysozyme owes its name to its initial identification in egg whites of the Embden goose (Canfield and McMurry 1967). Since then, it has been characterized in several avian species such as chicken, black swan, ostrich, cassowary and rhea (table 2). It is remarkable that in the eggs of some bird species, g-type is the major lysozyme, while in others it is the c-type. The dominant lysozyme reported in the egg whites from species belonging to the order of Galliformes (e.g. chicken, pheasant) is of the c-type, while in the order of Anseriformes (e.g. the black swan), c-type, g-type, or both types of lysozymes can occur in the egg whites, depending on the species. Hikima *et al.* (2001) were the first to demonstrate the occurrence of g-type lysozymes outside the class of birds. They reported a cDNA of g-type lysozyme in the Japanese flounder, and their work was soon followed by similar reports for other fish species (table 2). A database search reveals additional g-type lysozyme homologues in other vertebrates including mammals, fish and amphibians (table 2; *www.ncbi.nlm.nih.gov/*, December 2009). Although g-type lysozyme was initially considered to be restricted to vertebrates, functional g-type genes have recently been identified in invertebrates such as some bivalve mollusk scallops and in members of the tunicates (= urochordates) (table 2). Analysis of complete genome sequences available in NCBI (December 2009) shows that g-type lysozyme is absent from *Anopheles gambiae*, *Apis mellifera*, *Drosophila melanogaster*, *Drosophila pseudoobscura* (all belonging to the Arthropoda), and from *Caenorhabditis briggsae* and *Caenorhabditis elegans* (both belonging to the Nematoda).

2.3 *I-type lysozymes*

The third type of lysozyme found in the animal kingdom is designated the invertebrate type (i-type) lysozyme, and this type has recently gained an increased interest. The existence of this type of lysozyme, based on comparison of the N-terminal amino acid sequence of a lysozyme isolated from the starfish *Asterias rubens* (belonging to the Echinodermata) with the other lysozyme types, was already proposed in 1975 (Jollès and Jollès). However, the first complete amino acid sequence of an i-type lysozyme was reported only in 1999 for the marine bivalve *Tapes japonica* (TjL; Ito *et al.* 1999). Later, several complete or N-terminal sequences of i-type lysozymes from invertebrates have been determined (table 3).

Table 2. Reports of g-type lysozymes in the animal kingdom (AA sequence = amino acid sequence determination)

	Organism	Type of identification	Reference
Birds	Goose	AA sequence	Canfield and McMurry 1967; Simpson and Morgan 1983
	Black swan	AA sequence	Simpson *et al.* 1980
	Ostrich	AA sequence	Jollès *et al.* 1977; Schoentgen *et al.* 1982
	Cassowary	AA sequence	Thammasirirak *et al.* 2002
	Rhea	AA sequence	Pooart *et al.* 2004
	Chicken	cDNA isolation	Nakano and Graf 1991
Fish	Japanese flounder	cDNA isolation	Hikima *et al.* 2001
	Common carp	cDNA isolation	
	Orange-spotted grouper	cDNA isolation	Yin *et al.* 2003
	Chinese perch	cDNA isolation	Sun *et al.* 2006
	Brill	cDNA isolation	Jiménez-Cantizano *et al.* 2008
	Atlantic salmon	cDNA isolation	Kyomuhendo *et al.* 2007
	Atlantic cod	cDNA isolation	Larsen *et al.* 2009
Mammals	Human	Similarity search to chicken lysozyme g in molecular databases	Irwin and Gong 2003
	Mice	Similarity search to chicken lysozyme g in molecular databases	Irwin and Gong 2003
	Rat	Similarity search to chicken lysozyme g in molecular databases	Irwin and Gong 2003
Mollusks	Bivalve scallop	cDNA isolation	Zou *et al.* 2005; Zhao *et al.* 2007
Urochordates	*Ciona intestinalis*	cDNA (EST) isolation	Irwin and Gong 2003; Nilsen *et al.* 2003
	Oikopleura dioica	cDNA (EST) isolation	Irwin and Gong 2003; Nilsen *et al.* 2003

Destabilase (EC 3.5.1.44)[1] from the medicinal leech (an annelid), earlier picked up in a screen for homologues of the *Chlamys islandica* i-type lysozyme (Nilsen *et al.* 1999), appeared to be a member of the i-type lysozyme family too, as was confirmed by the detection of lysozyme activity upon expression of the destabilase gene in *E. coli* (Zavalova *et al.* 2000; Zavalova *et al.* 2004).

Reports of similarity searches and our own BLAST searches reveal putative i-type lysozymes in the Annelida, Echinodermata, Crustacea, Insects, Mollusca and Nematoda (Ito *et al.* 1999; Paskewitz *et al.* 2008; *www.ncbi.nlm.nih.gov/*, December 2009). In addition, we found that all completely sequenced insect genomes contain i-type lysozyme homologues, suggesting that these enzymes are widespread, if not universally present, in insects. BLAST analysis in NCBI further shows that i-type lysozyme is absent in all available vertebrate genomes (the mammals *Bos taurus*, *Canis lupus familiaris*, *Equus caballus*, *Homo sapiens*, *Macaca mulatta*, *Monodelphis domestica*, *Mus musculus*, *Ornithorhynchus anatinus*, *Pan troglodytes*, *Rattus norvegicus, Sus scrofa*; the fish *Danio rerio*; the birds *Gallus gallus* and *Taeniopygia guttata*). In summary, current knowledge confirms that i-type lysozyme occurs (at least) in the phyla of molluscs, annelids, echinoderms, nematods and arthropods.

[1]Destabilase is an enzyme that hydrolyses isopeptide bonds formed between a glutamine γ-carboxamide and a lysine ε-amino group. In the leech, destabilase has been known for a long time as the enzyme in the salivary gland secretion that prevents ingested blood from clotting (Baskova and Nikonov 1991). This is further discussed in section 5.3.

3. Catalytic mechanism of the different lysozyme types related to their primary and tertiary structures

3.1 *Amino acid sequence*

Like other c-type lysozymes, HEWL is produced with an N-terminal signal sequence for secretion (table 4;

Table 3. Reports of i-type lysozymes in the animal kingdom (AA sequence = amino acid sequence determination)

Phylum	Organism	Type of identification	Reference
Annelida	*Eisenia andrei* Earthworm	cDNA isolation	Josková *et al.* 2009
	Eisenia foetida Earthworm	N-terminal AA sequence	Ito *et al.* 1999
	Medicinal leech	Experimental evidence for lytic activity of the destabilase protein	Zavalova *et al.* 2000; Zavalova *et al.* 2004
	Nephthys hombergi	N-terminal AA sequence	Périn and Jollès 1972
Arthropoda	*Anopheles gambiae* (mosquito)	cDNA isolation	Paskewitz *et al.* 2008
Echinodermata	Sea cucumber	cDNA isolation	Cong *et al.* 2009
	Starfish	cDNA isolation and AA sequence	Bachali *et al.* 2004
Mollusks	*Tapes japonica*	cDNA isolation and AA sequence	Ito *et al.* 1999; Takeshita *et al.* 2004
	Chlamys islandica	cDNA isolation	Nilsen *et al.* 1999
	6 bivalve species, e.g. *Mytilus edulis*	cDNA isolation	Bachali *et al.* 2002
	Crassostrea gigas	cDNA isolation	Matsumoto *et al.* 2006; Itoh and Takahashi 2007
	Ostrea edulis	cDNA isolation	Matsumoto *et al.* 2006
	Crassostrea virginica	cDNA isolation	Xue *et al.* 2007
	Lunella coronata Marine conch	N-terminal AA sequence	Ito *et al.* 1999
	Purple washington clam *Saxidomus purpurata*	N-terminal AA sequence	Miyauchi *et al.* 2006
Porifera	*Suberites domuncula*	cDNA isolation	Thakur *et al.* 2005

http://www.cbs.dtu.dk/services/SignalP/). The goose egg white lysozyme (GEWL) sequence was initially deduced from purified lysozyme (Simpson and Morgan 1983), which was found as a secreted protein in bird eggs, and thus this lysozyme is also expected to have a signal peptide. With the exception of the g2 chicken lysozyme, bird and mammalian g-type lysozyme genes indeed contain predicted signal sequences for secretion. The former is thought to be secreted by the non-classical secretory pathway (SecretomeP; Nile *et al.* 2004). Most fish g-type lysozymes (except in the zebrafish, cod and the salmon), in contrast, do not have the characteristic secretion signal features at their N-terminal sequence (including a charged n-region, a hydrophobic h-region, and a polar c-region; *www.cbs.dtu.dk/services/SignalP/*), suggesting that they are not secreted from cells (Irwin and Gong 2003; Kyomuhendo *et al.* 2007; Larsen *et al.* 2009). Kyomuhendo *et al.* (2007), however, found indications for alternative splicing of the lysozyme gene in a few fish (e.g. salmon and zebrafish), and likewise, Larsen *et al.* (2009) detected a different codon usage for cod g-type lysozymes, resulting in proteins carrying or lacking a secretion signal, respectively. This could be a strategy to fulfil the need for both intracellular as well as extracellular lysozymes. Similarly, for one of the three completely known tunicate g-type lysozyme amino acid sequences, a secretion signal is predicted, while the other two seem to be produced intracellularly (Nilsen *et al.* 2003). The Zhikong scallop *Chlamys farreri* g-type lysozyme also has a signal peptide (Zhao *et al.* 2007).

cDNA sequence determination of the *Tapes japonica* i-type lysozyme gene predicted a protein of 136 amino acids (Takeshita *et al.* 2004), while the mature TjL protein consists of only 123 amino acids (Ito *et al.* 1999). The N-terminal sequence (11 amino acids) and the C-terminal sequence (2 amino acids) might be processed *in vivo*. For other i-type lysozymes, signal sequences with great variability in length and sequence are predicted (Bachali *et al.* 2002, 2004; Olsen *et al.* 2003; Itoh and Takahashi 2007; Xue *et al.* 2007; Paskewitz *et al.* 2008), and comparison of the predicted protein sequences from cDNA with the mature protein reveals, for some lysozymes, an additional region at the N-terminal end, which is thought to be cleaved off from a possibly inactive pro-form (Olsen *et al.* 2003; Bachali *et al.* 2004; Paskewitz *et al.* 2008).

The overall amino acid identities of c-, g- and i-type lysozymes are low. They amount to 24% (over 111 aligned amino acids) for HEWL and TjL, 19% (over 129 aligned residues) for HEWL and GEWL, and 16% (over 121 aligned residues) for GEWL and TjL. Table 4 summarizes some of the molecular characteristics of HEWL, GEWL and TjL. Typical g-type lysozymes are significantly larger (~20–22 kDa) than c- and i-type lysozymes (~11–15 kDa). In general, c- and g-type lysozymes are basic proteins; this is reflected in their high isoelectric point (pI) values, while i-type lysozymes

Table 4. Characteristics of representatives of the three types of animal lysozymes, namely, the number of amino acids in the mature protein, the predicted signal sequence, the predicted molecular weight (MW) and isoelectric point (pI) of the mature protein, and the number of cysteine residues (Cys) in the mature protein

Lysozyme	Amino acids (mature protein)	Signal/ processed sequence	MW (Da) (mature protein)	Predicted pI (mature protein)	Cys (mature protein)
HEWL	129	18	14 313	9.32	8
GEWL	185	-	20 373	9.53	4
TjL	123	11 + 2	13 696	9.03	14

have quite variable pIs (Xue *et al.* 2004). However, some c-type and i-type lysozymes that have evolved to function as digestive enzymes are notable exceptions. For this function, a primary requirement seems to be a reduction of the pI to a neutral or acidic value (cfr section 5.2). Nilsen *et al.* (2003) observed that g-type lysozymes in urochordates also cover a wide range of pI values (varying from 4.4 to 9.9), and suggested that this may reflect a specialization or adaptation to function in specific tissues. Similarly, some g-type lysozymes from fish have low, or at least not high, (predicted) pI values (Yin *et al.* 2003; *www.expasy.org* [data not shown]), which may be an adaptation to their intracellular location or presence in different tissues. Also, for the i-type lysozymes, a relationship between the varying pI values and different possible functions of the lysozymes has been suggested (Xue *et al.* 2004).

3.2 *Three-dimensional structure*

Although the similarity in primary structure between the three lysozyme types is limited, their three-dimensional structures show striking similarities (figure 3). HEWL was the first enzyme to have its three-dimensional structure determined by X-ray crystallography (Phillips 1966). It is divided into two domains by a deep cleft containing the active site. One domain mainly consists of the β-sheet structure, while the other domain is more helical in nature. Similarly, the tertiary fold of GEWL is also an α/β structure with a pronounced active-site cleft separating a small β-strand domain from a larger α-helical domain (Weaver *et al.* 1995). Moreover, structural comparison of TjL and HEWL led Goto *et al.* (2007) to conclude that the tertiary structure of TjL, which is characterized by six α-helices, one β-sheet and a large cleft for substrate binding, is similar to the overall structure of HEWL. The availability of the three-dimensional structures of these lysozymes paved the way for further research and hypotheses on their working mechanism.

3.3 *Catalytic mechanism*

Since the catalytic mechanism of HEWL has been most intensively studied, it is used here as a model for comparison of the working mechanisms of the different lysozymes. The crystal structure of a HEWL–$(NAG)_3$ complex (Cheetham *et al.* 1992) showed that the binding of the substrate to the enzyme positions the atoms of the target C–O bond in the vicinity of two potential catalytic groups, notably glutamic acid at position 35 (Glu35) and aspartic acid at position 52 (Asp52). The active site of HEWL consists of six subsites A, B, C, D, E and F, which bind up to six consecutive sugar residues. In this configuration, the glycosidic bond between the NAM at subsite D and the NAG at subsite E is weakened by steric distortion of the sugar ring in subsite D, and is the target for the hydrolytic cleavage. In 2001, Vocadlo *et al.* finally delivered experimental evidence for the exact working mechanism of HEWL. The hydrolysis of the β-(1,4)-glycosidic bond between NAM and NAG occurs through a double displacement reaction (figure 4). In a first step (reaction step A in figure 4), the carboxylate group of Asp52 acts as a nucleophile to form the glycosyl intermediate, which leads to an inversion of configuration. Here, Glu35 acts as a general acid donating a proton to the glycosidic oxygen, which facilitates bond cleavage. Second, this enzyme carboxylate is removed from the glycosyl–enzyme intermediate by water, again with an inversion of configuration and thereby restoring the original configuration (reaction step B in figure 4). Interactions between the acetyl groups from the hexasaccharide glycan strand with the amino acids in the long groove of HEWL are important for substrate binding, which explains the lack of HEWL enzymatic activity on deacetylated peptidoglycan (Vocadlo *et al.* 2001). The presence of acetyl groups at the C6 hydroxyl of muramoyl residues in the modified peptidoglycans of some bacteria such as *Staphylococcus aureus* (Bera *et al.* 2006), on the other hand, is thought to prevent binding in the active site by steric hindrance. The roles of Glu35 and Asp52 in the catalytic mechanism of HEWL were further investigated by mutagenesis of each residue to its corresponding amide. The Asp52Asn mutant enzyme showed some residual activity, while the Glu35Gln lysozyme was completely inactive, confirming the importance of both residues (Malcolm *et al.* 1989). Also, the conservation of both acidic residues among c-type lysozymes illustrates their functional importance (Prager 1996; Hikima *et al.* 1997, 2003; Jain *et al.* 2001; Obita *et*

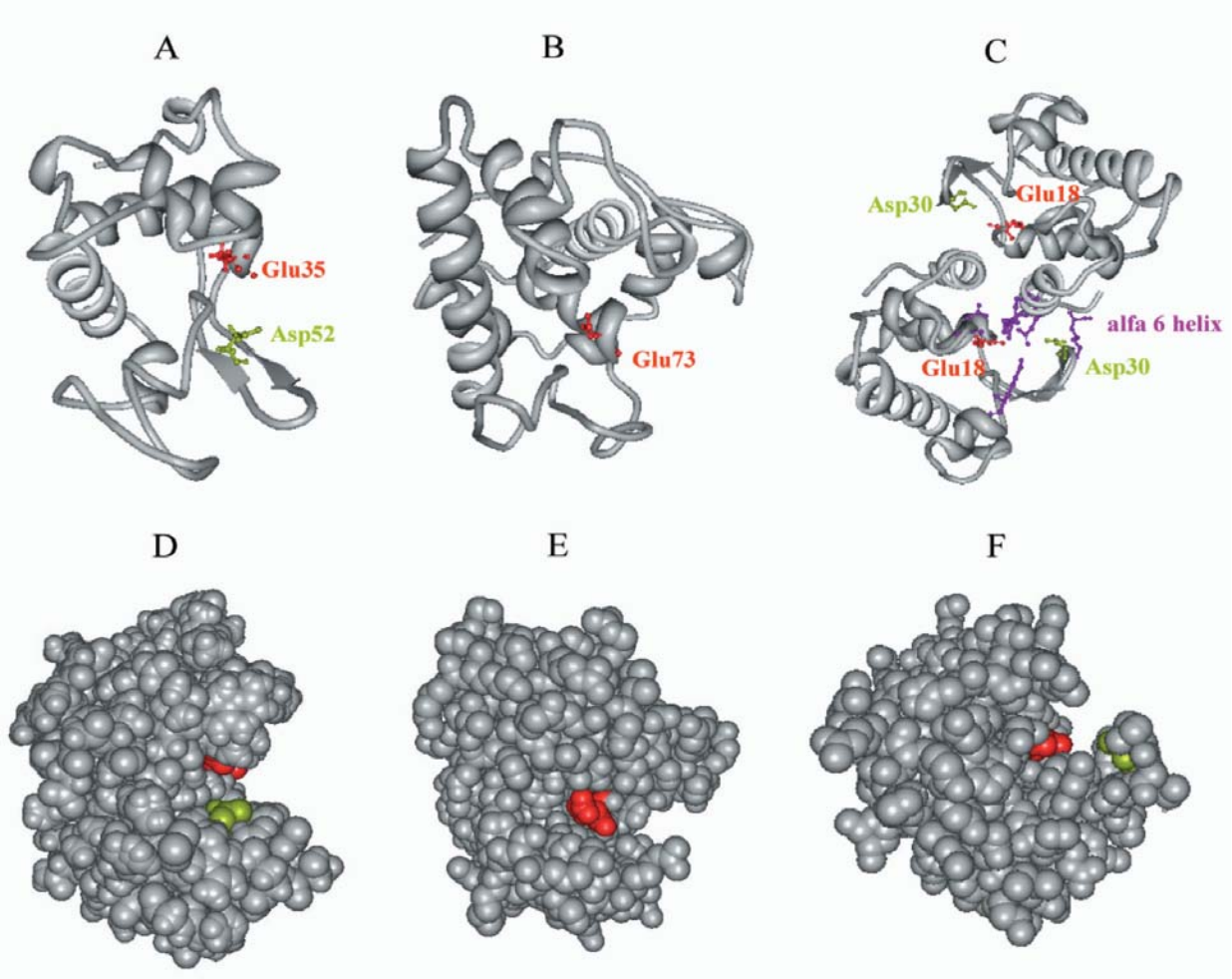

Figure 3. Ribbon diagrams of three-dimensional structure of **(A)** HEWL (Protein Data Bank, *http://www.rcsb.org/pdb/*, entry 2VB1), **(B)** GEWL (PDB, entry 153L) and **(C)** dimer of TjL (PDB, entry 2DQA), with corresponding space-filling models in **(D)**, **(E)** and **(F)** (only showing a monomer of TjL), all viewed facing into the active site. Orientation of the respective ribbon and space-filling models of each lysozyme are identical. Spirals and arrows in the ribbon diagrams represent α-helix and β-sheet structures, respectively. Catalytic residues (Glu and/or Asp) are highlighted. The residues involved in dimer formation of TjL are highlighted in purple in **(C)**.

al. 2003; Irwin 2004; Jiménez-Cantizano *et al.* 2008; Ursic-Bedoya *et al.* 2008). In this context, there is a remarkable observation that random co-polymers of phenylalanine and glutamate are able to mimic the lytic activity of lysozyme to some degree (Naithani and Dhar 1967; Robson and Marsden 1987). These polypeptides have carboxyl functions in hydrophobic as well as hydrophilic regions, and some of these were suggested to represent an equivalent for the lysozyme active site (*see above*). However, the induction of bacterial autolysis may be a more likely explanation for the effect of these co-polymers.

The refined structure of GEWL in complex with $(NAG)_3$ revealed that Glu73 of GEWL corresponds with Glu35 of HEWL, not only in an alignment of both amino acid sequences (data not shown), but also in the spatial arrangement (Weaver *et al.* 1995). This glutamic acid residue is conserved in all g-type lysozymes of birds, mammals, fish, urochordates and mollusks, except for one predicted human lysozyme and one predicted urochordate lysozyme, for which lytic activity has not yet been investigated (Nilsen *et al.* 2003; Pooart *et al.* 2004; Zhao *et al.* 2007; Zheng *et al.* 2007). Kawamura *et al.* (2006) experimentally confirmed the importance of Glu73 in the catalytic activity of g-type lysozyme of ostrich by site-directed mutagenesis.

In TjL, Glu18 appears to be the counterpart of the catalytic glutamic acid residue, both in the alignment (data not shown) and in the spatial structure of TjL in complex with $(NAG)_3$ as determined by X-ray crystallography (Goto *et al.* 2007). In other i-type lysozymes for which lytic activity was proven, this amino acid is conserved. However, this is not the case in some other predicted i-type lysozymes. Whether these enzymes are inactive, or whether this glutamic acid

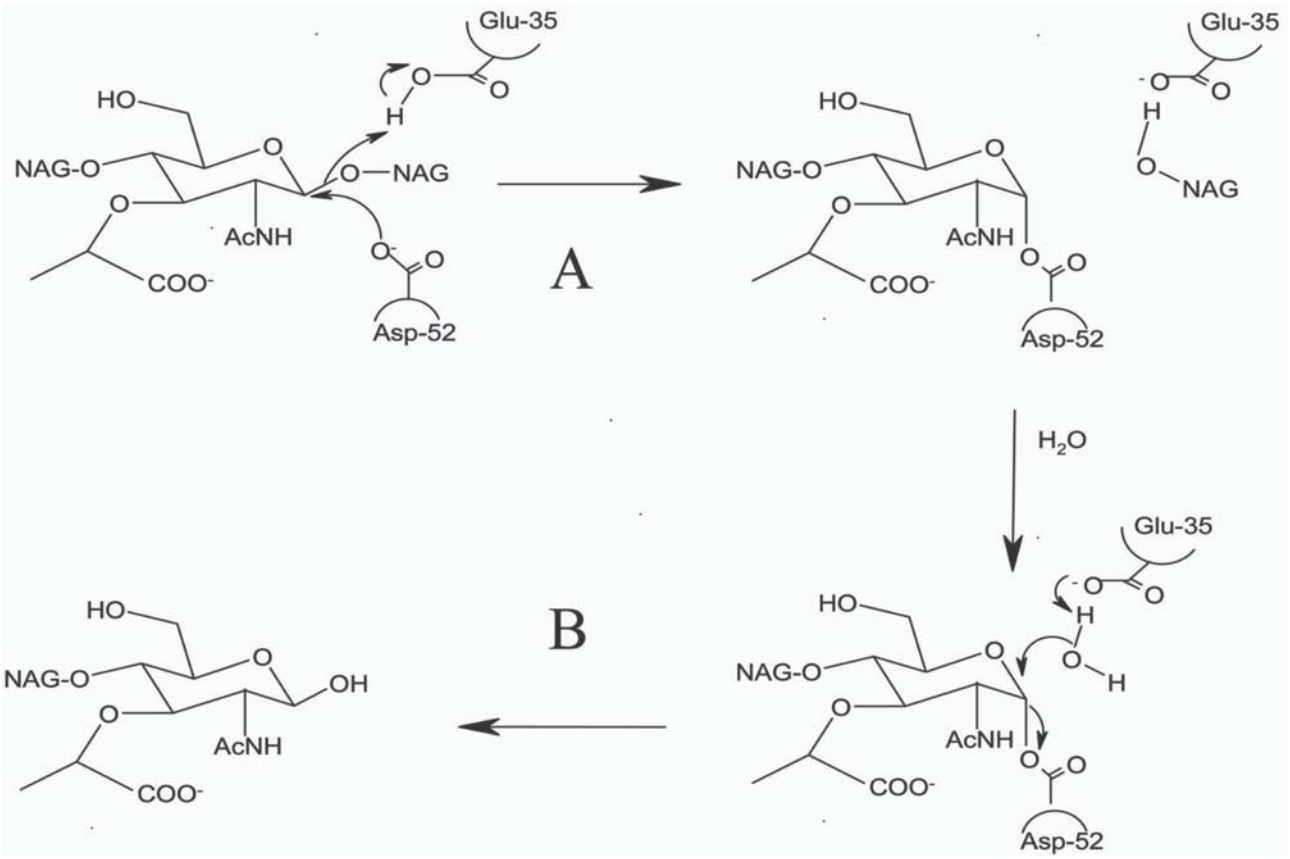

Figure 4. The double displacement catalytic mechanism of HEWL according to Kirby (2001). The mechanism, essentially consisting of two reaction steps (**A** and **B**) is discussed in detail in the text.

residue is not essential for their lysozyme activity, is not yet clear, but site-directed mutagenesis confirmed the catalytic importance of Glu18 in TjL (Goto *et al.* 2007; Paskewitz *et al.* 2008). In the spatial structure of TjL, Asp30, which is also well conserved in i-type lysozymes, is proximal to Glu18 and, therefore, was presumed to function in the same way as Asp52 in HEWL. The role of this residue in the catalytic mechanism of TjL was also confirmed by mutagenesis (Goto *et al.* 2007). However, there seems to be no obvious counterpart for Asp52 of HEWL in GEWL. Although some side-chains of GEWL might be considered as possible counterparts for the catalytic aspartate, neither of these come sufficiently close to the target glycosidic bond upon binding of GEWL with $(NAG)_3$ to anticipate a catalytic role. This raises questions on the details of the catalytic mechanism of GEWL and suggests that a second acidic residue is not essential for the catalytic activity of g-type lysozymes (Weaver *et al.* 1995). If this is the case, an inverting mechanism, rather than the retaining mechanism of HEWL, can be proposed for GEWL. In this context, Kuroki *et al.* (1999) found that this lysozyme does, in fact, change the chirality. Based on their results from molecular dynamics simulations, Hirakawa *et al.* (2008) also suggest an inverting mechanism for GEWL. Inverting enzymes may require a second carboxylate which is further away from the substrate, thereby leaving room for an attacking water molecule between the carboxylate and the substrate. According to Hirakawa *et al.* (2008), Asp97 is a good candidate for this function. Helland *et al.* (2009) examined the crystal structure of the g-type lysozyme from Atlantic cod, and their observations support the hypothesis of g-type lysozymes being inverting enzymes that use the Asp101 (counterpart of Asp97 in GEWL) as a second catalytic residue. However, this still needs further confirmation for the GEWL by site-directed mutagenesis. For TjL, in contrast, the catalytic mechanism was elucidated together with its crystal structure. According to Goto *et al.* (2007), it retains the original conformation and proceeds through a covalent sugar–enzyme intermediate, just like the above-described catalytic mechanism for HEWL (figure 4).

Another difference between the $(NAG)_3$ complexes of GEWL and HEWL is that substrate binding appears to not induce distortion of the sugar ring in subsite D in the case of GEWL (Weaver *et al.* 1995). However, this difference may not necessarily be extrapolated to the binding of a true peptidoglycan substrate, since $(NAG)_n$ polymers are very

poor substrates for g-type lysozymes when compared with $(NAM–NAG)_n$ polymers with NAM residues substituted with a peptide moiety (Jollès *et al.* 1968; Arnheim *et al.* 1973). The saccharide in subsite D is positioned deeper in the active site in HEWL than in GEWL. Weaver *et al.* (1995) suggested that the additional energy for transition from the 'stable productive' GEWL–$(NAG)_3$ complex to a fully penetrated 'reactive' complex must be provided by interactions between a peptide side chain and the enzyme. This may possibly explain the preference of GEWL for $(NAM–NAG)_n$ polymers with peptide-substituted NAM residues. In general, the lysozyme types show varying specificities for different peptidoglycan substrates, suggesting differences in their catalytic mechanisms (Nakimbugwe *et al.* 2006, together with unpublished results). As opposed to g-type lysozymes, c-type and some, but not all, i-type lysozymes also have chitinase activity, i.e. they are able to hydrolyse $(NAG)_n$ substrates (Ito *et al.* 1999; Nilsen *et al.* 1999; Xue *et al.* 2004; Miyauchi *et al.* 2006).

In terms of quaternary structure, TjL differs from HEWL and GEWL in its occurrence as a dimer under low salt conditions (Goto *et al.* 2007). The crystal structure revealed a dimer formed by the electrostatic interactions of catalytic residues (Glu18 and Asp30) and residues proximal to the active site (Asp95 and Lys42) from one molecule with residues Lys115, Lys120, Lys108 and Glu111 of an α-helix (α6) at the C terminal from the other molecule (figure 3). This dimer dissociated to monomers in a high salt environment (500 mM NaCl), presumably by disruption of the electrostatic interactions. Correspondingly, with increasing salt concentrations, chitinase activity [since the substrate here was $(NAG)_n$] increased because more active sites became available. Activities of other i-type lysozymes (the blue mussel and the oyster lysozyme) are also modulated by salt concentration (Olsen *et al.* 2003; Xue *et al.* 2004; Xue *et al.* 2007), although in different ways, depending on the lysozyme and the pH, suggesting that dimer formation by electrostatic interactions might be a common feature of (some) i-type lysozymes, and might represent a simple mode of activity modulation. However, the residues from the TjL α-helix participating in the dimer formation are not highly conserved among i-types. Also, i-type lysozymes exhibit different salt concentration sensitivities (Olsen *et al.* 2003; Xue *et al.* 2004, 2007), which may reflect variations in dimer interactions due to the observed sequence variation at the particular α-helix (Goto *et al.* 2007). Since the environment of marine bivalves is seawater, this type of modulation of activity can allow a fast conversion of lysozyme to its active form. Regarding the role of lysozyme in feeding, as well as for its potential in the antibacterial defence of the bivalve (cfr section 5), the uptake of seawater in specific tissues, either by swallowing or through injuries, will augment the salt concentration, leading to the conversion of the lysozyme from the less active dimer to the active monomer (Goto *et al.* 2007).

Finally, the presence of intramolecular disulphide bridges may also be important for the structure and the enzymatic activity of lysozymes. HEWL and other c-type lysozymes contain eight conserved cysteines (Cys) which form four disulphide bonds (table 4). Reduction of these disulphide bridges decreases the enzymatic activity (Proctor and Cunningham 1988; Touch *et al.* 2004). Within g-type lysozymes, on the other hand, there is a striking variation in Cys content. In avian g-type lysozymes, four conserved Cys residues form two intramolecular disulphide bonds in the mature proteins (Thammasirirak *et al.* 2002; Pooart *et al.* 2004; Irwin and Gong 2003). Mammalian g-type lysozymes share the same four residues but have up to three additional Cys residues, two of which are in locations that could participate in disulphide bridge formation. This would leave one free cysteine residue, which possibly forms a disulphide bridge with another g-type lysozyme or a different protein with a free cysteine residue (Irwin and Gong 2003). The g-type lysozymes found in fish, in contrast, have either no Cys, as in flounder and grouper (Hikima *et al.* 2001; Yin *et al.* 2003), one, as in carp and salmon (Savan *et al.* 2003; Kyomuhendo *et al.* 2007), or two, as in zebrafish, but with no potential to form an intramolecular disulphide bond (Irwin and Gong 2003). Invertebrate g-type lysozymes, finally, have six to thirteen Cys residues, but lack the four conserved Cys in birds and mammals (Nilsen *et al.* 2003; Zou *et al.* 2005; Zhao *et al.* 2007). The presence and location of the disulphide bonds in invertebrate g-type lysozymes have not yet been determined. The role of the disulphide bonds for g-type lysozyme activity has been documented only for ostrich egg-white lysozyme. Although neither of these bonds is crucial for the correct folding into the enzymatically active conformation, they are essential for the structural stability of this g-type lysozyme (Kawamura *et al.* 2008). High Cys content (e.g. fourteen in the TjL) is characteristic of i-type lysozymes. Goto *et al.* (2007) revealed by X-ray crystallography that all of the fourteen Cys present in TjL form disulphide bonds. Whether these are essential for the catalytic activity of this lysozyme is not yet known. The high levels of Cys residues in mollusk lysozymes (both g- and i-types) is remarkable and, by promoting a compact structure, possibly makes them more stable in the high osmolarity conditions of seawater and protects them from the proteases coexisting in the digestive organs (Ito *et al.* 1999).

3.4 *Antibacterial activity of lysozymes*

As described above, lysozymes (muramidases) exhibit their catalytic activity by cleaving the β-(1,4)-bond between the NAM and NAG residues of the bacterial peptidoglycan. This

cell wall polymer, unique to bacteria, determines the shape of the cells and provides protection against cellular turgor pressure. Loss of peptidoglycan integrity, therefore, results in rapid cell lysis in a hypo-osmotic environment. However, the peptidoglycan of Gram-negative bacteria is not directly accessible for lysozymes, because it is surrounded by a lipopolysaccharide-containing outer membrane (figure 5B, Masschalck and Michiels 2003). Nevertheless, this barrier can be breached by components of the innate immune systems of animals such as lactoferrin, defensins and cathelicidins, which permeabilize the outer membrane. Further, cell wall modifications of the glycan strands such as *N*-deacetylation, *N*-glycolylation and *O*-acetylation, or the covalent linkage of other cell-wall polymers such as teichoic acid to the peptidoglycan, have been described in several Gram-positive and Gram-negative bacteria (Zipperle *et al.* 1984; Clarke and Dupont 1992; Raymond *et al.* 2005; Bera *et al.* 2007, for extensive review *see* Vollmer 2008). *N*-deacetylation and *O*-acetylation occur frequently in pathogenic bacteria including *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Bacillus anthracis*, *S. aureus*, *Neisseria meningitidis, Campylobacter jejuni, Helicobacter pylori* (Vollmer 2008) and contribute to lysozyme resistance, at least in *L. monocytogenes* and *S. aureus* (Boneca *et al.* 2007; Bera *et al.* 2006). Possibly, these pathogens evolved mechanisms to evade lysozyme action. From this point of view, the pathogenicity of these strains argues in favour of a role of lysozyme in bacterial defence (*see* section 5.1).

A different bacterial strategy which has emerged more recently to ward off the bactericidal action of lysozyme is the production of lysozyme inhibitors (Monchois *et al.* 2001; Callewaert *et al.* 2008a, b). Evidence is accumulating that c-type lysozyme inhibitors are an important attribute of the bacterial defence against lysozyme of the animal innate immune system (Deckers *et al.* 2004; Abergel *et al.* 2007; Callewaert *et al.* 2008a,. 2009). In pathogenic bacteria they may even contribute to virulence, which would make them an attractive novel target for antibacterial drug development. In support of such a role, we found that the c-type lysozyme inhibitor Ivy is required for the ability of *E. coli* to grow in human saliva, which is naturally rich in lysozyme (Deckers *et al.* 2008). In *Salmonella typhi*, the homologue of the c-type lysozyme inhibitor *mliC* was induced in macrophages (Daigle *et al.* 2001), which are known to produce a set of antibacterial peptides including lysozyme. Furthermore, both *ivy* and *mliC* are induced by challenge with lysozyme in *E. coli* (Callewaert *et al.* 2009). However, the inhibitory spectrum of the known lysozyme inhibitor families (Ivy family and PliC/MliC family) seems restricted to the c-type lysozymes (Callewaert *et al.* 2005, together with unpublished results). From that point of view, the recent isolation and identification of bacterial g-type and i-type lysozyme inhibitors comes up to expectations (Vanderkelen *et al.* 2008; Van Herreweghe *et al.* 2010). Further research will have to point out the functional importance of these additional lysozyme inhibitors.

Besides the well known lytic activity of lysozymes based on their enzymatic activity, there is substantial evidence for a non-enzymatic bactericidal activity of HEWL (Masschalck and Michiels 2003). This non-enzymatic activity is ascribed to the activation of bacterial autolysins upon interaction of the cationic lysozyme molecule with the cell wall (Laible and Germaine 1985; Ibrahim *et al.* 2001), or to a direct interaction of lysozyme with the cell membrane resulting in membrane leakage without hydrolysis of the peptidoglycan (Ibrahim *et al.* 2001; Masschalck *et al.* 2002).

Only a few studies have addressed the antibacterial properties of arthropod c-type lysozymes but, besides the expected activity against Gram-positive bacteria, some reports (Abraham *et al.* 1995; Yu *et al.* 2002) of anti-Gram-negative activity of insect lysozymes are available. Moreover, Hikima *et al.* (2003) showed that the c-type lysozyme of kuruma shrimp (belonging to the family Crustacea) displays lytic activity against several *Vibrio* species (including a shrimp pathogen). Even though not extensively studied, Thammasirirak *et al.* (2006) reported some antimicrobial activity of c-type turtle lysozymes against *Vibrio cholerae* and very weak activity against *Pseudomonas aeruginosa*. However, the enzymes apparently lack lytic activity against *Salmonella typhi* and *Aeromonas hydrophila,* the latter being an important turtle pathogen.

A remarkable case of a c-type lysozyme with anti-Gram-negative activity is that of the rainbow trout. One of the two c-type lysozymes isolated from the kidney of this organism was surprisingly bactericidal to all seven tested strains of the five Gram-negative species *Vibrio anguillarum*, *Vibrio salmonicida*, *Aeromonas salmonicida*, *Yersinia rucken* and a *Flavobacterium* species (Grinde 1989). Amino acid sequence determination of these two lysozymes revealed that they only differ by the amino acid at position 86, where the bactericidal lysozyme has an alanine residue, in contrast to an aspartic acid in the other lysozyme (Dautigny *et al.* 1991). Karlsen *et al.* (1995), who determined the crystal structure of a mixture of the two lysozymes, suggested that the difference in bactericidal activity of the two lysozymes is probably due to subtle differences in the hydrophobicity of a small surface region.

Even though not as substantial as in the case of the rainbow trout, some other fish lysozymes such as the c-type lysozymes from ayu fish, coho salmon eggs and Japanese flounder, and the g-type lysozymes from yellow croaker, orange-spotted grouper and Japanese flounder, were reported to have antibacterial activity against Gram-negative bacteria as well (Itami *et al.* 1992; Yousif *et al.* 1994; Hikima

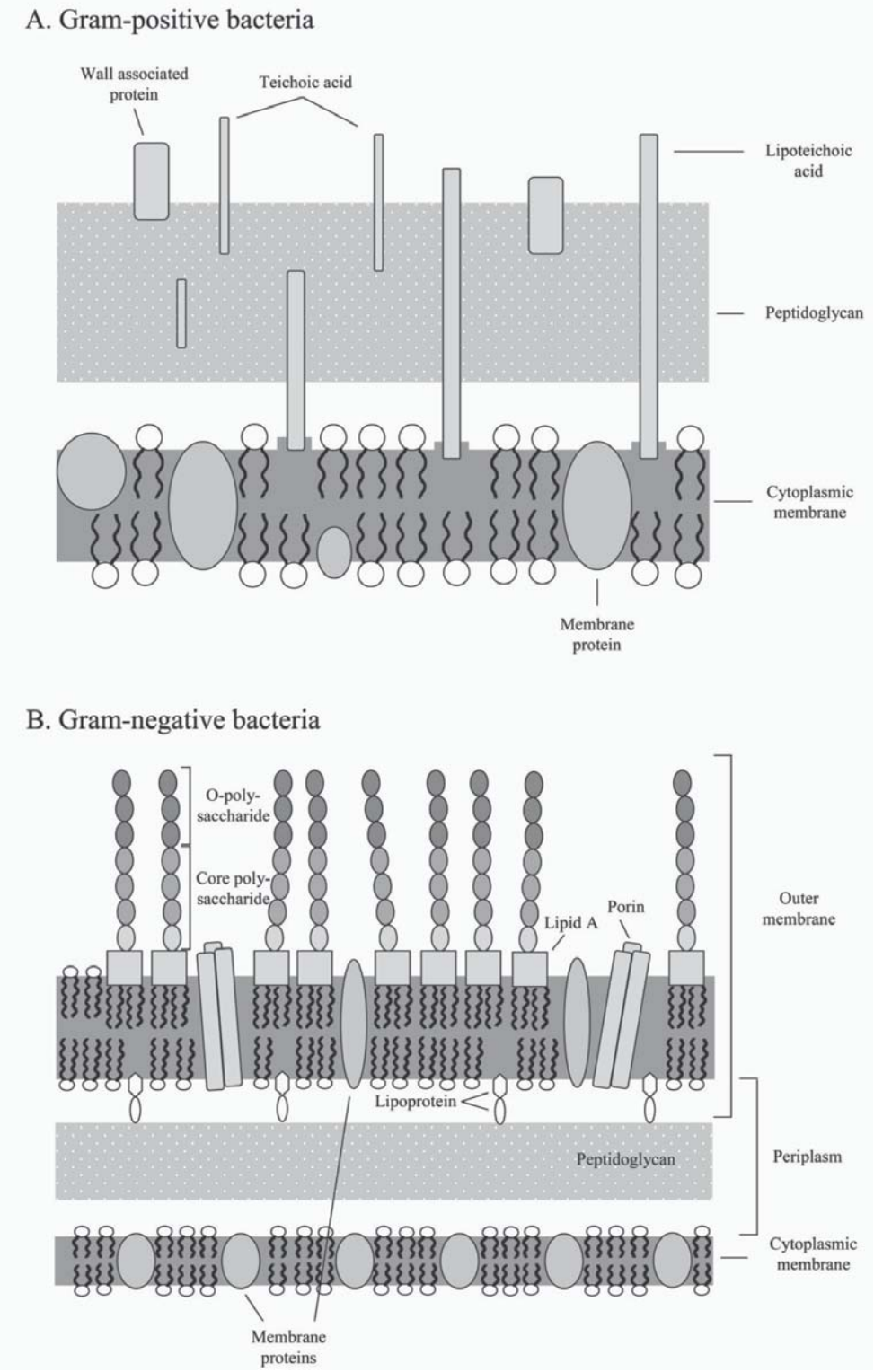

Figure 5. Cell envelope of Gram-positive **(A)** and Gram-negative **(B)** bacteria, based on Madigan *et al.* (2000).

et al. 2001; Minagawa *et al.* 2001; Zheng *et al.* 2007). Remarkably, the activity of these fish lysozymes inversely correlated with the virulence of the target bacteria for that particular species (Saurabh and Sahoo 2008). Similarly, the g-type lysozyme from the scallop *Chlamys farreri* showed, besides the obvious lytic effect on Gram-positive bacteria,

weak lytic activity against the Gram-negative bacteria *V. anguillarum*, *Vibrio splendidus* and *Vibrio parahaemolyticus* (Zhao *et al.* 2007).

Finally, some i-type lysozymes also have significant antibacterial activity against Gram-negative bacteria. The antibacterial activity of the i-type lysozyme chlamysin from a bivalve species was tested on seven strains of bacteria, representing both Gram-positive (*L. monocytogenes*, *Staphylococcus epidermidis*, *Bacillus cereus* and *Enterococcus faecalis*) and Gram-negative (*E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *V. salmonicida*) bacteria. Growth of all tested strains was completely inhibited by moderate concentrations (2–10 μM) of chlamysin (Nilsen *et al.* 1999). Xue *et al.* (2007) reported two i-type lysozymes in the eastern oyster, both of which significantly inhibited the growth of *E. coli*, *Vibrio vulnificus* and *Pediococcus cerevisiae*, but at different concentrations. Zavalova *et al.* (2006) detected high antimicrobial activity of heat-treated destabilase/lysozyme that lacked glycosidase activity towards both *Micrococcus luteus* and *E. coli*. They considered this as evidence for a non-enzymatic antibacterial mode of action of the destabilase/lysozyme from medicinal leech besides its enzymatic activity. Similarly, Cong *et al.* (2009) have very recently indicated that the sea cucumber i-type lysozyme has both enzymatic and non-enzymatic antibacterial action. Interestingly, remarkable activities of this lysozyme were observed against the pathogens *P. aeruginosa* and *V. parahaemolyticus*.

The observation that lysozymes from some animals show direct bactericidal activity against pathogens for these animals is a strong indication of the importance of this enzyme in antibacterial defence (*see* section 5.1).

4. Genomic organization and evolution of lysozyme genes

C-type lysozymes can be divided into non-calcium-binding and calcium-binding subtypes. The latter are found in some birds and mammals and are phylogenetically closely related to α-lactalbumins. Alpha-lactalbumins and c-type lysozymes share about 40% identical amino acids in their sequence, have conserved disulphide bridges and a similar intron–exon gene organization, and possess common secondary and tertiary structures (Phillips 1966; Acharya *et al.* 1991). Yet, they greatly differ in expression profile and function. In contrast to the widespread occurrence of lysozyme in different body fluids of animals (cfr section 5), α-lactalbumins are only found in mammalian milk and colostrum. Moreover, α-lactalbumin does not have catalytic activity, but it alters the specificity of galactosyl transferase, a widely distributed enzyme that catalyses the transfer of galactose units from UDP-galactose to (mainly) *N*-acetyl-D-glucosamine. The interaction of α-lactalbumin with galactosyl transferase results in a preference of the latter for D-glucose over *N*-acetyl-D-glucosamine, leading to synthesis of lactose. Gene duplication of a common ancestor and subsequent divergent evolution are thought to be the evolutionary events leading to the coexistence of lysozymes and α-lactalbumins in mammals (Qasba and Kumar 1997). Although in general α-lactalbumins do not possess lysozyme activity, and lysozymes do not interfere with lactose synthesis, a protein in the milk of an echidna (spiny anteater) was shown to function both as an α-lactalbumin (although weakly) and a lysozyme (Hopper and McKenzie 1974). This supports the general assumption that these two proteins evolved from a common ancestor.

The widespread occurrence of lysozymes among animals and the coexistence of different types of lysozymes within many taxonomic units (e.g. c- and g-type in vertebrates, c- and i-type in arthropods, g- and i-type in mollusks; figure 2) raises questions about their evolutionary relationship. The structural correspondence between the different types of animal lysozymes (cfr section 3.2) raises the possibility that these lysozymes evolved from a common precursor, but the low primary sequence identity makes this uncertain. Several hypotheses concerning the relationship between c-, g-, and i-type lysozymes have been proposed. While Grütter *et al.* (1983) and Hikima *et al.* (2003) put the c-type lysozyme forward as the common precursor of g- and i-type lysozyme, Bachali *et al.* (2002) rather believe, based on the similarity of active lysozyme domains, that c- and i-type lysozymes evolved from a common ancestor. Other authors suggest that g-type lysozyme takes a central position in the lysozyme superfamily, and accordingly propose a g-type-like common ancestor (Thunissen *et al.* 1995). Liu *et al.* (2006) constructed a phylogenetic tree of c-type, g-type and i-type lysozymes. This analysis revealed that i-type and g-type lysozymes are strongly clustered and more closely interrelated than either is to c-type, suggesting that c-type lysozyme is ancestral to i- and g-type lysozymes. As a consequence, this favours the hypothesis of c-type lysozymes being closest to the lysozyme ancestor.

In figure 6, a phylogenetic tree of c-type lysozymes (both vertebrate and invertebrate), g-type lysozymes (from birds, mammals, fish, tunicates and invertebrates), and i-type lysozymes (from nematodes, arthropods, poriferans, echinoderms, annelids and mollusks) is shown. The lysozyme sequences used for this analysis, together with their Genbank accession numbers are listed in table 5. In comparison with the tree constructed by Liu *et al.* (2006), a broader selection of sequences is included. Nevertheless, the relatedness Liu *et al.* (2006) noticed between i-type and g-type lysozymes is confirmed in this phylogenetic tree. On the other hand, the specific divergence of some of the lysozymes is not as expected by the evolutionary relatedness of the corresponding species (e.g. human g-type lysozyme

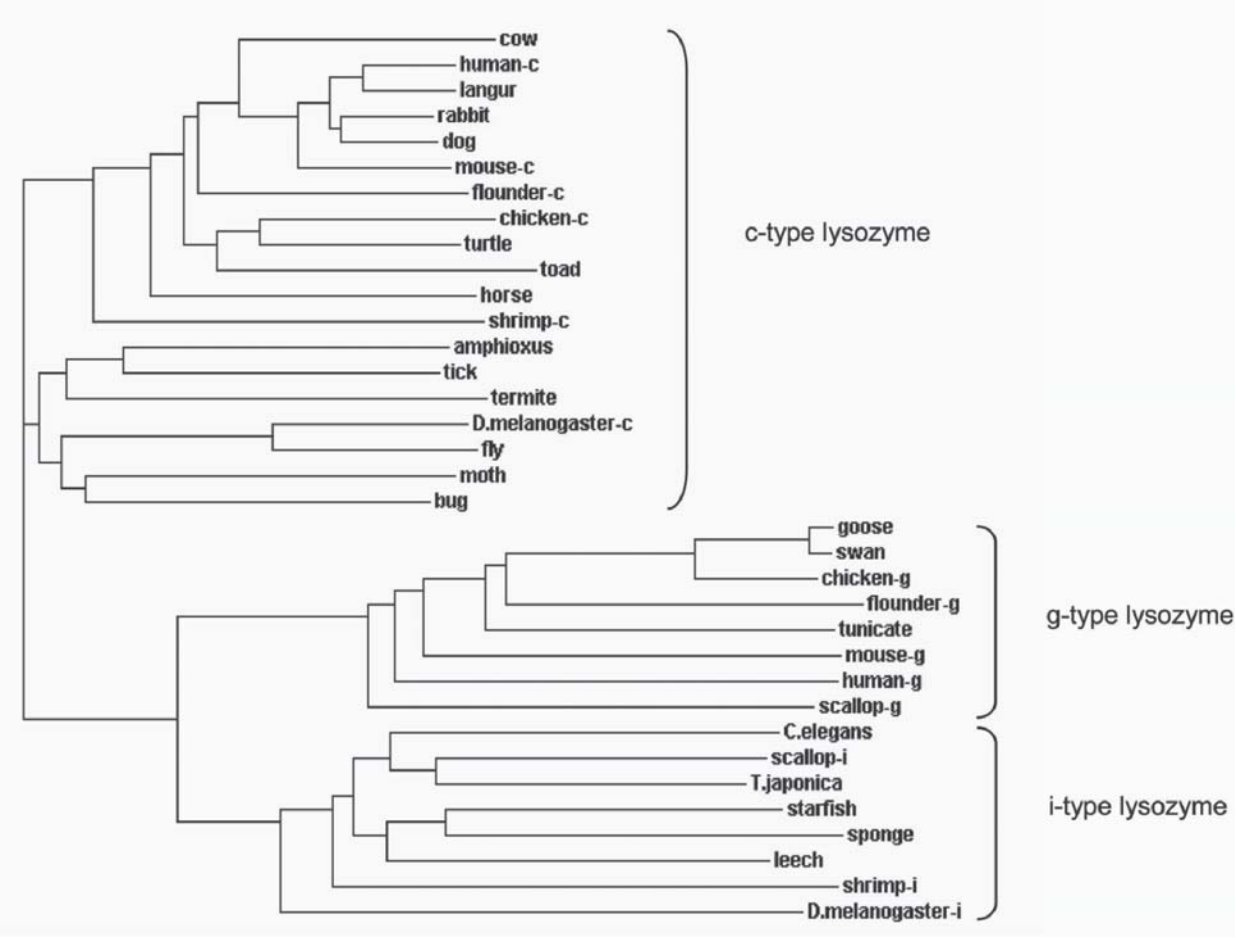

Figure 6. Phylogenetic analysis of the protein sequences of various c-, g- and i-type lysozymes, listed in table 2.

branches from the bird g-types lysozymes before the latter branches from the tunicate lysozyme). An in-depth analysis, using several lysozyme sequences of each animal order, should further clarify this. However, this is beyond the scope of this paper.

Evolutionary events such as gene duplications and gene losses are important in the origin, diversification and spread of the different types of lysozymes. Gene duplication enables the animal to diversify a lysozyme to a specialized function, or to vary its expression and activity in specific tissues without losing its original function. In this way, gene duplications within the lysozyme family have led to the evolution of a novel biological function for the stomach lysozymes in digestion (Irwin 1996). Ruminant artiodactyls (e.g. cow and sheep) have approximately ten lysozyme-like genes, while non-ruminant artiodactyls (e.g. pig and peccary) have only a single lysozyme gene (Irwin and Wilson 1989, 1990; Yu and Irwin 1996). Multiple c-type lysozyme genes are also found in fish (Grinde *et al.* 1988; Ng *et al.* 2005) and rodents such as rats and mice. In the latter, two lysozyme genes with different expression profiles (cfr section 5.1) have been described (Cross and Renkawitz 1990; Yeh *et al.* 1993). Furthermore, some lysozyme-like genes have been found in the genome of the mouse, just like in the human genome. However, these genes have not been further characterized and it is not known whether they encode functional lysozymes. The cephalochordate amphioxus has two copies of c-type lysozyme (Liu *et al.* 2006), while *Drosophila* possesses thirteen c-type lysozyme genes, of which at least eight are expressed in different parts of the digestive tract and at different stages of development (Kylsten *et al.* 1992; Hultmark 1996). Furthermore, two different g-type lysozyme genes exist in humans, mice, rats and zebrafish (Irwin and Gong 2003). The latter is probably the result of a lineage-specific gene duplication, since in other fish species only one g-type lysozyme has been found (Hikima *et al.* 2001; Savan *et al.* 2003; Yin *et al.* 2003; Sun *et al.* 2006). In humans and mice, the g-type lysozymes exhibit tissue-dependent expression (Irwin and Gong 2003; cfr section 5). To date, the presence of multiple i-type lysozymes has been reported for a few mollusk species (Ito *et al.* 1999; Olsen *et al.* 2003; Xue *et al.* 2004; Xue *et al.*

Table 5. Sequences used for phylogenetic analysis

Species	Common name	Lysozyme type	Accession number in GenBank
Bos taurus	Cow	Lysozyme c LYSC 2 Digestive lysozyme	Q06283
Branchiostoma belcheri tsingtauense	Amphioxus	Lysozyme c; from digestive tract	AY175372
Bufo andrewsi	Toad		P85045
Canis lupus familiaris	Dog	Spleen lysozyme	P81709
Drosophila melanogaster	Fruit fly	Lysozyme c LYS D Digestive lysozyme	P3715
Equus caballus	Horse	Milk lysozyme	P11376
Gallus gallus	Chicken	Lysozyme c	AAL69327
Homo sapiens	Human	Lysozyme c	P00695
Hyalophora cecropia	Moth	Lysozyme c	P05105
Marsupenaeus japonicus	Shrimp		BAC57467
Mus musculus	Mouse	Lysozyme c M	P08905
Musca domestica	Fly	Lysozyme c Digestive lysozyme	AAQ20048
Ornithodoros moubata	Tick	Lysozyme c, from digestive tract	AAL17868
Oryctolagus cuniculus	Rabbit	Kidney	P16973
Paralichthys olivaceus	Flounder	Lysozyme c	BAB18249
Pelodiscus sinensis	Turtle		Q7LZQ1
Presbytis entellus	Langur	Lysozyme c Digestive lysozyme	CAA42795
Reticulitermes speratus	Termite		BAC54261
Triatoma infestans	Bug		AAP83129
Argopecten irradians	Scallop	Lysozyme g	AAX09979
Anser anser	Graylag Goose	Lysozyme g	P00718
Ciona intestinalis	Tunicate	Lysozyme g	XP_002120319
Cygnus atratus	Black swan	Lysozyme g	P00717
Gallus gallus	Chicken	Lysozyme g	P27042
Homo sapiens	Human	Lysozyme g	AAH29126
Mus musculus	Mouse	Lysozyme g (lys 1)	XP_194692
Paralichthys olivaceus	Flounder	Lysozyme g	Q90VZ3
Asterias rubens	Starfish	Lysozyme i	AAR29291
Caenorhabditis elegans	Nematode	Lysozyme i (lys 1)	AAC19179
Chlamys islandica	Little scallop	Lysozyme i	CAB63451
Drosophila melanogaster	Fruit fly	Lysozyme i	CAA21317
Hirudo medicinalis	Medicinal leech	Lysozyme i	AAA96144
Litopenaeus vannamei	Shrimp	Lysozyme i	ABD65298
Suberites domuncula	Sponge	Lysozyme i	CAG27844
Tapes japonica	Mollusks	Lysozyme i	BAB33389

2007; Itoh and Takahashi 2007), the mosquito *Anopheles gambiae* (Paskewitz *et al.* 2008) and the medical leech *Hirudo medicinalis* (Zavalova *et al.* 1996, 2000).

Besides gene duplications, gene losses are also an important evolutionary mechanism that has contributed to the present spread of the different lysozymes (cfr section 2 and figure 2). Since c-type lysozymes are present in both vertebrates and invertebrates (i.e. in the Arthropoda), their existence in the basal chordates including urochordates and cephalochordates, which are intermediary between invertebrates and vertebrates, was to be expected. However, when Nilsen *et al.* (2003) found that the urochordates *Ciona intestinalis* and *Oikopleura dioica* only have g-type lysozymes, they postulated a loss of the c-type lysozyme gene in this subphylum as an explanation.

Similarly, the i-type lysozyme gene, which to date has not been identified in any chordate, has probably been lost in a common ancestor of all chordates. Its occurrence in both protostomia (including Nematoda, Arthropoda, Mollusca and Annelida) and deuterostomia (including the starfish belonging to the Echinodermata) suggests that the i-type lysozyme gene was present in the bilaterian ancestor. In the same way, the presence of a g-type lysozyme in the invertebrate scallop *Chlamys farreri* may indicate that their origin goes back to the divergence of deuterostomia from protostomia (Zhao *et al.* 2007).

Furthermore, knowledge of the genomic organization of lysozyme genes contributes to a better understanding of the evolutionary events leading to their diversity. C-type lysozyme genes in vertebrates such as chicken, human, cow, rat, pig, and flounder fish are relatively small genes, and are all organized similarly in four exons interrupted by relatively large introns (figure 7, Jung *et al.* 1980; Peters *et al.* 1989; Irwin *et al.* 1993; Yeh *et al.* 1993; Yu and Irwin 1996; Hikima *et al.* 2000).

Even the cephalochordate amphioxus lysozyme gene is composed of four exons (Liu *et al.* 2006). Invertebrate c-type lysozyme genes, in contrast, are more compact, usually consisting of three exons, as in the giant silk moth (Sun *et al.* 1991), tobacco hornworm (Mulnix and Dunn 1994) and malaria mosquito (Kang *et al.* 1996). Their introns are generally smaller, although intron sizes greatly differ: the giant silk moth lysozyme gene has large introns of 1.6 and 0.6 kb (Sun *et al.* 1991), while the mosquito *Anopheles gambiae* gene contains introns of only 70 bp (Kang *et al.* 1996). Even more extreme in this context are the intronless invertebrate c-type lysozyme genes of *Drosophila melanogaster* and the termite *Reticulitermes speratus* (Daffre *et al.* 1994; Fujita *et al.* 2002). Comparison of the organization of the c-type lysozyme genes from vertebrates and invertebrates revealed correspondence of the first two exons, while exons three and four in vertebrates are combined in a single third exon in the invertebrate genes (figure 7; Nilsen and Myrnes 2001; Liu *et al.* 2006). The intron–exon structure of the cephalochordate amphioxus gene is clearly of the vertebrate type, indicating that the split of exon three of invertebrate c-type genes into the two exons (three and four) of vertebrate c-type genes occurred before the cephalochordate/vertebrate divergence (Liu *et al.* 2006).

Within the g-type lysozyme genes, the number of exons varies, from five in the fish g-type lysozyme genes (Hikima *et al.* 2001; Sun *et al.* 2006; Kyomuhendo *et al.* 2007) to a maximum of seven for the human g1 gene (Irwin and Gong 2003). The chicken and other known mammalian lysozyme g genes each have six exons. The varying exons are all located in the 5′ untranslated region of the lysozyme mRNA. Irwin and Gong (2003) proposed the absence of at least one exon in fish g-type lysozyme as a possible explanation for their lack of a signal peptide. However, this seems contradictory with the recent publication of Kyomuhendo *et al.* (2007), which describes the presence of a signal peptide in salmon g-type lysozyme. In general, the structure of the lysozyme g gene has been largely maintained within vertebrates (Irwin and Gong 2003). Nevertheless, Nakano and Graf (1991) point out that the structural similarities between the c- and g-type lysozymes in chicken are not reflected by their genomic organization, since the exon–intron pattern of their genes is very different.

Finally, some studies have addressed the genomic organization of i-type lysozyme genes. Some of these

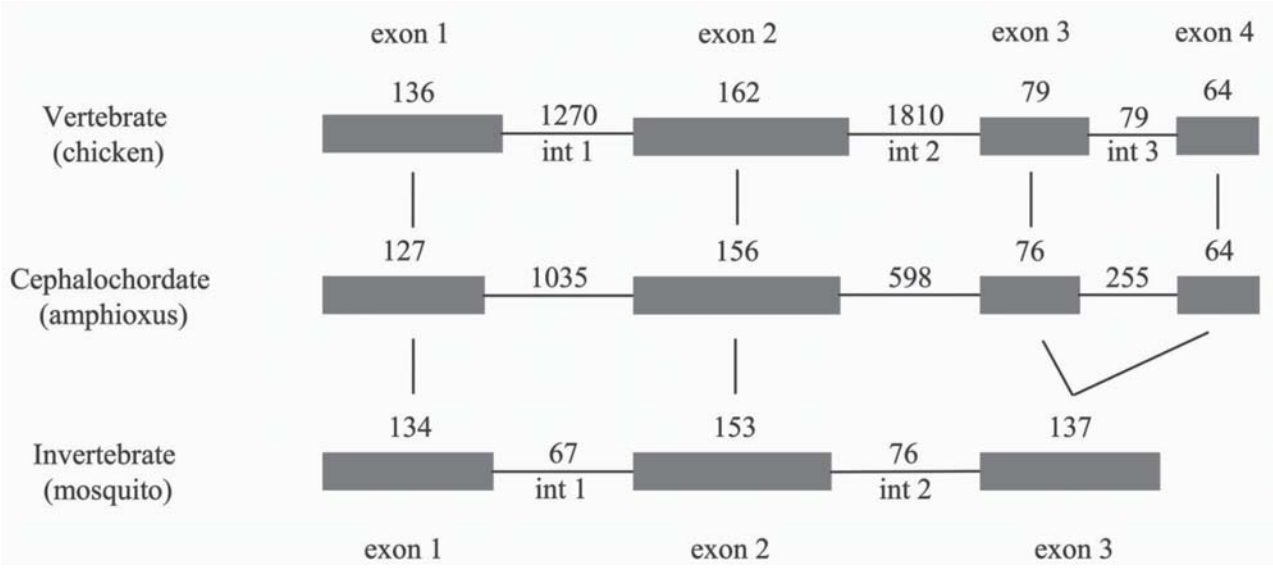

Figure 7. Genomic organization of c-type lysozymes from vertebrate (chicken, data from Jung *et al.* 1980), cephalochordate (amphioxus, data from Liu *et al.* 2006) and invertebrate (mosquito *Anopheles gambiae*, data from Kang *et al.* 1996), based on Liu *et al.* (2006).

lysozymes share the genomic organization of four exons with three introns with the vertebrate c-type lysozymes, like an i-type lysozyme from *Anopheles gambiae* (Paskewitz *et al.* 2008) and the scallop *Chlamys islandica* (Nilsen and Myrnes 2001). However, other configurations exist, for example, in *Mytilus edulis* and *Anopheles gambiae* which have five (Bachali *et al.* 2002) and two exons (Paskewitz *et al.* 2008), respectively. More i-type lysozyme genes need to be investigated to get a better overview on the structural organization and evolutionary relationships of the genes of this lysozyme group.

In conclusion, the study of the genomic organization (gene duplications, gene losses and gene structure) supports the idea that c, g- and i-type lysozymes diverged from a common ancestor, but still leaves room for alternative scenarios. Either way, the three lysozyme types currently form distinct groups, each present in certain animal orders. G- and i-type lysozymes are more closely related than either of them is to c-type lysozymes, and within c-type lysozymes, vertebrate and invertebrate lysozymes clearly share a common ancestor. Gene duplications have contributed to the diversification of lysozyme function in some cases, while in others the functional importance of this event still needs to be explored.

5. Biological role of lysozyme in different animals

As discussed in section 2, lysozymes of different types are widespread among animals. Most organisms have the genetic capacity to produce multiple lysozymes of different types, and it is presumed that these may have complementary or even different functions. For some lysozymes, the biological significance is well established, while the precise function of others remains to be unravelled. A widely recognized function of lysozymes is their contribution to antibacterial defence. Additionally, lysozymes function as a digestive enzyme in some animals. In general, indications for the function of lysozyme can be derived from the spatial expression pattern and regulation of the gene, and from functional adaptations of the enzyme. Lysozymes contributing to antibacterial defence are generally expressed in tissues and body fluids exposed to the environment or involved in bacterial clearance, while high expression levels of lysozyme in the stomach or gut rather points to a digestive function. Further, an upregulation of lysozyme after bacterial infection indicates a defensive role for the enzyme. Finally, defensive lysozymes typically have a neutral pH optimum and pI values of 8.0 or higher, while digestive lysozymes tend to have a low pH optimum and low pI value, and a higher resistance to proteases.

However, the precise role of some lysozymes, such as the g-type lysozymes in mammals, is not yet well understood. Irwin and Gong (2003) reported that neither of the two human lysozyme g genes are widely or highly expressed, and moreover, that there is variation in the site and level of g-type lysozyme expression within mammals. As a consequence, further research is needed to point out the significance of these lysozymes.

In the following part, the evidence for the role of lysozymes in antibacterial defence, digestion, and some other possible functions in various animals will be discussed.

5.1 *Lysozyme in the defence against bacteria*

5.1.1 *Vertebrates:*

(i) Mammals

In humans, c-type lysozyme is found in various body fluids (e.g. tears, saliva, airway fluid, breast milk, urine, serum, cerebrospinal fluid, cervical mucus and amniotic fluid), in tissues including the respiratory tract, intestinal tract, and in the lysosomal granules of neutrophils and macrophages (Brouwer *et al.* 1984; Firth *et al.* 1985; Lewis *et al.* 1990; Cole *et al.* 2002; Hein *et al.* 2002; Faurschou and Borregaard 2003; Song and Hou 2003; Akinbi *et al.* 2004; Dommett *et al.* 2005; Kucheria *et al.* 2005; Fox and Kelly 2006; Sariri and Ghafoori 2008). Due to methodological difficulties, information on the contribution of lysozyme to the innate immune defence in man is often indirect. However, some studies have directly demonstrated the role of lysozyme in the innate immune defence of both humans and other mammals, and these will be discussed here.

Recently, Deckers *et al.* (2008) depleted human saliva of lysozyme by affinity chromatography with a specific bacterial inhibitor of c-type lysozyme as a ligand, and in this way demonstrated that salivary lysozyme suppresses growth of *E. coli* in the saliva. Growth of *P. aeruginosa* was not suppressed, and neither was growth of *E. coli* or *P. aeruginosa* in human milk, indicating that the antibacterial activity of lysozyme may be important in some tissues and secretions, but not in others, probably depending on the composition of the surrounding fluid.

Using a similar approach, Cole *et al.* (2002) provided evidence for the biological activity of lysozyme in airway secretion. Removal of cationic polypeptides (not only lysozyme) from nasal fluid ablated its activity against *E. coli*, *L. monocytogenes*, and a mucoid cystic fibrosis isolate of *P. aeruginosa*. The addition of physiological concentrations of lysozyme was sufficient to restore the antibacterial activity of the cationic polypeptide-depleted nasal fluid, confirming the important role of lysozyme in the antibacterial activity of airway secretion.

Akinbi *et al.* (2000) assessed the role of lysozyme in pulmonary host defence *in vivo* by using transgenic mice expressing rat lysozyme in respiratory epithelial cells. These mice exhibited significantly enhanced killing of group B streptococci, *E. coli* and a mucoid strain of *P. aeruginosa* in the lung. Moreover, a decreased systemic dissemination

of group B streptococci, and an increased survival of the *P. aeruginosa* strain following infection was reported for the transgenic mice.

Furthermore, the house mouse has two genes encoding a c-type lysozyme itself (lysozyme M and P). The lysozyme M gene is strongly expressed in leukocytes and several epithelial tissues, while high levels of P lysozyme transcripts are restricted to intestinal Paneth cells (Cross *et al.* 1988; Obita *et al.* 2003; Nile *et al.* 2004). Markart *et al.* (2004b) found that deficiency of the M lysozyme in the lungs of transgenic mice increased their susceptibility to *Klebsiella pneumoniae* infection, whereas increased expression of this lysozyme conferred resistance to infection and enhanced survival. A similar finding was reported later with *P. aeruginosa* (Cole *et al.* 2005). Likewise, Shimada *et al.* (2008) reported that lysozyme M deficiency in mice led to an augmented susceptibility to middle ear infection caused by *Streptococcus pneumoniae* and moreover resulted in severe middle ear inflammation compared to wild-type mice. Interestingly, the lack of M lysozyme in LysM null mice was partially compensated for by an upregulation of P lysozyme in the lung tissue (25% of wild-type muramidase activity). Furthermore, M lysozyme was found to be more effective against Gram-negative pathogens than P lysozyme, while both were equally effective against Gram-positive pathogens (Markart *et al.* 2004a). Despite the production of P lysozyme, a significantly better survival of the Gram-positive *M. luteus* in these lysozyme M-deficient mice was revealed, which indicates the importance of both proteins in host innate defence (Ganz *et al.* 2003).

The above investigations clearly point out that lysozymes are part of the mammal innate immune system. The *in vivo* studies particularly provide strong direct experimental evidence for the importance of lysozyme in the defence against different types of pathogens.

The influence of breast milk lysozyme on the bacterial flora in the gastrointestinal tract has been investigated *in vivo* by Maga *et al.* (2006) and Brundige *et al.* (2008), using transgenic goats expressing human lysozyme in the mammary gland. Pasteurized milk from transgenic goats was fed to young pigs who subsequently developed fewer numbers of total coliforms and *E. coli* in their gut microflora than those feeding on milk from non-transgenic control animals, thereby demonstrating that milk lysozyme can modulate the bacterial population of the gut in these pigs (Maga *et al.* 2006). Brundige *et al.* (2008) performed a comparable feeding trial followed by challenge with a porcine-specific enteropathogenic *E. coli* (EPEC) using young pigs. They also found that challenged pigs receiving the milk from transgenic goats had fewer total coliforms and EPEC in their ileum than the control animals feeding on milk from non-transgenic animals, indicating a protective effect of the milk from transgenic animals against EPEC infection. This study shows that oral administration of lysozymes can be used to suppress gastrointestinal pathogens, and also that it influences the entire intestinal microbial ecosystem, an observation that certainly deserves further investigation.

Besides the direct bacteriolytic activity, the modulation of the immune response and inflammation may be an additional mechanism by which lysozyme contributes to antibacterial defence. The innate immune system of mammals recognizes microorganisms through different types of pattern recognition receptors, such as the peptidoglycan recognition proteins (PGRPs), the cytoplasmic nucleotide-binding oligomerization domain (NOD) proteins, and the transmembrane toll-like receptor (TLR) proteins (Dziarski and Gupta 2005; Royet and Dziarski 2007). However, pathogen detection by peptidoglycan receptors depends on how the peptidoglycan is presented. Therefore, hydrolysis of peptidoglycan by lysozyme and other muralytic enzymes can modulate the activation of the immune response and inflammation pathways induced by these receptors (Chaput and Boneca 2007). Ganz *et al.* (2003) illustrated this by showing that lysozyme M-deficient mice incurred much more severe tissue injury than wild-type mice after challenge by *M. luteus*. The deficiency in lysozyme activity apparently delayed the degradation of peptidoglycan, a process which normally leads to the termination of the inflammatory responses. As a consequence, this contributed to a prolonged and more severe inflammatory response. Moreover, Lee *et al.* (2009) recently investigated the effect of HEWL supplementation on dextran sodium sulphate-induced colitis in pigs. Besides an attenuation of the symptoms of this colitis, treatment with HEWL significantly reduced the local expression of pro-inflammatory cytokines, indicating that HEWL has potent anti-inflammatory activity and may function as an immunomodulator (Lee *et al.* 2009).

In conclusion, the defensive role of c-type lysozymes in mammals is experimentally well founded, in sharp contrast to the complete lack of experimental studies regarding the g-type lysozyme function in mammals.

(ii) Birds

Bird lysozymes are predominantly found in the eggs. However, there is a lot of variation in the expression of lysozymes among bird species. Some birds' egg whites have only g-type lysozyme (e.g. Embden goose), while others contain only c-type (e.g. chicken) or both (e.g. black swan) lysozyme types (Prager and Wilson 1974). In other tissues as well, considerable variation has been observed in the expression of lysozyme types. This is illustrated by a study of Nile *et al.* (2004), who investigated the intestinal expression of the c-type lysozyme and the two g-type lysozyme genes retrieved in chicken using reverse transcriptase polymerase chain reaction (PCR). Although c-type lysozyme expression was detected in the small intestine of young (up to 8-day-old) birds, no lysozyme c mRNA was

detected in the intestine of older birds (Nile *et al.* 2004). On the other hand, lysozyme g1 transcripts were found in the intestine of 4–35-day old chickens, while lysozyme g2 mRNA was identified in the intestine from chickens of all ages (Nile *et al.* 2004). Therefore, the authors suggest a complementary role for these lysozymes in protecting the intestine against pathogens. Apart from these studies indicating the possibility of a defensive role of lysozyme, solid experimental evidence for such a function in birds seems to be lacking.

(iii) Fish

Fish live in water, an environment that can contain a wide range, and sometimes high numbers, of both pathogenic and non-pathogenic microorganisms. Thus, not surprisingly, their innate immune system is particularly important (Hikima *et al.* 2001). During the past decades, lysozyme activity has been widely reported in fish tissues including the head kidney, which is a leukocyte-rich key organ in immunity, and in the gill, skin, gastrointestinal tract and eggs, where the risk of bacterial invasion is high (Saurabh and Sahoo 2008). Moreover, several c- and g-type fish lysozymes have been reported to kill both Gram-positive and Gram-negative bacteria (Grinde 1989; Itami *et al.* 1992; Yousif *et al.* 1994; Hikima *et al.* 2001; Minagawa *et al.* 2001; Zheng *et al.* 2007; cfr section 3.4), thus highlighting their capacities as antibacterial proteins even more than those from other animals.

Evidence for an antimicrobial function also stems from the molecular characterization and analysis of expression profiles. Expression of g-type fish lysozymes is reported (predominantly) in haematopoietic tissues, skin and intestine tissues, head kidney, peritoneum, spleen and gills for different fish (Hikima *et al.* 2001; Zheng *et al.* 2007; Larsen *et al.* 2009). This wide expression was proposed to represent an adaptation to the constant exposure of fish to high numbers of opportunistic pathogens in the water (Hikima *et al.* 2001). Particularly relevant in this context are the high expression levels in organs exposed to the external environment, such as the skin, intestine and gills.

Furthermore, upregulation of mRNA levels of c- or g-type lysozymes after challenge with fish pathogens or stimulation with lipopolysaccharides have been reported in different fish species (Hikima *et al.* 2001; Yin *et al.* 2003; Zheng *et al.* 2007; Caipang *et al.* 2008; Fernández-Trujillo *et al.* 2008; Jiménez-Cantizano *et al.* 2008). This strongly suggests a function of fish lysozymes in their antibacterial immune response.

More direct evidence for the potential relevance of lysozyme in fish immunity was provided by a challenge experiment with *Flavobacterium columnare* and *E. tarda* on transgenic zebrafish expressing c-type chicken lysozyme (Yazawa *et al.* 2006). Significantly reduced mortality was observed in the transgenic zebrafish 15 h after *F. columnare* challenge (by immersion) and *E. tarda* challenge (by intramuscular injection) when low bacterial loads were used (10^4 and 10^3 CFU/ml, respectively).

(iv) Reptiles

Only limited information is available on the expression pattern of reptile lysozymes. Two studies report the isolation and characterization of lysozymes isolated from the egg white (Araki *et al.* 1998b; Thammasirirak *et al.* 2006), but whether these lysozymes are expressed in other tissues has not yet been reported. Nevertheless, these enzymes are also considered as important players in the interaction with bacteria, since Thammasirirak *et al.* (2006) suggested that the lack of lytic activity of reptile lysozymes towards *A. hydrophila* (cfr section 3.4) might contribute to the virulence of this bacterial species for reptiles. Obviously, without direct experimental evidence, such a statement remains speculative. Among six tested Gram-negative bacteria, *V. cholerae* was substantially lysed by the reptile lysozymes, *P. aeruginosa* was weakly lysed, and the others were insensitive.

5.1.2 *Invertebrates:*

(i) Arthropods

Opposed to the highly specific adaptive immune system of vertebrates, the immune defence in insects cannot rely on a memory effect to combat intruders. However, insects have a very competent inducible immune defence system, which is regarded as a model for innate immune reactions in general. Upon bacterial challenge, insects synthesize a number of bactericidal proteins and peptides in the haemolymph. Inducible (probably c-type) lysozyme activity in the haemolymph has been demonstrated for many insect orders, including the Diptera (e.g. mosquitos, Hernandez *et al.* 2003; Gao and Fallon 2000), the Lepidoptera (e.g. moths, Hultmark *et al.* 1980; Lee and Brey 1995; Abraham *et al.* 1995; Fujimoto *et al.* 2001; Bae and Kim 2003; Kim and Yoe 2003; Gorman *et al.* 2004; Gandhe *et al.* 2006), the Orthoptera (e.g. crickets, Schneider 1985; Adamo 2004) and the Coleoptera (e.g. beetles, Ourth and Smalley 1980). In addition to their activity against Gram-positive bacteria, some insect c-type lysozymes are antibacterial against Gram-negative bacteria (cfr section 3.4). Moreover, other antimicrobial proteins and peptides that are also induced by bacterial infection can broaden the antibacterial spectrum of lysozyme through synergistic effects. In insects, examples of such components are cecropins, defensins and attacin-like proteins, all known to affect bacterial cell membranes (Boman 1998; Bulet *et al.* 1999). Therefore, together with these molecules, at least the c-type lysozyme is likely to play an important role in the insect's defence against bacteria.

In holometabolous insects, basically all larval tissues are degraded and replaced by new structures of the adult animal during metamorphosis (Hultmark 1996). Since this is a vulnerable stage in the insect's development, several

immune functions, including lysozyme production, are upregulated to prevent the spread of bacteria. Accordingly, high lysozyme levels are detected in the midgut of full-grown larvae in tobacco hornworm, *Drosophila,* Hessian fly and the cotton bollworm (Russel and Dunn 1991; Daffre *et al.* 1994; Mittapalli *et al.* 2006; Zhang *et al.* 2009). Lysozyme is stored in granules in the midgut cells, and released into the gut lumen just before metamorphosis is initiated (Russel and Dunn 1991). Although the process of metamorphosis is less radical for hemimetabolous insects, upregulation of lysozyme expression in the bug and soft tick was also found immediately after the moult (Kopáček *et al.* 1999; Kollien *et al.* 2003).

Regardless of the lysozyme levels upon bacterial challenge, a significant baseline concentration exists (Dunn *et al.* 1985). Next to a role in the first-line defence against bacteria, constitutively expressed lysozyme is possibly involved in the modulation of the immune response. Together with other macromolecular components, peptidoglycan fragments released by lysozyme from bacterial cell walls initiate an antibacterial response in insects, since they are captured and transmitted by PGRP. PGRPs also occur in mammals but, in insects, they defend host cells against infection through very different mechanisms (Royet and Dziarski 2007). In insects, two different peptidoglycan recognition systems are present, one for the induction of antimicrobial peptides, the other for activating the prophenoloxidase (proPO) cascade leading to melanization (Kim *et al.* 2008). Many PGRPs have been identified in different insects (Dziarski and Gupta 2006). The necessity of partially degrading bacteria (e.g. by lysozyme) to generate peptidoglycan fragments that can function as signal molecules in insects was already suggested by Dunn *et al.* (1985) and Iketani and Morishima (1993), but a clear demonstration of the role of lysozyme in enhancing the access of PGRPs to peptidoglycan was provided by Park *et al.* (2007). Indeed, partial lysozyme digestion of peptidoglycan drastically increased its binding to the PGRPs of both *Drosophila melanogaster* and *Tenebrio molitor*. This enhanced interaction is expected to lead to an activation of both the Toll and proPO pathways. This was already confirmed *in vivo* by the observation of a stronger and faster melanin synthesis in *Tenebrio molitor* larvae injected with partially digested peptidoglycan than in larvae injected with untreated peptidoglycan. Activation of the proPO pathway eventually leads to production of melanin and subsequent deposition of this brown–black pigment at the site of the damaged tissues and on the surfaces of invading pathogens (Christensen *et al.* 2005). Since a *Plasmodium*-resistant strain of *Anopheles gambiae* melanizes ookinetes of *Plasmodium* before they develop to the oocyst stage (Collins *et al.* 1986), this immune response, which is apparently unique to arthropods and some other invertebrates, has received considerable attention because of its potential exploitation to control mosquito-borne diseases. However, melanin production is also crucial for other physiological processes such as hardening of the egg chorion, wound healing and cuticle tanning, thus complicating the elucidation of gene functions and biochemistry regarding this pathway (Christensen *et al.* 2005). Recently, Li and Paskewitz (2006) discovered an unexpected role for lysozyme in mediating melanization of foreign targets in the mosquito *Anopheles gambiae*. A factor that was deposited on Sephadex beads (used as targets) and which protected these targets from melanization was purified. N-terminal sequence determination identified this factor as lysozyme. Thus, under certain circumstances, lysozyme may play a role in limiting melanization, but the mechanisms by which lysozyme exerts this function are not yet understood.

(ii) Bivalve mollusks

Because of their muramidase activity and, in some cases, broad antibacterial activity with strong growth-inhibiting effects against both Gram-positive and Gram-negative bacteria (Nilsen *et al.* 1999; cfr section 3.4), bivalve i-type lysozymes are believed to play a role in host defence (McHenery *et al.* 1986; Chu and La Peyre 1989; Chu and La Peyre 1993; Carballal *et al.* 1997; Cronin *et al.* 2001). Apart from this, mRNA of the recently identified g-type lysozyme of the scallop *Chlamys farreri* is highly expressed in the gills and haemocytes. The latter contribute to bacterial clearance by phagocytosis and, similarly, as in fish, the gills are constantly flushed with water and thus exposed to pathogens. The high expression levels in these cells and in this tissue suggest a contribution of the lysozyme in warding off bacterial infecton of the scallop; however, direct evidence for such a role is still lacking.

(iii) Nematodes

Besides a family of lysozymes homologous to those of the amoeboid protozoon *Entamoeba histolytica* (not further discussed here), *Caenorhabditis elegans* possesses several putative i-type lysozyme genes (cfr section 2). The latter were upregulated after infection by feeding the nematod with *Microbacterium nematophilum* and, moreover, knockdown of the expression of one of the i-type lysozymes made the worms suffer more from the pathogen. These data prove the importance of i-type lysozymes in the response of *C. elegans* to infection, and suggest a role for these lysozymes in worm defence (Mallo *et al.* 2002; O'Rourke *et al.* 2008).

5.2 *Lysozyme as a digestive enzyme*

In some animals, lysozyme has been recruited as a digestive enzyme, enabling them to use bacteria as a food source. This additional role for lysozymes has been well discussed the past twenty-five years for vertebrates, while the debate on a digestive function of lysozymes in invertebrates is more recent (Fujita 2004). However, examples of this

adaptation of lysozyme exist both in vertebrates and invertebrates.

5.2.1 *Vertebrates:* Although mammals do not have the ability to digest cellulose, ruminants can break down this structural component of plant cell walls by their unusual mode of digestion. They have developed a symbiotic relationship with microbial consortia in the foregut (rumen) chambers of the stomach, which convert cellulose to end-products such as acetate that can be used by the host. However, these microbes use approximately 10% of the carbon, 80% of the nitrogen, and 60% of the phosphorus of the ruminant diet for producing their own microbial biomass. Thus, to benefit from the digestion of cellulose by the microbes, ruminants need to recover the microbial biomass as a part of their diet. Therefore, one of the factors contributing to the evolutionary success of the ruminants is the acquisition of lysozymes that contribute to the digestion of bacteria in the stomach (Dobson *et al.* 1984).

Within the mammals, an independently acquired but similar adaptation from lysozyme to a digestive enzyme has been reported in a leaf-eating monkey (langur) (Stewart *et al.* 1987).

Interestingly, Kornegay *et al.* (1994) described a lysozyme expressed at high levels in the foregut of the hoatzin, the only known avian foregut fermenter. This bird can survive on a diet of leaves, thanks to its enlarged crop with an active fermenting microbiota.

The regular vertebrate lysozyme which functions as a shield against bacterial invasion, is typically produced in, for example, white blood cells, milk and tears (cfr section 5.1), and accordingly has a neutral pH optimum (pH 5.5–7.5). Since the recruitment of lysozyme as a digestive enzyme described in the above cases implies the production and enzymatic activity of this protein in the true stomach of foregut-fermenting vertebrates, an acidic compartment, both regulatory and structural adaptations of the lysozyme protein were needed. In ruminants, the langur and hoatzin, high levels of lysozyme are produced in their true stomach (lysozyme concentration in the stomach mucosa of ruminants is about seventy times higher than in that of monogastric animals). Additionally, gene duplications, as observed in the ruminants (about 10 lysozyme genes) and hoatzin (5 lysozyme genes) also allow an increase in lysozyme production, and are thought to have facilitated the recruitment of lysozyme to the new function as a digestive enzyme, without loss of its function as an immune-related antimicrobial protein in other parts of the body. Specific mutations in some of the lysozyme alleles have allowed the enzymes to function in the acidic stomach fluid containing pepsin and the fermentation product diacetyl. The loss of some pepsin-sensitive bonds and a decreased electrostatic interaction with pepsin has presumably resulted in resistance of the digestive lysozyme to pepsin cleavage. Comparison of the charge distribution of the cow digestive lysozyme with those of hen and human lysozymes revealed that the surfaces of the former bear more negative charges, probably resulting in a decreased electrostatic interaction with the negatively charged pepsin. Indeed, the crystal structure of the recombinant bovine stomach lysozyme 2, recently gathered by Nonaka *et al.* (2009), revealed the presence of negatively charged surfaces. Together with a shortened loop and salt bridges in the lysozyme molecule, this provides structural stability, which in turn results in resistance to pepsin (Nonaka *et al.* 2009). The disappearance of the acid-sensitive Asp-Pro bond usually present in lysozymes, together with a decrease in other acid labile amino acids (i.e. Asp, Asn or Gln residues) illustrates the adaption of resistance to an acidic medium. Moreover, these digestive lysozymes can function in this low pH compartment by virtue of their low pH optima of 4.8–5.2 for the ruminant, 4.8 for the langur and <5 for the hoatzin stomach lysozyme. A decrease in pI value (to 6.2–7.7) due to a diminished amount of basic amino acids could account for the low pH optimum (Prager 1996). Since the nucleophilic character of Asp52 is thought to be important for the catalytic mechanism of lysozyme (cfr section 3.3), the presence of basic amino acids in its surrounding can cause a significant deviation on the pK_a value (= the negative logarithm of the acid dissociation constant) of this residue, and thereby influence the catalytic activity of lysozyme at different pH values. However, Nonaka *et al.* (2009) found that although bovine lysozyme 2 has an acidic optimal pH, its relative activity level is lower than that of HEWL, even at acidic pH. Further analyses, based on the crystal structure of the bovine lysozyme, revealed that the pK_a values of the catalytic amino acids are not reduced, suggesting that the catalytic activity of the bovine lysozyme 2 is not adapted to acidic conditions (Nonaka *et al.* 2009). Finally, a reduction in the number of arginine residues is hypothesized to decrease the susceptibility to modification by the fermentation product diacetyl, which results in a partial inactivation of the lysozyme (Patthy and Smith 1975; Dobson *et al.* 1984; Kornegay *et al.* 1991; Jollès *et al.* 1996; Prager 1996; Irwin 2004).

Besides the foregut fermenters, hindgut fermenters such as rabbits also consume large amounts of cellulose. These animals ferment cellulose by means of a bacterial consortium in their hindgut. As in ruminants, this fermentation releases additional nutrients, but these cannot be resorbed in the hindgut or further downstream. Hindgut fermenters solve this problem by reingesting their so-called soft 'night-faeces', which are rich in energy- and carbon-containing metabolites, but also in minerals, vitamins and proteins. Eventually, hard faecal pellets are the final waste product after redigestion of these soft pellets. Reflecting this nutritional strategy called coprophagia, rabbit lysozyme is expressed at a high level in the distal part of the colon

to function as a digestive enzyme that releases nutrients from the bacteria. The higher level of lysozyme in the stomach contents of rabbits when they have reingested their soft faeces indicates that the colon lysozyme is passed to the stomach. This suggests a function in the digestion of reingested caecal bacteria in the upper gastrointestinal tract for this lysozyme (Cámara and Prieur 1980; Cámara and Prieur 1984; Cámara *et al.* 1990; Fujita 2004). The narrow acidic pH optimum of the colonic lysozyme is in agreement with this digestive function (Ito *et al.* 1994). Recently, Jiménez-Cantizano *et al.* (2008) raised the possibility of a digestive function for a fish lysozyme. They investigated the expression profile of the c-type and g-type lysozyme in brill. While their results indicated that the g-type lysozyme was involved in the response against bacterial infections (cfr section 5.1), a role in digestion seemed more plausible for the c-type lysozyme, since this lysozyme was expressed mainly in the stomach and liver, and mRNA transcripts did not increase after intraperitoneal injection with bacteria. In view of the fact that brill primarily feed on small crustaceans (particularly shrimps), and c-type lysozymes generally show chitinase activity (cfr sections 3.3 and 5.3), degradation of the exoskeleton of these feed animals by hydrolysis of the β-(1,4)-bonds between the NAGs of chitin is a plausible function for this lysozyme.

Based on their distinctive molecular characteristics and expression profile, the contribution of specialized lysozymes in digestion is thus well established for foregut fermenters, hindgut fermenters and fish. However, direct evidence for such a function, for example, by the construction of lysozyme knockout animals, is lacking.

5.2.2 *Invertebrates:*

(i) Arthropoda class Insecta

In the class of the insects, several studies have revealed a digestive function of lysozymes in the higher species of the dipteran order (Terra 1990; Lemos *et al.* 1993; Daffre *et al.* 1994; Regel *et al.* 1998; Kollien *et al.* 2003). Although the food sources of adult Diptera Cyclorrhapha are more diverse, most Diptera Cyclorrhapha larvae are saprophagous, feeding largely on bacteria. Therefore, the capacity of the flies *Drosophila* and *Musca* to digest bacteria is not surprising (Terra 1990). The characterization and analysis of the biological function of the lysozyme genes of *Drosophila* and, more recently, the complete genome sequence, revealed that this insect has at least twelve lysozyme genes, of which eight are known to exhibit different expression profiles. As discussed for the ruminants and the hoatzin, gene duplication is believed to be at the basis of specialization of gene function, and the same mechanism possibly allowed *Drosophila* to recruit lysozyme for a digestive function. The Lys D family (five closely related genes named Lys A, B, C, D and E) are strongly expressed in the midgut of larvae and adults, while Lys P is expressed in the salivary glands of adults. Lys S, on the other hand, has the larval gastric caecae as the main expression site, while Lys X (as discussed above) is mainly expressed in the metamorphosing midgut of late larvae and early pupae (Daffre *et al.* 1994; Hultmark 1996; Regel *et al.* 1998). Surprisingly, none of the lysozyme genes is expressed in the haemocytes or in fat body cells, both important players of the insect immune system. Moreover, bacterial infection of *Drosophila* severely represses the Lys D family and Lys S genes, possibly reflecting a malaise reaction in the digestive tract (Daffre *et al.* 1994). Apparently, these lysozymes of *Drosophila* are recruited as digestive enzymes, rather than being a part of the immune defence against bacteria. Two c-type lysozymes, lysozyme 1 and lysozyme 2, were isolated and purified from the larval midguts of *Musca domestica* (Lemos *et al.* 1993). Besides their expression in tissues relevant for digestion (Lemos *et al.* 1993; Daffre *et al.* 1994), both these lysozymes of *Musca domestica* and the Lys D family of *Drosophila* show molecular characteristics of an adaptation to a digestive function. First of all, these lysozymes in general have lower pI values, varying from 5.5 (Lys D family of *Drosophila*) to 7.9 and 8.2 (lysozymes of the midguts of *Musca domestica*) (Daffre *et al.* 1994; Regel *et al.* 1998; Lemos *et al.* 1993; Cançado *et al.* 2007, 2008). Moreover, their pH optimum is shifted towards acidic pH values in media with physiological ionic strengths, reflecting the acidic region in the midgut, which is characteristic for these flies (Lemos *et al.* 1993; Regel *et al.* 1998; Fujita 2004; Cançado *et al.* 2008). Cançado *et al.* (2007) calculated the pK_a of the catalytic groups in a lysozyme c from the housefly, and found lower values than for HEWL. The crystal structure of this lysozyme further allowed them to correlate these results with the observed conformation of the protein. Finally, these lysozymes are resistant to the proteolytic activity of cathepsin D, an acid proteinase present in the middle region of the midgut of Diptera Cyclorrhapha (Lemos *et al.* 1993; Regel *et al.* 1998). Additionally, expression of the basic Lys P of *Drosophila* in the salivary glands of adults may promote early bacterial lysis in the anterior midgut (Regel *et al.* 1998).

Mosquitoes are members of the lower Diptera, which do not seem to have recruited lysozyme as a digestive enzyme. However, Kang *et al.* (1996) noticed a striking similarity between the *Anopheles gambiae* lysozyme c-1 and the Lys P of *Drosophila.* Reverse transcriptase PCR analysis of the *Anopheles gambiae* lysozyme gene revealed much higher expression levels in sugar-fed adults compared to animals fed on blood. The rationale for this may be that, when feeding on a nectar diet, these mosquitoes probably ingest bacteria and fungi, while blood is usually sterile. The elevated lysozyme expression with the nectar diet would thus allow the capture of nutrients from the bacteria in addition to the nectar sugars. Li *et al.* (2005), in contrast, described this lysozyme c-1, together with the lysozyme c-2 as the best candidates for

an immune function (cfr section 5.1). Since their research revealed highest transcript levels for lysozyme c-3 and c-8 in larvae that ingest bacteria as part of their diet, Li *et al.* (2005) propose a digestive function for these lysozymes. Adaptations resulting in an acidic pI were not present (pI value of 8.46 for both lysozymes c-3 and c-8), but also not expected here, since larval mosquito guts (in contrast to those in higher flies) are weakly to strongly alkaline (Zhuang *et al.* 1999). Besides the eight c-type lysozyme genes that exist in *Anopheles gambiae* (Li *et al.* 2005), Paskewitz *et al.* (2008) characterized two i-type lysozyme genes in this mosquito. An increased transcript level of these lysozymes was found in the midguts after blood-feeding compared to non-blood-fed mosquito midguts. Since bacterial populations increase in mosquito midguts after blood-feeding (DeMaio *et al.* 1996), a potential digestive role for these i-type lysozymes is possible.

Besides the described digestive role of lysozyme in the higher dipteran species, the possibility that lysozyme enables wood-feeding termites (belonging to the isopteran insects) to digest hindgut bacteria transferred by trophallaxis (exchange of food among colony members) was raised by Fujita *et al.* (2001). The diet of these termites mainly consists of wood and other dead plant material. Although these only contain 0.03–0.7% nitrogen, termite tissues contain 8–15% nitrogen, and the question how termites can survive on such a low-nitrogen diet has been raised. A mechanism adopted by termites to countervail this lack is nitrogen fixation by intestinal microorganisms. Tayasu *et al.* (1997) showed that about 50% of the body nitrogen of the wood-feeding termite *Neotermes koshunensis* comes from atmospheric nitrogen. However, the mechanism by which the fixed nitrogen is incorporated in termite tissues remained unexplained since fermentation by microorganisms in termites always takes place in the hindgut (Fujita *et al.* 2001; Fujita 2004). Investigation of the lysozyme distribution in the digestive tract of a wood-feeding termite revealed high specific lysozyme activity in the salivary gland and foregut, while very low activity was detected in the hindgut (Fujita *et al.* 2001). Comparable lysozyme distribution (i.e. mostly in the salivary glands) has been found in three higher termites, *Macrotermes annandalei*, *Pericaproitermes nitobei* and *Nasutitermes takasagoensis* (Fujita and Abe 2002). Therefore, ingestion of its own faecal material, or that of a nestmate, would be unavoidably required for a termite to efficiently digest these microorganisms. This behaviour (proctodeal trophallaxis) is well known in termites, and thought to be important for their nutritional economy (Fujita 2004).

(ii) Arthropoda class Arachnida

In the phylum Arthropoda, the Arachnida represent another class in which lysozyme is believed to function as a digestive enzyme. Among acaridid mites, lysozyme is widely distributed (Erban and Hubert 2008). These animals thrive in a range of nutrient-rich habitats, including decaying plant and animal tissues, which carry high numbers of microorganisms. Although they did not reveal the type of lysozyme (presumably c- or i-type), Erban and Hubert (2008) observed a correlation between lysozyme activity and population growth of eight mite species, and concluded that lysozyme action may enable mites to utilize bacteria as a food source, thus accelerating their growth. Moreover, the digestive function of the implicated lysozymes was further supported by the correspondence of their observed pH optimum (pH 4.5) with the pH of the anterior midgut of acaridid mites, which is around 5.0. Although synanthropic mites produce a wide spectrum of cysteine and serine proteases in their digestive system, the investigated lysozymes remained unaffected, again supporting their digestive functionality (Erban and Hubert 2008).

In the lysozyme from the gut of the soft tick *Ornithodoros moubata*, features of an antibacterial activity seem to be combined with a digestive function. Grunclová *et al.* (2003) reported an upregulation of the lysozyme at the transcriptional level by a bloodmeal uptake, and Kopáček *et al.* (1999) cite work by Podboronov and Berdyev (original paper in Russian) showing an 8–10-fold increase in lysozyme activity in whole body homogenates 24 h after feeding on blood infected with different bacterial species than after sterile blood-feeding. Although phylogenetic analysis showed a stronger relatedness of this lysozyme to the family of the *Drosophila* digestive lysozymes than to other c-type lysozymes, and the pH optimum of the tick lysozyme is rather in the acidic range (pH 6.0), some characteristics, including a more acidic pI attributed to digestive lyszoymes are not present (Kopáček *et al.* 1999; Grunclová *et al.* 2003).

(iii) Bivalve mollusks

Besides c-type lysozymes, i-type lysozymes can also fulfil a digestive function. To benefit from the large amounts of bacteria ingested as a food source, bivalve mollusks need a digestive system capable of breaking down prokaryotic cells. Hence, the high lysozyme activities detected in the digestive systems of many bivalve mollusks have triggered speculation about a possible digestive role for these lysozymes (Nilsen *et al.* 1999; Bachali *et al.* 2002; Takeshita *et al.* 2004; Matsumoto *et al.* 2006; Itoh and Takahashi 2007; Xue *et al.* 2007). Jollès *et al.* (1996) have already suggested a digestive role for lysozymes in deep water bivalve mollusks that rely on symbiotic bacteria in their gills for nutrition. Again, a molecular adaptation in the evolution of bivalve lysozymes to this digestive function has been postulated (Jolles *et al.* 1996; Xue *et al.* 2007). Xue *et al.* (2007) compared the function of the cv lysozyme 2 and the previously described (Xue *et al.* 2004) cv lysozyme 1 of *Crassostrea virginica* (the eastern oyster). The predominant expression of the cv lysozyme 2 gene in digestive gland tissues and in the epithelia of the digestive tubules, the inhibition of this

lysozyme at high ionic strengths, and the presence of fewer protease-cutting sites in cv lysozyme 2 compared with cv lysozyme 1 indicated that the main role of the former is in digestion. Although the pH optima for the two lysozymes are in the same range (5.4–6.4 for cv lysozyme 2 compared with 5.5–6.5 for cv lysozyme 1), the lower pI value (6.33) favours the function of cv lysozyme 2 in an acidic environment over that of cv lysozyme 1 with its pI of 8.95. Also, the CGL-2 lysozyme of the Pacific oyster *Crassostrea gigas* is expressed in digestive cells, has a low pI value and a low number of arginine residues and protease cleavage sites, suggesting adaptation to a digestive function similar to the cv lysozyme 2 of *Crassostrea virginica* (Itoh and Takahashi 2007). Olsen *et al.* (2003) demonstrated the presence of four different i-type lysozymes in the blue mussel (*Mytilus edulis*), and proposed that these serve different biological functions, one possible function being the digestion of bacteria. Bivalves also produce g-type lysozymes (cfr section 2) and, in the scallop *Chlamys farreri,* a role as digestive enzyme has been proposed for this enzyme next to its potential immune function (cfr section 5.1, Zhao *et al.* 2007).

In conclusion, many invertebrates from different phyla have lysozymes that show the typical molecular adaptations and expression profiles of digestive lysozymes. However, solid direct experimental evidence for a digestive role is lacking.

5.3 *Other functions of lysozyme*

5.3.1 *Isopeptidase activity:* Besides their lysozyme activity, some i-type lysozymes also have isopeptidase activity, i.e. they hydrolyse isopeptide bonds formed between a glutamine γ-carboxamide and a lysine ε-amino group (Zavalova *et al.* 2000; Takeshita *et al.* 2003). Such bonds are formed, for example, between fibrin molecules in the final steps of blood coagulation by specific transglutaminase enzymes. This cross-linking results in stabilized fibrin, the building block of blood clots. Due to their capability to dissolve stabilized fibrin, isopeptidases are also called destabilases. Since destabilases can reverse the critical final step in blood coagulation, they have drawn a lot of attention in the context of inhibition of thrombus formation (Takahashi *et al.* 1986; Baskova and Nikonov 1991; Fradkov *et al.* 1996; Zavalova *et al.* 1996). In fact, destabilase from the leech has been known for a long time as the enzyme in the salivary gland secretion of the leech that prevents ingested blood from clotting (Baskova and Nikonov 1991). However, the discovery of the lysozyme activity of this enzyme, and its designation as an i-type lysozyme, is of a more recent date (Zavalova *et al.* 2000). The i-type lysozyme from *Tapes japonica* and from *Eisenia andrei* also have both lysozyme and isopeptidase activities (Takeshita *et al.* 2003; Takeshita *et al.* 2004; Jasková *et al.* 2009), and it is in fact assumed that this is true for all i-type lysozymes since, in all these enzymes, a 'destabilase domain' (PF05497, *http://pfam.sanger.ac.uk/*; Finn *et al.* 2008) is present. However, the destabilase activity of i-type lysozymes other than those from the leech and *Tapes japonica* has not been investigated, and the molecular mechanism of isopeptide bond hydrolysis remains unknown till date. Whether all predicted proteins containing the destabilase domain also have lysozyme activity seems questionable, since they are not all designated to the lysozyme clan (CL0037, *http;//pfam.sanger.ac.uk/*).

The functional significance of lysozymes with destabilase activity is not yet clear for all the different invertebrate animals. Lysozyme activity might be involved in preserving blood in the leech stomach where it can remain fresh for months (Zavalova *et al.* 2000).

The increased i-type lysozyme (both Lys i-1 and Lys i-2) expression in the midgut of the mosquito *Anopheles gambiae* after blood-feeding suggests a similar function for these enzymes in breaking down the blood clot. Whether these putative lysozymes truly have muramidase and/or isopeptidase activity has not been investigated (Paskewitz *et al.* 2008).

Remarkably, an isopeptide bond formed between a glutamic acid γ-carboxyl and a lysine α-amino group exists also in the peptidoglycan of many Gram-positive bacteria. The bond is not identical to the fibrin cross-link, however, since the glutamic acid residue has the D-conformation in peptidoglycan. Whether the destabilase activity is able to hydrolyse this bond, and thereby contributes to the bacteriolytic activity of i-type lysozymes, has not yet been demonstrated. However, if this is the case, the muramidase and isopeptidase activities of i-type lysozymes may have both evolved because of their antibacterial action, and may in fact reinforce each other.

5.3.2 *Chitinase activity:* C-type lysozymes and some i-type lysozymes can hydrolyse the β-(1,4)-glycosidic bond in a NAG-polymer, and are therefore said to have chitinase activity (cfr section 3.3). The degradation of chitin takes place with retention of the stereochemistry at the anomeric carbon. The initial distortion of the sugar ring and protonation of the glycosidic oxygen by a protonated acidic residue is similar to the lysozyme catalytic mechanism (*see* section 3.3), but the subsequent nucleophilic attack differs from the lysozyme mechanism since here it involves the N-acetyl group of the sugar which functions as nucleophile, as opposed to a carboxylate side chain on the protein in the case of lysozyme (van Aalten *et al.* 2001). Chitin is the main component of the exoskeletons of crustaceans, arachnids and insects. Since, in these animals, c-, i-, or both c- and i-type lysozymes occur, a role for lysozyme in the recovery of lesions in the chitinous body has been proposed (Li *et al.* 2005).

5.3.3 *Antibacterial agents in animal feed preservation:* HEWL is used as an antibacterial pre-servative in some

industrially produced foods but, in fact, one could argue that this application of lysozyme has been invented first by certain invertebrates who feed on blood. Blood has a very favourable nutrient composition, and thus creates an ideal environment for the multiplication of bacteria and other microorganisms, especially when it is stored before final digestion. Lysozymes might contribute to suppress bacterial growth and degradation of ingested blood by bacteria in these animals (Kopáček *et al.* 1999; Kollien *et al.* 2003; Zavalova *et al.* 2000) by virtue of their antibacterial activity as described in sections 3.3 and 3.4.

6. Conclusion and perspectives

Although the first studies on lysozyme date from the sixties, we can conclude that this enzyme is still a current subject of many research projects (as reflected by the many manuscripts published every year), and knowledge on lysozyme is still growing. While lysozymes from some animal phyla have been well studied and characterized, other animal branches remain little explored regarding the presence, activity and function of lysozyme. Nevertheless, the phylogenetic tree of animals already suggests which types of lysozymes are most likely to be present in a particular species.

Direct antibacterial activity of purified lysozymes seems limited in many cases; however, *in vivo* they often occur together with other permeabilizing molecules, which might be the key to their activity *in vivo*. Lysozyme resistance mechanisms such as O-acetylation and N-deacetylation of peptidoglycan and the production of lysozyme inhibitors possibly allow bacteria (in particular, pathogens) to evade lysozyme antibacterial activity. Further study of lysozymes belonging to different animal classes and, in particular, to little explored animal branches, together with a profound investigation of the spread and functional importance of lysozyme resistance mechanisms should further elucidate the mechanisms of lysozyme action *in vivo*.

Eighty years after its discovery and forty years after the resolution of its three-dimensional structure, lysozyme continues to attract the interest of scientists in disciplines such as structural enzymology, evolutionary biology, animal physiology and immunology, and microbiology. The occurrence and distribution of different types of lysozyme in animals indicates an early origin, but it remains a challenge to clarify their precise genealogy, and this will require more comparative studies. Due to its highly specific antibacterial action and (probably) universal presence in all lineages of animals, lysozyme has become a paradigm for the functioning of the innate immune system. Although the role of lysozyme in antibacterial defence is now well established in some experimental models (e.g. using lysozyme knockout animals), a more detailed insight in the spatio-temporal expression of lysozyme and the precise contribution of lysozyme to antibacterial defence in different tissues is lacking. Furthermore, the observation that most animals have two different types of lysozyme, or at least the genes to produce them, raises the question of whether these would have a complementary role in antibacterial defence. An interesting development is the increasing number of indications that lysozyme may have acquired other functions besides its role in innate immunity. For example, there is good evidence that, following one or more events of gene duplication, the enzyme has been recruited for a digestive function in several animals, both invertebrate and vertebrate. Furthermore, lysozyme-specific peptidoglycan fragments released by the action of lysozyme may act as agonists of inflammatory pathways by binding to molecular pattern receptors of the innate immune system. Finally, the important role of lysozyme is also reflected by the discovery of an increasing number of specific lysozyme inhibitor proteins in bacteria. The role of these inhibitors in bacteria–host interactions needs to be further established, but in view of their specific inhibition of either c- g- or i-type lysozyme, these inhibitors will be very useful tools to help in distinguishing the role of the different lysozymes produced by most animals.

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