Review

Invertebrate lysozymes: Diversity and distribution, molecular mechanism and *in vivo* function

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Lysozymes are antibacterial enzymes widely distributed among organisms. Within the animal kingdom, mainly three major lysozyme types occur. Chicken (c)-type lysozyme and goose (g)-type lysozyme are predominantly, but not exclusively, found in vertebrate animals, while the invertebrate (i)-type lysozyme is typical for invertebrate organisms, and hence its name. Since their discovery in 1975, numerous research articles report on the identification of i-type lysozymes in a variety of invertebrate phyla. This review describes the current knowledge on i-type lysozymes, outlining their distribution, molecular mechanism and *in vivo* function taking the representative from *Venerupis philippinarum* (formerly *Tapes japonica*) (Vp-ilys) as a model. In addition, invertebrate g-type and ch-type (chalaropsis) lysozymes, which have been described in molluscs and nematodes, respectively, are also briefly discussed.

[Van Herreweghe JM and Michiels CW 2012 Invertebrate lysozymes: Diversity and distribution, molecular mechanism and *in vivo* function. *J. Biosci.* **37** 327–348] **DOI** 10.1007/s12038-012-9201-y

1. Introduction

Lysozymes (EC 3.2.1.17), discovered in 1921 by Alexander Fleming, are a heterogeneous family of enzymes, all sharing the capacity of specifically hydrolysing the β-1,4-glycosidic bond between the *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues in the glycan moiety of peptidoglycan (figure 1). Peptidoglycan is an essential and unique cross-linked bacterial cell wall heteropolymer. By forming a mesh-like structure surrounding the entire bacterial cell, it provides structural strength and protects the osmotically sensitive protoplast. Since cleavage of peptidoglycan by lysozymes and other hydrolases results in bacterial cell lysis, the peptidoglycan layer is considered as the Achilles' heel of bacteria (Coyette and van der Ende 2008). Based on differences in amino acid sequence as well as biochemical and enzymatic properties, lysozymes can be classified into several types, and each type shows a particular phylogenetic distribution. The major lysozymes that have been found in animals up to now belong to the c-type (chicken or conventional type), g-type (goose type), i-type (invertebrate type) and ch-type (chalaropsis). Vertebrate animals have only the former two types, while all four types have been found in invertebrates. Additional lysozyme types exist in plants, bacteriophages (viruses) and microorganisms, but since this review focuses on invertebrates, these will not be further discussed. In the classification system of the carbohydrate active enzymes database (CAZy, *http://www.cazy.org/*) (Cantarel *et al.* 2009), g-type lysozyme is in the glycoside hydrolase (GH) family 23 and ch-type lysozyme is part of the GH family 25, while c- and i-type lysozyme are part of the GH family 22.

Both in vertebrates and invertebrates, lysozymes are believed to contribute to antibacterial defence and, in some cases, to digestion. Further, additional functions may be associated with the isopeptidase activity which some i-type lysozymes exhibit besides lysozyme activity. For further reading on the function of lysozymes in vertebrate animals, we refer you to Callewaert and Michiels (2010).

2. Invertebrate-type lysozyme: Discovery and phylogenetic distribution

The existence of a typical invertebrate lysozyme type (i-type) was first proposed in 1975 based on the comparison of the

Keywords. Catalytic mechanism; crystal structure; *in vivo* function; invertebrates; lysozymes; phylogeny

Published online: 1 May 2012

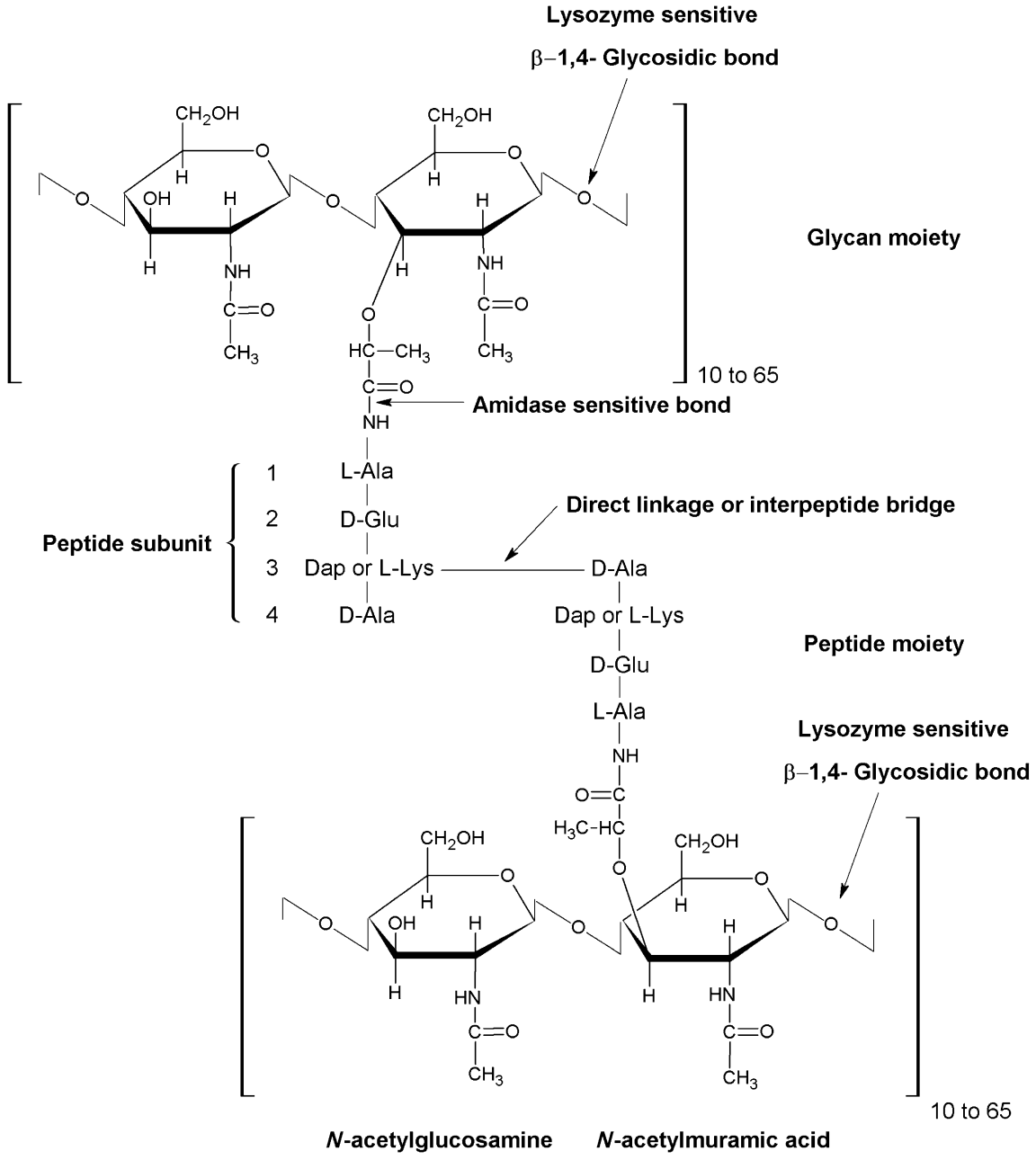

Figure 1. Structure of a fragment of the peptidoglycan layer representing two cross-linked disaccharide tetrapeptide units. An important variation in the peptidoglycan is observed at the third residue of the peptide unit (L-Lys in Gram-positive bacteria and diaminopimelic acid in Gram-negative bacteria and *Bacilli*), and in the cross-link between the peptide side chains, which can either occur by direct linkage or by an interpeptide bridge.

N-terminal sequence of a novel lysozyme isolated from the marine invertebrate *Asterias rubens* (starfish, echinodermata) with those from other, at that time, known lysozyme types including chicken, goose and phage type (Jollès and Jollès 1975). Only in 1999, amino acid sequence analysis of lysozymes from the marine bivalve *Venerupis philippinarum* (complete sequence), the marine conch *Lunella coronata* (N-terminal sequence) and the earthworm *Eisenia fetida* (N-terminal sequence), and the occurrence of homologs in nucleic acid databases, fully confirmed the i-type lysozymes as a unique invertebrate lysozyme widely distributed along different invertebrate phyla including molluscs, annelids, echinoderms and nematodes (figure 2) (Ito *et al.* 1999). As illustrated in figure 2 not only i-type lysozymes

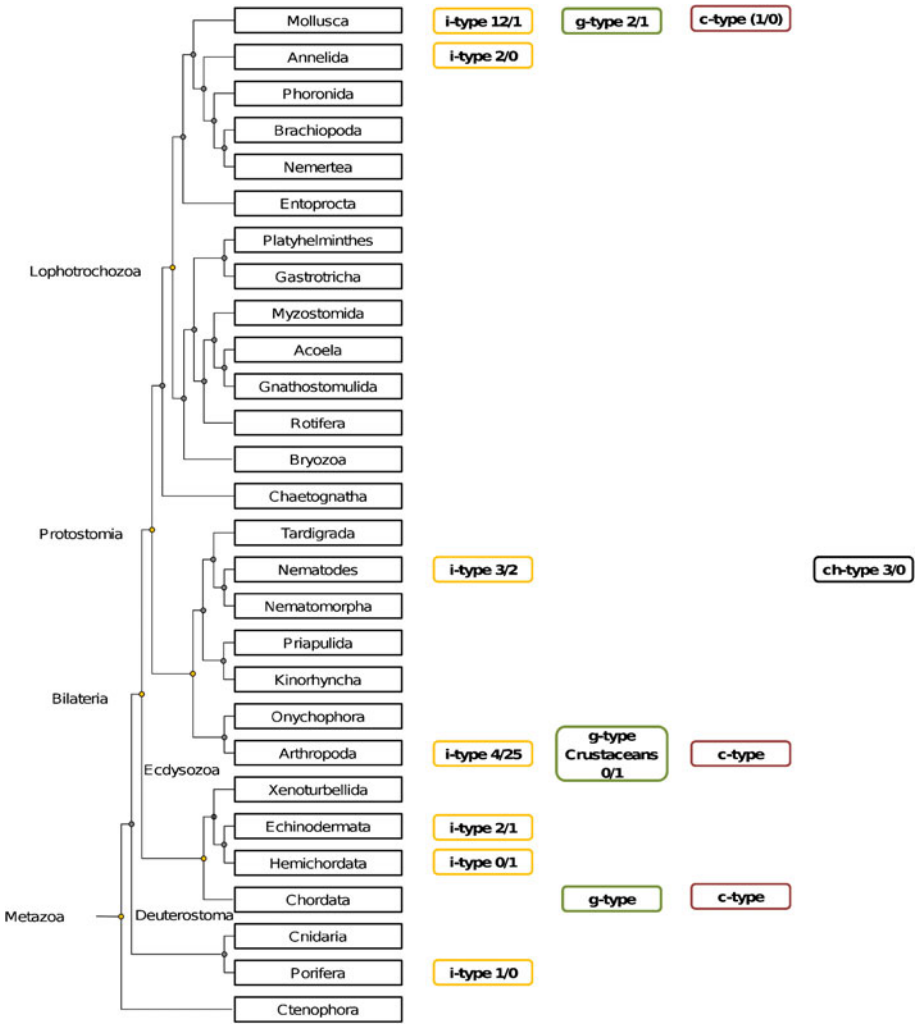

Figure 2. Phylogenetic distribution of the different lysozyme types along the invertebrate phyla indicated on a cladogram adapted from Dunn *et al.* (2008). Indication of the presence of a certain lysozyme type in a certain phylum is based on one or more of the following: (i) publications describing the isolation and characterization; (ii) literature describing the cloning and/or expression; (iii) existence of annotated lysozyme genes in publicly available databases and (iv) presence of putative lysozyme homologs as based on BLAST analysis of the NCBI non-redundant protein database (conducted may 2011). The numbers (#/#) indicate the number of species in which a certain lysozyme type has been experimentally studied (table 1)/ the number of species in which the same lysozyme type is found based on a BLAST search, but not studied (table 2 for a complete list of the latter).

occur in invertebrates, but within some phyla c-type (molluscs and arthropods), ch-type (nematodes) (section 6.2) or g-type lysozymes (molluscs and arthropods) are additionally found. During a search for homologs with the complete protein sequence of the *Venerupis philippinarum* lysozyme (Vp-ilys), 46% primary sequence identity was observed with a salivary gland protein (section 5.2) of the medicinal leech *Hirudo medicinalis* known as destabilase, which was later proven to be an i-type lysozyme (Hm-iLys) (Zavalova *et al.* 2000). Homology was also observed with several hypothetical proteins of the nematode *Caenorhabditis elegans*. A multiple alignment of Vp-ilys, Hm-iLys and a homolog from *C. elegans* revealed a high content and conservation of cysteines, and suggested the formation of intramolecular disulphide bridges, which would explain the observed structural stability of the proteins (Ito *et al.* 1999). Meanwhile, experimental studies reported on i-type lysozymes in various invertebrates, based on different types of evidence (see table 1 for an overview).

Combined with a BLASTp analysis with the Vp-ilys sequence in the NCBI database for i-type lysozymes not experimentally studied (see table 2 for an overview), this reveals the occurrence of i-type lysozyme homologs in the following phyla: porifers, molluscs, annelids, nematodes,

Table 1. List of experimentally studied i-type lysozymes

Phylum and species	Organ/cells	Lysozyme name	Type of evidence*					Accession number	Reference
			A	B	C	D	E		
Echinodermata									
Asterias rubens	whole body		X		X			AAR29291	Jollès and Jollès 1975
Apostichopus japonicus	extracted from body wall, intestine and digestive tract	SjLys		X		X		ABK34500	Cong *et al.* 2009
Molluscs									
Venerupis philippinarum (Tapes japonica)	hemocytes, gills and digestive gland lesser degree in foot, mantle and muscle	Vp-ilys	X	X	X	X		BAB33389	Ito *et al.* 1999 Takeshita *et al.* 2004 Zhao *et al.* 2010
Lunella coronata	viscera and muscles		X		X				Ito *et al.* 1999
Chlamys islandica	viscera, crystalline style	chlamysin	X		X			CAB63451	Nilsen *et al.* 1999
Mytilus edulis	soft body, digestive gland	Me-iLys-Bm	X	X	X			AF334662	Olsen *et al.* 2003 Bachali *et al.* 2002
	crystalline style	Me-iLys-sA, Me-iLys-sB, Me-iLys-sC,	X X X						McHenery and Birkbeck 1982; Olsen *et al.* 2003
	unknown	Me-ilys2						ABB76765	Caponera and Rawson**
Mytilus galloprovincialis	digestive gland	Mg-iLys1	X	X				AAN16210	Bachali *et al.* 2002
	unknown	Mg-iLys2						BAF63423	Itoh and Takahashi (2007)**
Bathymodiulus thermophilus	digestive gland		X	X				AF334664	Bachali *et al.* 2002
Bathymodiulus azoricus	digestive gland		X	X				AF334663	Bachali *et al.* 2002
Crassostrea virginica	digestive gland, crystalline style basophil cells of digestive tubules	Cv-iLys 2	X	X	X	X	X	BAE47520	Xue *et al.* 2007
	style sac midgut, digestive gland, gills labial palps, mantle, gonad	Cv-iLys 1	X		X	X	X	BAE93114	Xue *et al.* 2007
	digestive gland, mantle, labial palps, gills, style sac-midgut, hemoctyes	Cv-iLys3	X	X	X	X		BAG41979	Xue *et al.* 2010
Saxidomus purpurata	digestive gland		X		X				Miyauchi *et al.* 2006
Ostrea edulis	digestive organs			X				BAD19060	Matsumoto *et al.* 2006
Crassostrea gigas	digestive gland, gills, mantle, gonads, hemocytes basophil cells of digestive tubules	Cg-iLys1		X		X	X	BAD19059	
	digestive gland, gills	Cg-iLys2		X			X	BAF48044	Itoh and Takahashi 2007
	mantle	Cg-iLys3		X		X		BAF94156	Itoh *et al.* 2010
	unknown	Cg-iLys4							***

Table 1. (continued)

Phylum and species	Organ/cells	Lysozyme name	Type of evidence*					Accession number	Reference
			A	B	C	D	E		
Calyptogena sp.1	gills		X	X				AF334666	Bachali *et al.* 2002
Calyptogena sp.2			X	X				AF334667	
Nematodes									
Caenorhabditis elegans	unknown	Ce-ilys1	Genome analysis					CE17548	Schulenburg and Boehnisch 2008
	intestine	Ce-ilys2						CE17549	
	intestine	Ce-ilys3						CE24850	
	unkown	Ce-ilys4						CE3458	
	intestine	Ce-ilys5						CE04442	
Annelids									
Hirudo medicinalis	salivary gland	Hm-iLys	X	X				AAA96144	Zavalova *et al.* 1996
Eisenia andrei	coelomocytes	Ea-iLys		X		X		ABC68610	Josková *et al.* 2009
Eisenia fetida	homogenate whole body		X		X				Ito *et al.* 1999
Arthropods									
Anopheles gambiae	fat body and malphigin tubules	Ag-iLys 1				X		AAT51799	Paskewitz *et al.* 2008
	fat body	Ag-iLys2				X		ABP35929	
Penaeus monodon	digestive gland	Pm-iLys 1		X		X		ACZ63471.1	Supungul *et al.* 2010
	antennal gland, eyestalk, hemocytes and heart (highest expression)	Pm-iLys 2		X		X		ACZ63472.1	
Procambarus clarkii	hemocytes, heart and stomach	Pc-iLys1		X		X		Copied from article	Zhang *et al.* 2010
		Pc-iLys2		X		X			
Porifers									
Suberites domuncula	gray cells mesohyl****	Sd-iLys		X			X	CAG27844	Thakur *et al.* 2005

*__A__: Protein extraction and purification, __B__: RNA isolation and cDNA synthesis, __C__: N-terminal protein sequence determination, __D__: RT-PCRand __E__: *(in situ)* hybridization.

**Direct submission in NCBI protein databank.

***Direct submission in NCBI EST databank.

****Mesohyl: Gelatinous matrix within a sponge filling the space between the external pinacoderm (epidermis) and the internal choanoderm (cell layer that lines the inner central cavity of sponges).

Table 2. List of invertebrate g-type and i-type lysozymes found by a BLAST search in the NCBI protein database with the g-type lysozyme of *Anser anser* and i-type lysozyme of *Venerupis philippinarum*, which are not experimentally studied (see table 1 for an overview of the experimentally studied i-type lysozymes)

Phylum	Class	Species	Method	Accession number
g-type lysozyme				
Crustacea		*Caligus rogercresseyi*	*	ACO11114
Mollusca		*Argopecten irradians*	*	AAX09979
i-type lysozyme				
Echinodermata		*Strongylocentrotus purpuratus*	**	XP_788343
Hemichordata		*Saccoglossus kowalevskii*	****	XP_002733318
Nematoda		*Loa Loa*	****	EFO27626
		Brugia malayi	****	XP_001898972
Arthropoda	Crustacea	*Litopenaeus vannamei*	*	ABD65298
		Rimicaris exoculata	*	R23733
	Insecta	*Apis mellifera*	****	XP_393161
		Acyrthosiphon pisum	***	XP_001949212
		Aedes aegypti	****	XP_001653399.1 & XP_001654787.1
		Anopheles gambiae str. PEST	*	XP_309527
		Camponotus floridanus	*	EFN71839
		Culex quinquefasciatus	*	XP_001850854
		rosophila ananassae	*	XP_001960609
		Drosophila erecta	*	XP_001975295
		Drosophila grimshawi	*	XP_001995414
		Drosophila melanogaster	*	AAL49382
		Drosophila mojavensis	*	XP_002006619
		Drosophila persimilis	*	XP_002016415
		Drosophila pseudoobscura	*	XP_001360781
		Drosophila sechellia	*	XP_002035647
		Drosophila simulans	*	XP_002081833
		Drosophila virilis	*	XP_002048990
		Drosophila willistoni	*	XP_002063642
		Drosophila yakuba	*	XP_002092148
		Glossina morsitans morsitans	*	ADD20033
		Harpegnathos saltator	*	EFN89916
		Ixodes scapularis	*	XP_002410814
		Nasonia vitripennis	**	XP_001600829
		Pediculus humanus corporis	*	XP_002432292

*Conceptual translation.

**Genome annotation confirmed by EST.

***EST.

****Genome annotation.

echinodermates, hemichordates and arthropods (figure 2). Based on this distribution, occurrence of homologs in all invertebrate phyla is likely, but difficult to prove due to a lack of sequences in the database for some phyla. Remarkably, some invertebrates belonging to the phyla of the molluscs (e.g. *Mytilus edulis, Crassostrea gigas,* etc.), nematodes (*Caenorhabditis elegans*) and arthropods (e.g. *Anopheles gambiae,* etc.) encode multiple i-type lysozymes differing in their cellular localization and/or biochemical properties, suggesting a functional differentiation (section 6).

3. Structural basis for the catalytic mechanism of i-type lysozyme

3.1 *Crystal structure of Venerupis philippinarum i-type lysozyme (Vp-ilys)*

Chitinases, chitosanases and lysozymes are all glycoside hydrolases (E.C. 3.2.1.-) active on very similar substrates in which the monomer building blocks are all linked by a β-1,4-glycosidic bond (chitin: homopolymer of *N*-acetylglucosamine, chitosan: deacetylated chitin, and peptidoglycan: heteropolymer of *N*-acetylmuramic acid and *N*-acetylglucosamine). In spite of the low overall amino acid sequence similarity between these glycoside hydrolases, it was found that a particular group among them, including GH family 22 (i- and c-type lysozymes), GH23 (g-type lysozyme), GH24 (viral or v-type lysozyme), GH46 (chitosanases) and GH19 (chitinase), shares a common global fold in their tertiary structure (Strynadka and James 1996; Wohlkönig *et al.* 2010). As illustrated in figure 3 for the invertebrate-type lysozyme Vp-ilys and the vertebrate lysozymes HEWL and GEWL, this common fold is shaped by two domains. One structural domain mainly consists of at least two antiparallel β-strands forming a β-sheet structure, and is connected by a long α-helix to a second α-helix-based domain, comprising the N-terminal and C-terminal protein part. As such, both domains form a negatively charged deep active site cleft. All lysozymes that are proven to be enzymatically active have a conserved glutamate catalytic residue, and apart from g-type lysozymes, most other lysozymes additionally have a catalytically active aspartate residue. As illustrated in figure 3, the glutamate residue is embedded in the α-helical domain while the aspartate residue is part of the β-sheet, and both protrude into the active site cleft from opposite sides. Recently, by searching for structurally equivalent residue ranges using superpositioning, not only a common global fold, but also a common structural core consisting of two regions was discovered within the above mentioned GH families (figure 3) (Wohlkönig *et al.* 2010). One region is the C-terminal part of the central helix of the alpha domain, which contains the glutamate active site residue. The second region consists of a β-hairpin from the β-strand domain. Based on amino acid conservation profiling, this β-hairpin region was shown to display a family specific signature motif (figure 5 and section 4) (Wohlkönig *et al.* 2010). Additionally, the structural comparison of these glycoside hydrolases also confirmed the earlier observed close relation between c-type and i-type lysozyme (Bachali *et al.* 2002; Goto *et al.* 2007). Based on folding similarity between Vp-ilys and HEWL, and further supported by site-directed mutagenesis, the amino acids Glu18 and Asp30 were indicated as the catalytic residues of Vp-iLys (Goto *et al.* 2007). In addition, the crystal structure confirmed the earlier postulate that all cysteines present in Vp-ilys are involved in disulphide bridges (Nilsen *et al.* 1999).

3.2 *Catalytic mechanism of i-type lysozymes inferred from Vp-ilys-NAG$_3$ crystal structure*

Phillips and co-workers already proved in 1966 that an oligomer of *N*-acetylglucosamine residues, figuring as a substrate analogue of the glycan moiety of the peptidoglycan, binds to six NAG binding subsites (A–F) present in the active site cleft of Hen Egg-White lysozyme (HEWL, c-type) (Phillips 1966). Analogously, a NAG trimer (NAG_3) binds to subsites A–C of the six present subsites in the active site cleft of Vp-ilys, where it is fixed by hydrogen bonds, as well as Van der Waals forces and hydrophobic interactions (Goto *et al.* 2007). The actual hydrolysis of the β-1,4-glycosidic bond between NAM and NAG residues of the peptidoglycan by invertebrate-type lysozymes occurs by a double displacement

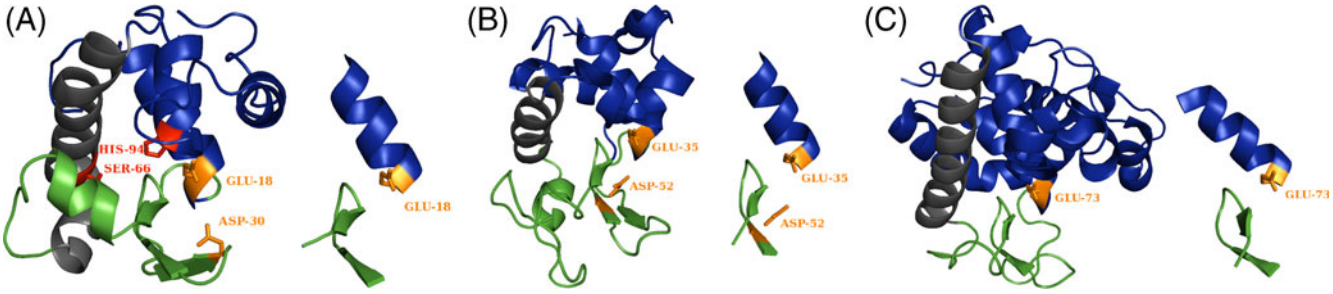

Figure 3. Ribbon diagram of the global fold of a *Venerupis philippinarum* lysozyme monomer (**A**), Hen Egg-White lysozyme (**B**) and Goose Egg-White lysozyme (**C**), constructed using PyMOL software from entries 2DQA, 153L and 1IEE in the PDB database (Berman *et al.* 2003). The β-sheet-based domain of the common glycoside hydrolase fold is shown in green, the α-helix domain in blue and the long interconnecting helix in grey. The residues involved in muramidase activity are represented in orange and those potentially involved in isopeptidase activity in red. The two regions forming the common structural core of glycoside hydrolases GH22, GH23, GH24, GH46 and GH19 (Wohlkönig *et al.* 2010) are duplicated from the structure and shown on the right-hand side.

mechanism proceeding through a covalent enzyme–substrate intermediate, as was described earlier for HEWL (Kirby 2001, Vocadlo *et al.* 2001) and recently proven for Vp-ilys (figure 4) (Goto *et al.* 2007). In the first step of the reaction, the carboxylate group of Asp30 acts as a nucleophile leading to the formation of a glycosyl-lysozyme intermediate with inversion of the anomeric configuration of the NAM residue (β- to α-anomer) (see step 1 and 2 in figure 4). Glu18 acts as a general acid by donating a proton to the glycosidic oxygen and thereby facilitating bond cleavage. In a second step the lysozyme is displaced from the covalent intermediate by water, restoring the original anomeric configuration (see step 3 and 4 in figure 4) and resulting in the protonation of the Glu18 carboxylate.

Based on the crystal structure of Vp-ilys in complex with NAG_3 as a substrate homolog, Goto *et al.* (2007) also identified the residues potentially involved in the interaction with the substrate by means of hydrogen bond formation (Gln40, Lys42, Tyr45, Asp48, Tyr77 and His94), or Van der Waals forces and hydrophobic interaction (Pro44, Tyr45, Tyr77, His94 and Pro98).

4. Multiple sequence alignment of invertebrate-type lysozymes

A multiple sequence alignment, represented as a logo sequence in figure 5A, of 35 experimentally studied invertebrate lysozymes (table 1; lysozymes with indicated accession number), belonging to different invertebrate phyla, confirms the nearly complete conservation of 14 cysteine residues. Together with the observation in the crystal structure of Vp-ilys that all of these residues are involved in disulphide bridge formation, this high number of disulphide bridges clearly distinguishes i-type lysozymes from c-type (4 disulphide bridges), g-type (mammalian: 2; fish: none; invertebrate: potentially 3 up to 6) and ch-type (1) lysozymes. Disulphide bridges often confer stability to proteins, and

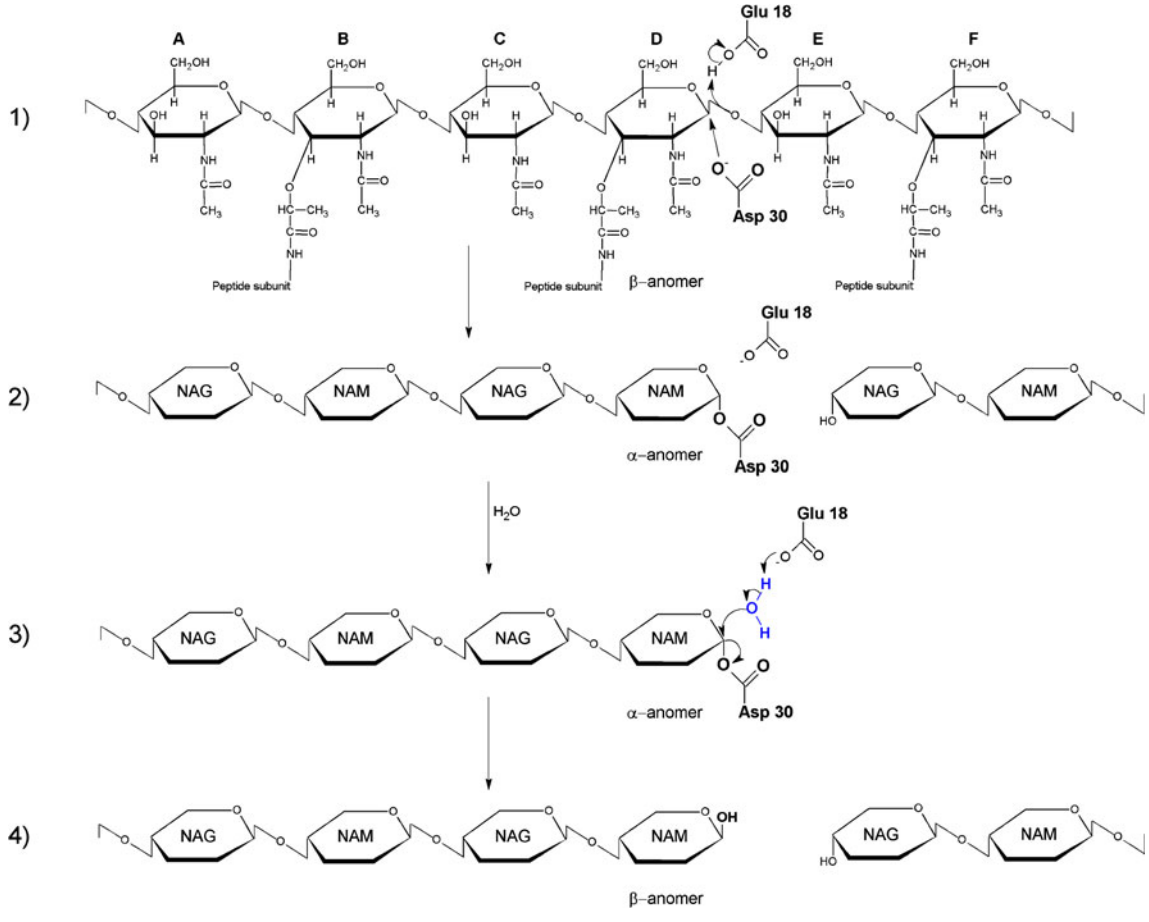

Figure 4. Scheme of the mechanism of hydrolysis of the β-1,4-glycosidic bond between NAM and NAG residues in peptidoglycan by invertebrate type lysozyme. Hydrolysis occurs by a double displacement mechanism proceeding by a covalent enzyme–substrate intermediate, as was earlier described for HEWL (Vocadlo *et al.* 2001) (Kirby 2001) and recently proven for Vp-ilys (Goto *et al.* 2007).

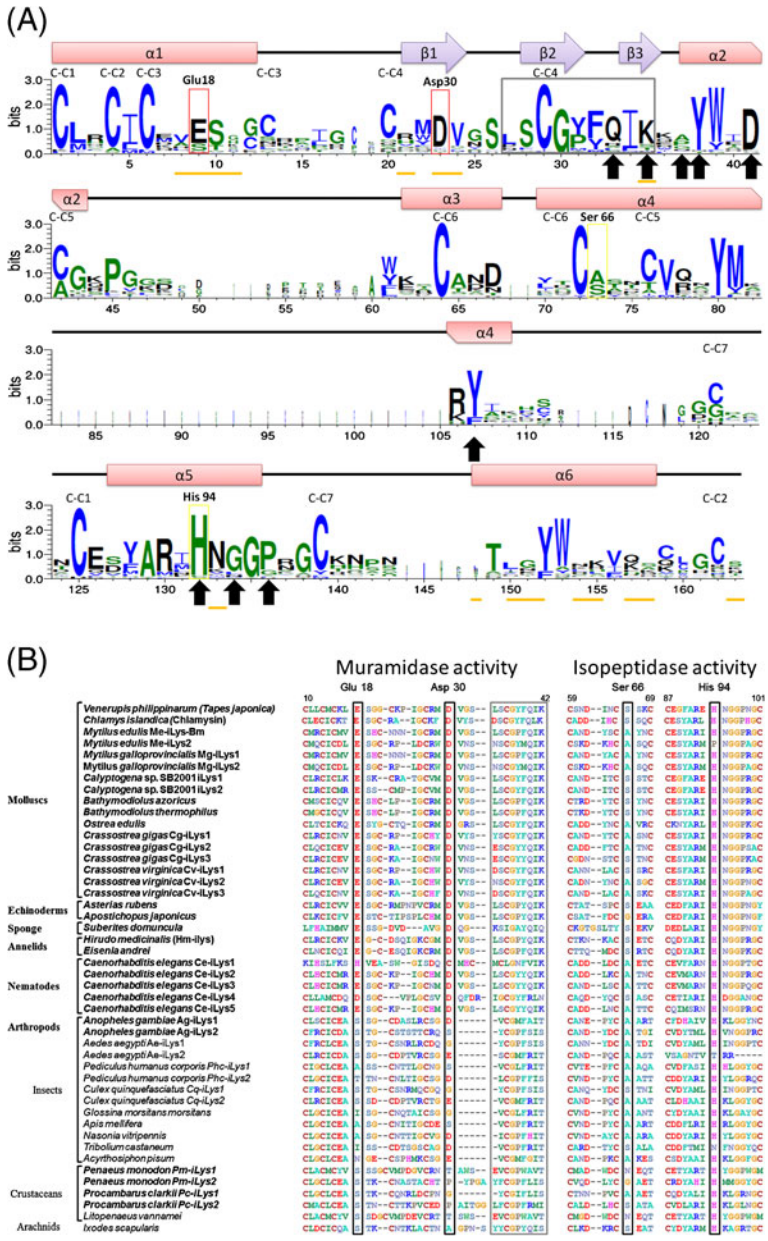

Figure 5. (**A**) Sequence logo built with Weblogo 3. (Crooks *et al.* 2004) , showing the conservation of amino acids in i-type lysozymes based on a multiple sequence alignment (built with Clustal X (Larkin *et al.* 2007)) of 35 invertebrate- type lysozymes with superposition of the secondary structure of Vp-ilys. Lysozymes included in the analysis are represented in boldface in (**B**); see table 2 for their corresponding accession numbers. Signal peptides were predicted by Signal P 3.0 (Bendtsen *et al.* 2004) and removed prior to alignment. Poorly aligning N-terminal and C-terminal parts were removed. Amino acids are coloured by hydrophobicity: hydrophobic, blue; neutral, green; hydrophilic, black. Disulphide bridge forming cysteines are indicated by c-cx, active site residues for muramidase activity by red boxes and for isopeptidase activity by yellow boxes, all based on those identified in Vp-ilys. Additional residues involved in the interaction with the substrate are indicated by a black arrow and those involved in lysozyme dimer formation by an orange line. The grey box illustrates the conservation of the GH22i (i-type lysozymes) specific signature (LSCGYFQIK for Vp-iLys) (**B**) Multiple sequence alignment (built with Clustal X (Larkin *et al.* 2007)) of the muramidase and isopeptidase active site region of experimentally studied i-type lysozymes (bold) and i-type lysozymes found by a BLASTp search in the NCBI database restricted to the phylum of the arthropods which are not experimentally studied (non-bold), showing the conservation of the active site residues for muramidase and isopeptidase activity in invertebrate lysozymes of several phyla based on those identified in Vp-ilys. The grey box illustrates the conservation of the GH22 i-type lysozymes specific signature (LSCGYFQIK for Vp-iLys).

Vp-ilys was indeed proven to resist better to denaturation than c-type lysozymes (HEWL and human) in a guanidine-HCl denaturation assay (Ito *et al.* 1999). Additionally, the alignment (figure 5) also confirms the complete conservation of the earlier identified active site residues responsible for the muramidase activity of Vp-ilys (Glu18 and Asp30 in Vp-ilys), in all characterized invertebrate-type lysozymes of the mollusc phylum, including the multiple lysozyme gene paralogs identified in the same species (*M. edulis*, *M. galloprovincialis*, *C. gigas* and *C. virginica*). The same observation is true for the lysozymes from echinoderm, porifer and annelid phyla, although with reservation since only a few lysozyme sequences are available in the databases for these phyla. The nematode *Caenorhabditis elegans* possesses a large repertoire of fifteen putative lysozyme genes including five i-type lysozymes. However, based on the multiple alignment (figure 5B), two among these (Ce-ilys1 and Ce-ilys4) are predicted to be inactive due to the absence of one (Ce-iLys4) or both (Ce-iLys1) active site residues.

Remarkably, predicted amino acid sequences from cDNA of the experimentally studied i-type lysozymes from the arthropod species *Anopheles gambiae* (Ag-iLys1 and 2) (mosquito), *Penaeus monodon* (Pm-ilys1 and 2) (giant tiger prawn) and *Procambarus clarkii* (Pc-iLys1 and 2) (red swamp crawfish) revealed that these enzymes are all missing the glutamate and all except Ag-iLys1 also the aspartate residue, both critical for muramidase activity of i-type lysozymes (Paskewitz *et al.* 2008). Inclusion of 13 additional sequences of arthropod i-type lysozyme homologs, identified by BLASTp search with Vp-ilys in the NCBI protein database, in the multiple sequence alignment (figure 5), confirmed that muramidase deficiency due to the absence of at least one of the catalytic residues might be a common feature of arthropod i-type lysozymes.

It is also worthwhile to note that the non-catalytic residues in Vp-ilys, earlier postulated by Goto *et al.* (2007) to be involved in the interaction with the substrate analogue NAG_3, are also fairly well conserved in i-type lysozymes. Finally, the alignment in figure 5 confirms that the specific signature motif discovered within the β-hairpin region of the common structural core of GH19, GH22, GH23, GH24 and GH46 families (section 3) is conserved in all i-type lysozymes (LSCGYFQIK for Vp-iLys) except for the arthropod representatives.

5. Invertebrate lysozyme as a multifunctional enzyme

5.1 *Muramidase/chitinase activity and its modulation by dimer formation*

Most lysozymes exhibit, besides muramidase activity, also chitinase activity, probably as a result of the similarity between peptidoglycan (heteropolymer of β-1,4 linked *N*-acetylmuramic acid and *N*-acetylglucosamine), the natural substrate of lysozymes, and chitin (homopolymer of β-1,4 linked *N*-acetylglucosamine), the natural substrate of chitinases. Moreover, some lysozymes and chitinases/chitosanases were found to be structurally related (section 3.1) (Wohlkönig *et al.* 2010). Besides warding off fungal infections there is no other clear function of the chitinase activity possessed by lysozymes. Among the i-type lysozymes, some have chitinase activity, while others, like chlamysin from *Chlamys islandica* and the i-type lysozyme from the sea cucumber *Asostichopus japonicus*, were shown to be unable of hydrolyzing chitin (Nilsen *et al.* 1999; Cong *et al.* 2009). The molecular basis for this difference is unknown. One of the i-type lysozymes which possess chitinase activity is the *Venerupis philippinarum* lysozyme (Vp-ilys). The quaternary structure in the Vp-iLys crystal revealed dimer formation by two Vp-ilys molecules, which was confirmed to be maintained in an aqueous solution with a low salt concentration (83 mM NaCl) by means of gel filtration chromatography (Goto *et al.* 2007). This dimer formation was proposed to be sustained by electrostatic interactions between the catalytic residues (Glu18 and Asp30) in one molecule and positive residues (Lys108 and 115) at the C-terminal helix 6 of the other molecule (marked by an orange line in figure 5A; figure 6). As a result, the active sites of both molecules involved are blocked by dimer formation, thereby suppressing chitinase and muramidase activity. The observation that high salt concentrations (≥ 133 mM) increased chitinase activity at low, but not at high protein concentration (> 10 μM), was assumed to result from the dissociation of the Vp-ilys dimer at high ionic strength. From these observations emerged the idea that the chitinase (and hence also muramidase activity) of Vp-ilys is modulated by environmental salt concentrations and by its own concentration. Whether this feature is common

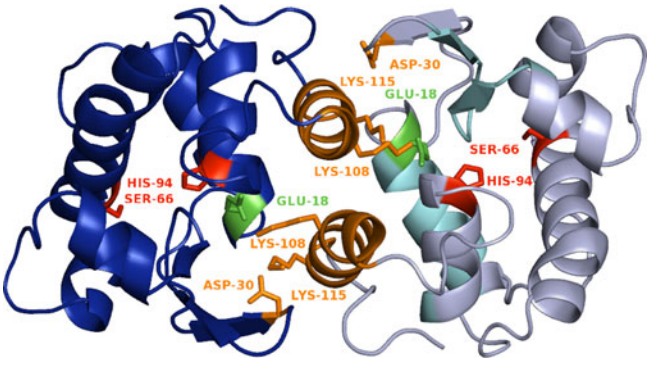

Figure 6. Ribbon diagram of a dimer of the *Venerupis philippinarum* lysozyme. The residues involved in muramidase activity are represented in green and those potentially involved in isopeptidase activity in red. Helix 6 and residues Lys 108 and 115 are shown in orange. Figure adapted from entry 2DQA 'Crystal structure of *Tapes japonica*' in the PDB database using PyMOL software.

for the invertebrate-type lysozymes is unknown. Some literature reports a modulation of lysozyme activity of some invertebrate lysozymes from mollusc species, such as the blue mussel and oyster, by differences in ionic strength and presence of divalent cations, however, without direct evidence for the involvement of dimer formation (Olsen *et al.* 2003; Xue *et al.* 2004; 2007). The residues involved in the electrostatic interactions sustaining the dimer formation in Vp-ilys, especially those in alpha helix 6, are not well conserved among i-type lysozymes (figure 5). From a physiological point of view, this salt and protein concentration-dependent quaternary regulation of lysozyme activity was proposed to allow rapid response of the organism to ingestion or invasion of bacteria suspended in seawater (500 mM NaCl), by the release of lysozyme stored in high concentrations in the inactive dimer form. Such a response could be important for digestion of bacteria as feed or for defence against bacterial pathogens (Goto *et al.* 2007).

5.2 *Isopeptidase activity*

The amino acid sequence of the first completely sequenced i-type lysozyme (Vp-iLys) (Ito *et al.* 1999) showed 46 % sequence identity with Hm-iLys present in the salivary gland secretion of the medical leech (*Hirudo medicinalis*). This enzyme was initially discovered based on its capacity to split isopeptide bonds[1] by means of endo-ε-(γ-Glu)-Lys isopeptidase activity (Fradkov *et al.* 1996; Zavalova *et al.* 1996). It owes its name to the ability to dissolve fibrin clots, in which isopeptide bonds occur as cross-links formed by transglutaminase (Factor XIIIa) between glutamine γ-carboxamide and ε-lysine amino groups in a final step of the blood clotting cascade. Besides its thrombolytic activity, Zavalova *et al.* (2000) demonstrated that lysozyme activity is an intrinsic property of destabilase, confirming that it can be equally well considered as an invertebrate type lysozyme (Hm-ilys). Catalytic residues potentially responsible for the isopeptidase activity of Hm-iLys, serine 62 and histidine 92 were predicted by Zavalova *et al.* (1996) based upon inhibition of Hm-iLys by a typical serine protease inhibitor (PMSF, phenylmethanesulphonylfluoride) and short motifs loosely homologous to serine protease consensus sequences (Fradkov *et al.* 1996). This prediction is supported additionally by indirect evidence showing that Vp-ilys, Ea-iLys (*Eisenia andrei,* annelids) and Pc-ilys2 (*Procambarus clarkii*, arthropods), in which both residues are conserved, possess isopeptidase activity, while Me-iLys-Bm (*Mytilus edulis*, body mass associated, molluscs) and Pc-ilys1, in which the serine residue is replaced by an alanine, are isopeptidase deficient (Bachali *et al.* 2002; Takeshita *et al.* 2003; Josková *et al.* 2009; Zhang *et al.* 2010). Moreover, upon specific inhibition of the isopeptidase activity of Vp-ilys, its muramidase activity was found to be unaffected, leading Takeshita *et al.* (2003) to conclude that Vp-ilys is a chimerically bifunctional protein with distinct activities exhibited by independent active sites. The multiple alignment of several i-type lysozymes (figure 5) clearly illustrates that the serine residue potentially involved in isopeptidase activity is not conserved, but replaced mainly by alanine in many i-type lysozymes. If this residue is indeed involved in catalysis, only a minority of the i-type lysozymes would possess isopeptidase activity. Within the crystal structure of Vp-iLys (Goto *et al.* 2007), the predicted isopeptidase catalytic residues (serine 66 and histidine 94) are not located in a groove as would be expected for a catalytic site (figures 3A and 6). Therefore, the issue of the isopeptidase activity remains enigmatic, and more rigorous evidence confirming the involvement of these catalytic residues in the isopeptidase activity of i-type lysozymes and a better understanding of the catalytic function should come from site-directed mutagenesis and/or crystallographic analysis of i-type lysozyme in complex with an isopeptide-type substrate.

Although blood clotting has little to do with bacteriolysis at first sight, there is a potentially interesting functional link between the lysozyme and isopeptidase activities of i-type lysozymes. In the peptidoglycan of some Gram-positive bacteria, the lysine and glutamate in the peptide side chain are cross-linked by an isopeptide bond (figure 1) that only differs from the isopeptide entity in fibrin in that the glutamine residue is a D-enantiomer as opposed to an L-enantiomer in stabilized fibrin clots, and that the bond is formed with the α-amino group of lysine as opposed to the ε-amino group of lysine in blood clots. Since Vp-ilys has isopeptidase activity against the chromogenic substrate D-γ-Glu-P-nitroanilide, the isopeptidase activity was proposed to potentially enhance the lytic activity of Vp-ilys in synergy with its muramidase activity. However, this has not been proven so far, since specific inhibition of the isopeptidase activity of Vp-ilys by AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride) did not affect the enzyme's lytic activity in a *Micrococcus* lysis assay (Takeshita *et al.* 2003).

5.3 *Non-enzymatic antimicrobial activity*

Besides the widely recognized enzymatic hydrolysis of the peptidoglycan layer of bacteria, some lysozymes are believed to display also a non-enzymatic antimicrobial activity. Until now such non-enzymatic activity of i-type lysozymes was suggested for Hm-iLys and Aj-ilys of the sea cucumber *Asostichopus japonicus*, based on the observation of residual antibacterial activity in muramidase-

[1] Isopeptide bond: amide linkage between a carboxyl or carboxamide group of one amino acid and an amino group of another amino acid in which at least one of these groups is not on the amino acid α-carbon.

deficient heat-inactivated enzymes (Cong *et al.* 2009), and in Pc-ilys1 of the red swamp crayfish *Procambarus clarkia*, which is naturally muramidase-deficient. As the non-enzymatic activity was only proven for a limited number of i-type lysozymes and the molecular basis for this activity is lacking, additional research is needed to confirm the consistency of this feature.

6. Contribution of lysozymes to the immune and digestive system of invertebrate organisms

Most vertebrate and invertebrate organisms share the environment in which they live with bacteria, with which they may enter into mutualistic, commensalic or parasitic interactions. Unlike vertebrate organisms, invertebrates lack an adaptive immune system and as a result solely depend on their innate immune system to cope with the threat of pathogenic bacteria. Lysozymes, together with other effector molecules, are considered to be key components of this host defence. Several invertebrates are known to feed on bacteria. For example, marine bivalves (including scallops, clams, oysters and mussels) are known as filter feeders. By drawing water over their gills, suspended food particles including bacteria are trapped on the mucus of the gills from where it is passed on to the gastrointestinal tract. Worms and flies often feed on decomposing organic matter including the large biomass of microorganisms causing the decomposition. This implies that some invertebrates not only require a well-developed immune system but also a digestive system capable of digesting bacteria. For this reason, i-type lysozymes of some invertebrates are also believed to be involved in digestion, similar to some c-type lysozymes in vertebrate organisms (for example ruminants). The following paragraphs give an overview on the current knowledge of the function of i-type lysozymes in several invertebrate phyla. Figure 7 shows a phylogenetic analysis of all i-type lysozymes discussed.

6.1 *Bivalves*

6.1.1 Bivalve i-type lysozymes: Based on the occurrence of lysozyme(−like) activity in hemolymph and crude extracts of specific cells (hemocytes) and organs (digestive gland, crystalline style, gills) of bivalves, lysozymes were hypothesized to take part in both digestion (McHenery *et al.* 1979) and defence (Carballala *et al.* 1997; Allam and Paillard 1998; Allam *et al.* 2000) in marine bivalves. Recent studies of bivalve i-type lysozymes including biochemical characterization of naturally extracted or by recombinant DNA-technology produced lysozymes, RT-PCR analysis and *in situ* hybridization revealed the existence in several organisms of multiple paralogous i-type lysozymes varying in biochemical characteristics and spatiotemporal expression patterns (Olsen *et al.* 2003). These findings suggest that bivalve invertebrates may have evolved a battery of lysozymes with specialized functions. Based on tissue-specific protein extraction, subsequent purification and biochemical characterization (pH, ionic strength, divalent cations), *M. edulis* was found to produce four i-type lysozymes, three crystalline-style-associated lysozymes (Me-ilys-sA, Me-ilys-sB, Me-ilys-sC) and one soft-body lysozyme (Me-ilys-Bm) considered as the major mussel lysozyme. The soft body lysozyme (Me-ilys-Bm) is believed to be a hemocyte-produced enzyme involved in antibacterial defence, while lysozymes from the digestive gland-associated crystalline style (figure 8) are believed to be involved in digestion. However, since Me-ilys-Bm was also purified from the digestive gland, a digestive role cannot be ruled out (Olsen *et al.* 2003). Independent from this study, a complete protein sequence of an i-type lysozyme from *M. edulis* (Me-iLys2), differing from Me-iLys-Bm, was submitted directly to the NCBI protein database (Bachali *et al.* 2002; Olsen *et al.* 2003). Since the primary sequences of the earlier mentioned crystalline-style-associated lysozymes, believed to be involved in digestion, are unknown, this might be one of these lysozymes or a fifth i-type lysozyme with a proposed digestive function in *Mytilus edulis*

Also from the Eastern oyster (*Crassostrea virginica*), initially two i-type lysozymes (Cv-iLys1 and Cv-iLys2), differing in molecular and biochemical parameters were purified and subsequently identified and sequenced by mass spectrometry. The tissue distribution of both lysozymes (figure 8) determined by RT-PCR, protein extraction and *in situ* hybridization revealed a different expression pattern for both lysozymes, suggesting a different function. Cv-iLys2 was mainly found in the digestive gland, in lower amounts in the crystalline style and was expressed in the basophil cells of digestive tubules, which is in line with a digestive function. In contrast, Cv-iLys1was mainly found in the labial palps, the mantle and in lower amounts in the gills, style sac, midgut, digestive gland and gonads. The epithelia of the latter organs are exposed to the external environment and can be used as a portal of entry by oyster pathogens, therefore suggesting a defence function for Cv-iLys1. Very recently, Xue and co-workers identified a third i-type lysozyme in *C. virginica* (Cv-iLys3) that, based on its biochemical characteristics, was proposed to represent a transitional form between the earlier identified defensive i-type lysozyme (Cv-iLys1) and digestive i-type lysozyme (Cv-ilys2) of this species (Xue *et al.* 2010). The molecular weight of Cv-iLys3 and N-terminal sequence resemble that of Cv-iLys1, while its other biochemical parameters including optimal ionic strength and spatial expression pattern resemble those of Cv-ilys2 (figure 8). The optimal pH of Cv-iLys 3 (7,5-8,5), however, clearly differs from that of Cv-iLys1 and 2 (5,5-6,5). Since Cv-iLys3 shares

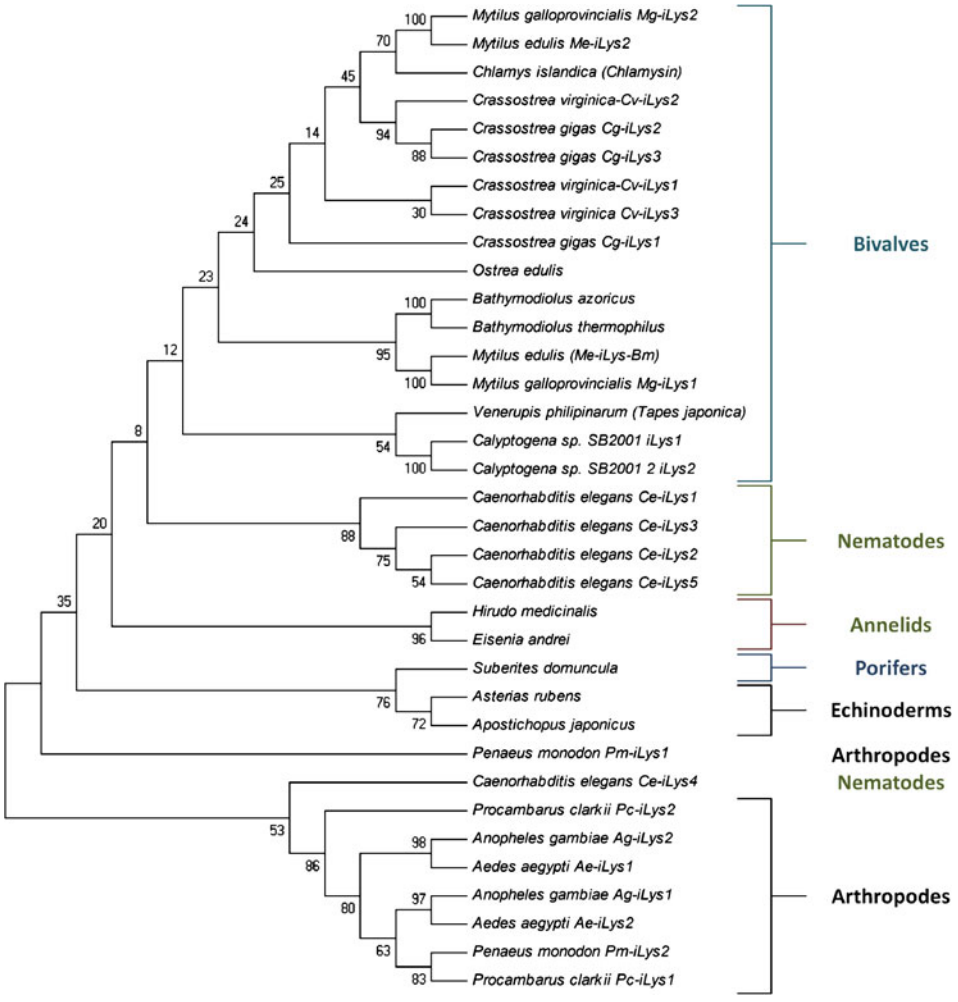

Figure 7. Phylogenetic analysis of experimentally studied i-type lysozymes (table 1). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.* 2007).

biochemical properties of both forms it might possibly function both in defence and digestion.

Also in the Pacific oyster (*Crassostrea gigas*), three cDNA's of distinct i-type lysozymes (Cg-iLys1, Cg-iLys2 and Cg-iLys3) were identified, and an expressed sequence tag (EST) of a fourth, encoding an ortholog of the transitional form Cv-ilys3, has also been deposited in the database (Matsumoto *et al.* 2006; Itoh and Takahashi 2007; Itoh *et al.* 2010). Cg-iLys2 expression was, based on *in situ* hybridization, only detected in the digestive cells of the digestive tubuli, suggesting a digestive function (figure 8). Moreover Cg-iLys2 resembles Cv-iLys2 from *Crassostrea virginica*, also a proposed digestive lysozyme (figure 8). Both Cv-iLys2 and Cg-iLys2 are produced within the digestive tubuli, by apparently a different cell type (Cv-ilys2 by the basophil cells and Cg-iLys2 by the digestive cells). The consequence of this difference on their function or mechanism remains elusive (Itoh and Takahashi 2007), although digestive cells are responsible for intracellular digestion, while basophil cells are known to be involved in extracellular digestion (secretion of digestive enzymes) in the digestive diverticulum of the bivalve stomach.

Cg-iLys1 expression was also detected in the digestive cells of the digestive tubuli. Thus, despite their different molecular and biochemical parameters, Cg-iLys1 and 2 may either have complementary roles in the digestive organs or exert a physiologically different function like digestion and protection of the intestine. A similar situation exists for Cg-iLys1 and Cg-iLys3, which were both found in the mantle tissue but have different biochemical characteristics. Cg-iLys1 has optimum lytic activity at neutral pH and low ionic strength (5 mM), compared to basic pH (8.5) and

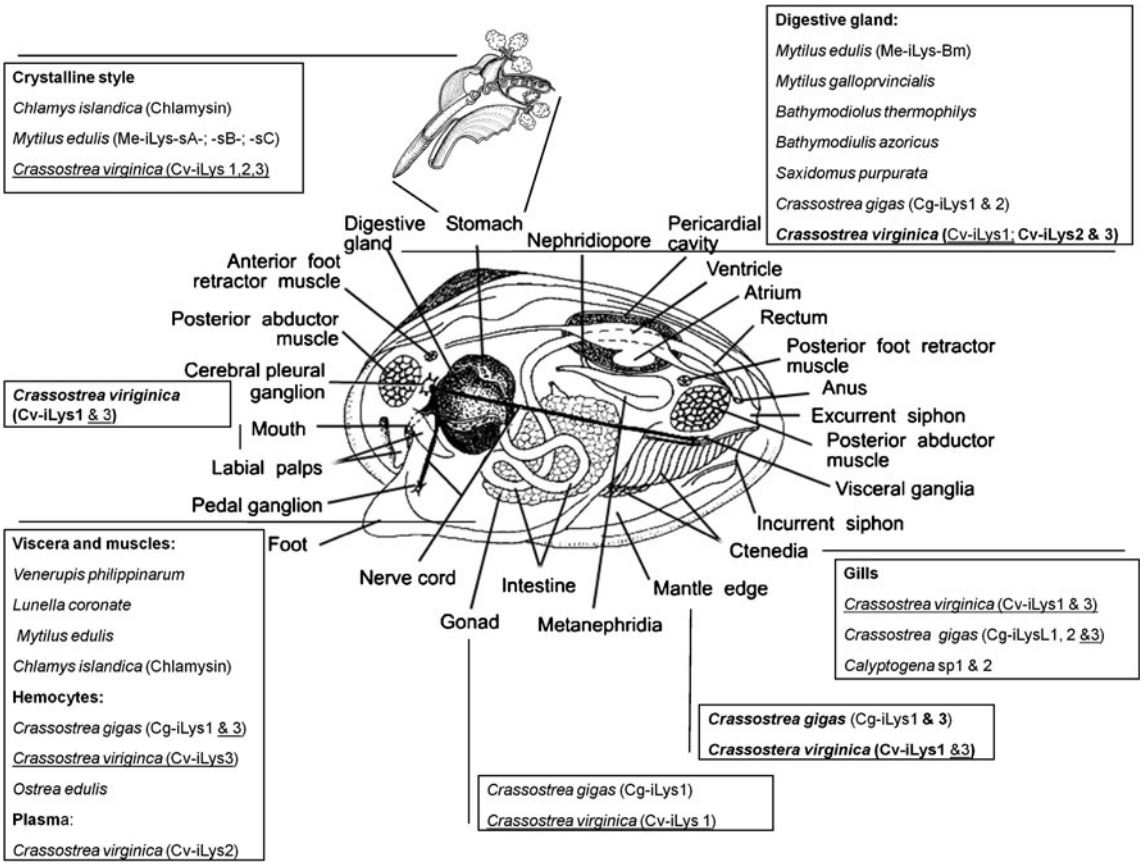

Figure 8. Representation of the general bivalve anatomy and indication of the occurrence of different i-type lysozymes in specific organs of different bivalve species. Major sites of expression are indicated by representing the name of the organism and lysozyme in boldface, sites of minor expression by underlining these names. Figure adapted from ©BIODIDAC (*http://biodidac.bio.uottawa.ca*, May 2011). Crystalline style: An elongated flexible rod in a diverticulum of the filter feeding bivalve stomach of which the anterior part juts into the stomach (Bailey and Worboys 1960). Upon rotating abrasion against the gastric shield, digestive enzymes are set free by dissolution of the style and ingested food particles are reduced in size (Horiuchi and Lane 1965) Palps (Labial palps): Large flap like folds located to either side of the mouth which secretes mucus that entangles suspended food and nutrient particles within the water to produce a ball of food and mucus called a bolus. Afterwards, cilia on the palps direct the bolus into the mouth(Barnes 1982). Gills: Organs consisting of long, folded filaments with cilia at the end, involved in respiration as well as filter feeding. Water is drawn over the gills through the beating of cilia. Suspended food (phytoplankton, zooplankton, algae and other water-borne nutrients and particles) are trapped in the mucus of a gill, and from there are transported to the mouth. Digestive gland: organ which secretes digestive enzymes, comparable to the pancreas in vertebrate organisms. Mantle: thin membrane surrounding the body.

higher ionic strength (20 mM) for Cg-iLys3. Therefore, Cg-iLys1 and 3 are considered to be both involved in the protection of the Pacific oyster's mantle tissue in a variety of conditions.

Recently an i-type lysozyme from *V. philippinarum* was newly identified and suggested to be a new member of the i-type lysozymes. BLAST and phylogenetic analysis (data not shown) indicate that this protein is identical to the earlier identified *V. philippinarum* lysozyme (Vp-iLys). In spite of that, the cellular localization studies performed by Zhao and co-workers suggest that Vp-ilys conducts a defensive function (Zhao *et al.* 2010).

Comparative analysis of c-type lysozymes in vertebrates (mainly ruminants) and invertebrates (insects) has shown that the hypothesized evolution of the lysozyme function from a defensive to a digestive function is paralleled by primary sequence changes in the proteins resulting in a shift from a basic, protease-sensitive protein (high pI, large number of trypsin cleavage sites) towards an acidic, more protease-resistant protein (low pI, less trypsin cleavage sites), rendering them more suited of working in an acidic environment (Prager 1996). Xue *et al.* (2010) postulate that bivalve i-type lysozymes evolved by a burst of positive selection from a defensive function to a digestive function

in a similar way c-type lysozymes did in vertebrates. However, some remarks can be made concerning this theory. First, Prager (1996) postulated that for digestive c-type lysozymes, their 'low isoelectric point could account for the low pH optimum in terms of the electrostatic model of lysozyme catalysis'. However, it is now known that c-type and i-type lysozyme catalysis is not based on an electrostatic model (stabilization of oxicarbonium ion by the Asp residue) but by a covalent lysozyme–substrate intermediate. More in general, Talley and Alexov recently disapproved any correlation between pH optimum and the isoelectric point of an enzyme (Talley and Alexov 2010). Furthermore, while the pH of the ruminant true stomach is highly acidic (pH 3.0–3.5), the pH of a bivalve stomach and crystalline style is only slightly acidic to neutral (pH 6.0–6.9) (Morton 1983). Finally, experimental evidence for a correlation between the number of trypsin cleavage sites and trypsin resistance of a protein is nonexistent, per our knowledge. Therefore, parameters as pI and number of trypsin cleavage sites were not considered as proper indications for either a digestive or defensive function.

6.1.2 Bivalve g-type lysozymes: Besides i-type lysozymes, a limited number of g-type lysozymes were identified in the bivalve class of the invertebrates. The first invertebrate g-type lysozyme (Cf-LysG) was discovered in *Chlamys farreri* and shown to be enzymatically active. A transcriptional study pointed to the digestive gland, gills and gonads as the major sites of expression. While the presence in the digestive gland points towards a digestive function, the expression in the gills points to a defensive function, leading to the suggestion that Cf-LysG is a multifunctional lysozyme (Zhao *et al.* 2007). The second identified g-type lysozyme (Cg-PGRP-L) is actually a Peptidoglycan Recognition Protein (PGRP) found in *Crassostrea gigas*. PGRPs are innate immune molecules currently known to occur in invertebrates (insects, molluscs and echinoderms, but not nematodes) as well as vertebrates and at least contain one C-terminal PGRP domain including a substrate binding site, Zn^{2+} binding residues and an amidase catalytic site. Based on their functionality, PGRPs can be classified into amidase active PGRPs (known in invertebrates and vertebrates), bactericidal PGRPs (only known in mammals) and amidase inactive activators of immune pathways (only known in insects) (Dziarski and Gupta 2006). An example of the latter can be found in the fruit fly *Drosophila melanogaster* where the Tracheal Cyto Toxin (TCT), a disaccharide tetrapeptide peptidoglycan fragment (NAG-NAM-1,6 anhydro L-Ala-D-γ-Glu-mesoDap-D-Ala), is known to activate the Imd (immuno deficiency) immune pathway[2] by interacting with an amidase inactive PGRP. The amidase activity in some PGRP's is anticipated to prevent overactivation of the immune response by removing the peptide side chains from peptidoglycan fragments (figure 1), thus rendering them immunologically inactive.

The newly identified PGRP in *Crassostrea gigas* (Cg-PGRP-L) is a unique member of the PGRP family since it is the only one known to combine a PGRP and a goose-type lysozyme domain, suggesting a dual functionality for this protein (Itoh and Takahashi 2009). Alignment with other g-type lysozymes confirmed the presence of the necessary active site residue for lysozyme activity while an alignment with other PGRPs showed that Cg-PGRP-L may have amidase and specific binding ability towards DAP-type peptidoglycan, which is typical for Gram-negative bacteria and Gram-positive bacilli. Expression of Cg-PGRP-L was mainly detected in the circulatory hemocytes by RT-PCR, and the presence of a signal peptide suggests that it is secreted. Its expression was shown to be up-regulated upon infection with a Gram-positive (*Marinococcus halophilus*) as well as a Gram-negative bacterium (*Vibrio tubiashii*). These findings suggest that Cg-PGRPL-L functions in bacterial recognition and bacteriolysis. However, further research that investigates the contribution of the amidase and lysozyme activity to its general function is needed to get a better understanding of the anticipated bifunctionality of this protein.

6.1.3 Bivalve c-type lysozyme: Only very recently, a cDNA of c-type lysozyme was identified for the first time in a mollusc species (*Haliotis discus hannai* Ino (Hd-LysC)) (Ding *et al.* 2011). Based on RT-PCR analysis, its expression was observed in hemocytes (lowest expression level), gills, mantle (highest expression level), digestive tract and muscle. Hd-LysC was produced in *E. coli* by recombinant DNA-technology, whereupon the purified and refolded enzyme was shown to possess lytic activity against both Gram-positive and Gram-negative bacteria. This finding suggests that molluscs are the first invertebrate phylum discovered in which i-, g- and c-type lysozymes coexist. However, based on BLASTp analysis, a g-type lysozyme of arthropod origin was found in a recent release of the NCBI sequence database, suggesting that some arthropods may also have the three lysozyme types (section 6.4.1).

6.2 *Nematodes*

6.2.1 Large repertoire of lysozymes in the Caenorhabditis genus: The bacteriovorous free living soil nematode *Caenorhabditis elegans* was the first multicellular organism with a fully sequenced genome (Consortium 1998). Genomic analysis of the congeneric nematode species *C. elegans, C. briggsae* and *C. remanei* uncovered a surprisingly large repertoire of up to 15 putative lysozyme genes, which

[2] Imd pathway: Immune pathway primarly involved in defence against Gram-negative bacteria in *Drosophila melanogaster.*

evolutionary analysis demonstrated to be the result of gene duplications and subsequent differentiation from one or more ancestor genes (Schulenburg and Boehnisch 2008). A minority of these genes encode i-type lysozymes (5 in *C. elegans*; 2 in *C. briggsae* and 3 in *C. remanei*), while the rest encodes a lysozyme type (ch-type; section 6.2.2), initially discovered in a fungus and meanwhile found to occur in amoebae and mainly (Gram-positive) bacteria and bacteriophages (10 in *C. elegans* and 7 in *C. briggsae* and *C. ramanei*). To our knowledge, *Caenorhabditis* is the only multicellular eukaryotic species known to encode this lysozyme type. Phylogenetic analysis has shown that the lysozymes from *C. elegans* fall into three main clades, one comprising the i-type lysozymes (Ce-ilys1-5) and two other comprising the ch-type lysozymes (Clade1: Ce-lys 1, 2, 3, 7, 8 and 9; Clade 2: Ce-lys 4, 5, 6 and 10) (Schulenburg and Boehnisch 2008).

6.2.2 ch-type lysozyme: an unexpected lysozyme type in the Caenorhabditis genus: All 10 non-i-type lysozymes of *C. elegans* contain a conserved GH 25 domain. Since the characterization of the first lysozyme with this signature in the fungus *Chalaropsis*, these lysozymes are better known as the ch-type lysozymes. Unlike all other lysozyme types, ch-type lysozymes exhibit β-1,4-*N*-,6-*O*-diacetyl muramidase activity in addition to β-1,4-*N*-acetyl muramidase activity, and hence are the only lysozymes capable of hydrolyzing 6-*O*-acetylated peptidoglycan. This modified peptidoglycan is produced in some Gram-positive bacteria like *Staphylococcus aureus* (Bera *et al.* 2005) and Gram-negative bacteria like *Neisseria gonorrhoeae* (Moynihan and Clarke 2010), where it contributes to virulence by rendering the bacteria insensitive to the lysozyme of the host. The first elucidated crystal structure of a ch-type lysozyme was that of Cellosyl from *Streptomyces coelicolor*, and it revealed an unusual lysozyme fold. While at least c-, g- i- and v-type lysozymes, together with chitinases and chitosanases, consist of two domains linked by a long α-helix (section 3), cellosyl consists of a single domain showing an unusual β/α-barrel fold, representing a new class of polysaccharide-hydrolyzing β/α-barrels (Rau *et al.* 2001, Wohlkönig *et al.* 2010). Similar to other lysozymes, the active site geometry reveals a groove culminating in a negatively charged deep hole lined by the acidic residues Asp and Glu, which were earlier proposed to be involved in catalysis of ch-type lysozymes (Fouche and Hash 1978).

6.2.3 Functional analysis of the nematode lysozymes: All nematodal lysozyme genes for which expression localization studies are available, are expressed in the intestine, while Cel-Lys1, 7 and 8 are additionally expressed in the nervous system, the muscle cells of L1 larvea and the terminal pharyngeal bulb, respectively (Schulenburg and Boehnisch 2008). The predicted physico-chemical properties of the deduced nematodal lysozymes, their pathogen-induced expression and regulation by the immune system differ distinctly for all three clades and even for the members within the clades (Schulenburg and Boehnisch 2008). The clade 1 ch-type lysozymes are considered as important immune effectors, since dependent on the infecting pathogen, specific members of this lysozyme clade are induced. In support of this hypothesis, RNAi knock-down or knock-out of clade 1 ch-type lysozymes resulted in an increased susceptibility against the following pathogens: *Microbacterium nematophilum* (Ce-lys7) (O'Rourke *et al.* 2006), *Bacillus thuringiensis* (Ce-lys1, 2, 7) (Boehnisch *et al.* 2011), *Pseudomonas aeruginosa* (Ce-lys2, 7) (Nandakumar and Tan 2008) , *Escherichia coli* LF82 (Ce-lys7) (Simonsen *et al.* 2011) and the fungus *Cryptococcus neoformans* (Ce-lys7) (Marsh *et al.* 2011), while increased resistance was observed upon clade 1 ch-type lysozyme overexpression for: *Bacillus thuringiensis* (Ce-lys 7) (Boehnisch *et al.* 2011) and *Serratia marcescens* (Ce-lys1) (Mallo *et al.* 2002, O'Rourke *et al.* 2006).

The clade 2 ch-type lysozymes and the i-type lysozymes, in contrast, are generally down-regulated upon pathogen infection or by immune regulatory pathways, as if their natural functionality interferes with the elicited immune response. A similar finding was observed for the digestive (c-type) lysozymes of the fruit fly *Drosophila melanogaster* during immune challenge (Hultmark 1996). Experimental evidence, however, indicates that a knock-down of Ce-ilys3 results in a higher susceptibility for *M. nematophilum*, while in the same study no effect was observed for a knock-down of Ce-ilys2 (O'Rourke *et al.* 2006).

The fact that induction of the different members of the clade 1 ch-type lysozymes depends on the infecting pathogen and different immune pathways leads to the assumption that *C. elegans* modulates its immune response in function of the bacteria that it encounters. The genetic diversification of pathogen recognition receptors like lectins and immune effectors, besides lysozymes, is believed to be responsible for this capacity (Du Pasquier 2006, Schulenburg and Boehnisch 2008, Schulenburg *et al.* 2007, Schulenburg *et al.* 2008). Further, special attention needs to be given to the potential synergistic interactions between several immune components, which contributes to a quick and effective immune response, as is well documented for lysozymes and membrane-active antimicrobial peptides (Hultmark 1996; Leippe 1999; Brown and Hancock 2006).

6.3 *Annelids*

The lysozyme Hm-iLys (section 5.2), present in the salivary gland secretion of the medical leech, is a unique polyfunctional representative of the i-type lysozymes, reported to

exhibit muramidase, isopeptidase, chitinase and a non-enzymatic antibacterial activity. In view of its isopeptidase-dependent ability to dissolve fibrin clots, it is believed to contribute to blood preservation in the crop of the leech, where the blood is stored between two meals for several months as a viscous intraluminal fluid of which small portions are gradually released into the intestinal tract. Kikuchi and Graf demonstrated that blood feeding is accompanied by a spatial and temporal change in the natural two-species (*Aeromonas veronii* bv. sobria and *Rikenella*-like bacterium) microbial community in the digestive tract of the medical leech (Kikuchi and Graf 2007). Briefly, the *A. veronii* population first expands and subsequently declines from day 3 post-feeding. The *Rikenella* population also increases and reaches a plateau on day 7 post-feeding. The latter form microcolonies encased in a polysaccharide *N*-acetylglucosamine matrix and may form an erythrocyte attached or granular (self-immobilized) biofilm in the intraluminal fluid. On day seven this biofilm breaks apart whereupon the microcolony size increases. In this dynamic process, the lysozyme Hm-iLys might conduct additional functions, for example, in controlling the expansion of the symbiotic population by its antibacterial activity and/or in dismantling the *N*-acetylglucosamine matrix around the encased *Rikenella* microcolonies by its chitinase activity.

The expression of i-type lysozyme from the earthworm *Eisenia andrei* (Ea-iLys) by the coelomocytes was studied by RT-PCR, and found to be up-regulated upon bacterial challenge with both the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Escherichia coli*. Since coelomocytes are considered as important immune cells in coelomate animals (molluscs, annelids and arthropods), these findings suggest Ea-iLys to function as an inducible immune effector providing protection upon infection with Gram-positive and negative bacteria (Josková *et al.* 2009).

6.4 *Arthropods*

6.4.1 Arthropod c-type lysozymes: Arthropods have c-type as well as i-type lysozyme genes (figure 2). This was considered to be a unique combination, but recently a c-type lysozyme was also documented in a mollusc (Ding *et al.* 2011). g-type lysozymes were long considered to be absent in the arthropods based on the absence of g-type lysozyme encoding genes in the available genomes of the most abundant class of arthropods: the insects. A BLASTp search with the g-type lysozyme from *Anser anser* in the NCBI protein database revealed a putative g-type lysozyme in the crustacean *Caligus rogercresseyi*. The dipterian insects *Drosophila melanogaster* (Hultmark 1996) and *Anopheles gambiae* (Kang *et al.* 1996, Li *et al.* 2005) are known to carry multiple c-type lysozyme genes, 11 and 9 , respectively. c-type lysozyme encoding genes were further reported in the arthropod orders lepidoptera (e.g. the silkworm (moth) *Bombyx mori*; Jolles *et al.* 1979; Lee and Brey 1995), hemiptera (e.g. the bug *Tritoma infestans*; Araujo *et al.* 2006; Waniek *et al.* 2009), isoptera (e.g. the Japanese termite *Reticulitermes speratus*; Fujita *et al.* 2002) as well as in the class of the arachnid (e.g. the soft thick *Ornithodoros moubata*; Grunclova *et al.* 2003) and the subphylum of the crustaceae (e.g. the giant tiger prawn *Penaeus mondon*; Supungul *et al.* 2010). Since the focus of this literature review is limited to the i-type lysozyme and some special observations on g-type and ch-type in invertebrates, the numerous studies on c-type lysozymes in invertebrates (Dunn and Dai 1990; Russell and Dunn 1991; Sun *et al.* 1991; Mulnix and Dunn 1994; Moreira-Ferro *et al.* 1998; Gao and Fallon 2000; Fujimoto *et al.* 2001; Hikima *et al.* 2003; Kollien *et al.* 2003; Bedoya *et al.* 2005; Cancado *et al.* 2008; Ren *et al.* 2009; Zhang *et al.* 2009) will not be discussed in this literature review.

6.4.2 Arthropod i-type lysozymes: Recently, two i-type lysozyme genes (Ag-iLys1 and Ag-iLys2) from the mosquito *Anopheles gambiae*, two cDNAs (Pm-iLys1 and Pm-iLys2) of the giant tiger prawn *Penaeus monodon* and two cDNAs (Pc-iLys1 and Pc-iLys2) of the red swamp crayfish *Procambarus clarkii* were identified by BLAST analysis within the *A. gambiae* genome database, the *P. monodon* EST database and a hemocyte cDNA library of *P. clarkii*, respectively. Analysis of the amino acid sequence of these six i-type lysozymes revealed that these enzymes are all missing the glutamate and all except Ag-iLys1 also the aspartate residue, both critical for muramidase activity of i-type lysozymes (cfr. section 4). Both i-type lysozymes from *Penaeus monodon* and *Procambarus clarkii* were expressed by recombinant DNA-technology in *E. coli*, purified and experimentally found to lack muramidase activity in a *Micrococcus lysodeikticus* turbidity assay (Supungul *et al.* 2010; Zhang *et al.* 2010). However, prokaryote expression systems are known to cause problems for proteins carrying multiple disulphide bonds, and thus, misfolding or malformation of disulphide bonds cannot be ruled out as a cause of enzymatic inactivity in these studies (Demain and Vaishnav 2009). A multiple sequence alignment of arthropod i-type lysozymes (section 4 and figure 5) confirms that muramidase deficiency is probably a common feature amongst arthropod i-type lysozymes. In this respect, it is noteworthy that Pm-iLys1 and 2 exhibit antimicrobial activity despite their muramidase deficiency. Antimicrobial activity was also reported for Pc-iLys1 (muramidase and isopeptidase deficient) but not for Pc-iLys2 (muramidase-deficient and isopeptidase active).

Paskewitz *et al.* (2008) postulated that the inactivity of i-type lysozymes in arthropods might be related to the fact that the functional redundancy with c-type lysozymes in

arthropods may have allowed diversification of i-type lysozymes to other (yet to be discovered) functional roles. On the contrary, such a functional diversification seems not to have occurred in other animals with redundant lysozymes, such as the molluscs, which have both active i- and g-type lysozymes, or vertebrates, which have active c- and g-type lysozymes. Interestingly, some of the g-type lysozymes discovered in molluscs are actually PGRPs which have a g-type lysozyme domain (section 6.1.2) and are therefore believed to be involved in bacterial recognition, signalling and bacteriolysis. In line with the postulate of Paskewitz, the presence of two lysozyme types in the same species might promote functional diversification but is not necessarily accompanied by a loss of enzymatic activity.

RT-PCR analysis revealed that Ag-iLys1 is constitutively expressed at all life stages, while Ag-iLys2 is only abundantly expressed in adults and late instar larvae, which might reflect a difference in dietary or environmental exposure of larvae and adults to bacteria. Ag-iLys1 is expressed in the fat body[3] and Malphighian tubules[4] while Ag-iLys 2 is only expressed in the fat body. Inoculation of bacteria into the haemocoel of adult female mosquitoes, however, did not result in an increased expression of any of these lysozymes, thus making a function in immunity unlikely. On the contrary, an increased transcription of Ag-iLys1 and 2 in the midgut of the mosquito *Anopheles gambiae* after blood-feeding was observed, suggesting a function in the digestion of blood (Paskewitz *et al.* 2008). However, because they lack the isopeptidase catalytic residues (figure 5), both i-type lysozymes from *A. gambiae* are predicted to be isopeptidase deficient, making this proposed function unlikely. An alternative function that has been proposed for Ag-iLys1 and 2 in relation to blood feeding, which is to control excessive outgrowth of bacteria after a blood meal, and this could be based on the non-enzymatic antibacterial effect of the proteins (DeMaio *et al.* 1996).

In *Penaeus monodon*, Pm-iLys1 is expressed in the hepatopancreas (digestive gland) and Pm-iLys2 in different tissues but not in the gills, lymphoid organs and intestine. Therefore, Pm-iLys1 possibly conducts a digestive function while that of Pm-iLys2 remains unknown.

Both *Procambarus clarkii* i-type lysozymes were found to have a similar tissue expression pattern (hemocytes, heart and stomach). They were proposed to have an immune function, because of hemocyte expression and increased expression upon challenge with *Vibrio anguillarum* and *Staphylococcus aureus*. However, since both lysozymes were also found in the stomach, a digestive function cannot be ruled out. In view of the bactericidal deficiency of Pc-iLys2, the authors speculated on a potential involvement of its isopeptidase activity in bacterial cell lysis. This seems rather unlikely, however, since Pc-iLys2 was not bactericidal towards Gram-positive bacteria known to have an isopeptide bond in their peptidoglycan (*Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus lysodeikticus*), and the observation that inhibition of the isopeptidase activity in mollusc i-type lysozyme Vp-ilys did not affect its bactericidal activity (section 5.2).

6.5 *Porifera*

The most obvious evidence of the involvement of an i-type lysozyme in the elimination of bacteria was obtained by studying the responsiveness of the marine demosponge *Suberitus domuncula* towards peptidoglycan fragments of Gram-positive bacteria. The elimination of Gram-positive bacteria from the mesohyl[5] compartment in this organism proceeds in two phases, the activation of the gray cells and the effector phase. In the activation phase, peptidoglycan fragments, released during bacterial lysis within the gray cells present in the mesohyl, act as an elicitor leading to the endocytosis of bacteria and further release of peptidoglycan. This causes an increased expression of an adaptor protein (AdaPTin-1) involved in endocytosis and subsequently, with a delay of one day, in an increased expression of i-type lysozyme by the gray cells present in the mesohyl. During the effector phase, bacteria are lysed extracellularly in the mesohyl by lysozyme (Thakur *et al.* 2005). AdaPTins are well-conserved proteins in both vertebrate and invertebrate metazoan, suggesting that similar mechanisms might be present in other organisms (Boehm and Bonifacino 2001).

7. Periplasmic inhibitor of i-type lysozyme

In response to lysozyme as an important effector molecule in the innate immune system of vertebrate as well as invertebrate organisms, bacteria have developed various strategies to prevent the lethal impact of lysozymes. In Gram-negative bacteria, one of these strategies comprises the production of specific periplasmic or membrane-linked lysozyme inhibitors. Such inhibitors were first discovered for c-type lysozyme in *E. coli* (**I**nhibitor of **V**ertebrate L**y**sozyme, Ivy; **M**embrane bound **l**ysozyme **i**nhibitor of **c-type** lysozyme, MliC) and *Salmonella* Enteritidis (**P**eriplasmic **l**ysozyme **i**nhibitor of **c-type** lysozyme, PliC)(Callewaert *et al.* 2008, Deckers *et al.* 2008, Deckers *et al.* 2004, Monchois *et al.* 2001). Recently also a specific, high-affinity **P**eriplasmic **l**ysozyme **i**nhibitor of **i-type** lysozyme (PliI) was discovered in *Aeromonas hydrophila* and a **P**eriplasmic **l**ysozyme

[3] Fat body: organ in insects similar in function to the vertebrate liver

[4] Malphighian tubules: excretory and osmoregulatory organ of insects that opens near the junction of the midgut and hindgut.

[5] Mesohyl: Gelatinous matrix within a sponge filling the space between the external pinacoderm (epidermis) and the internal choanoderm (cell layer that lines the inner central cavity of sponges).

inhibitor of **g-type** lysozyme (PliG) in *Escherichia coli* (Van Herreweghe *et al.* 2010, Vanderkelen *et al.* 2010).

For all these inhibitors, the molecular mechanism of interaction with their corresponding lysozyme has been investigated: Ivy (Abergel *et al.* 2007), MliC/PliC (Voet *et al.* 2011; Yum *et al.* 2009), PliI (Leysen *et al.* 2011) and PliG (unpublished results). Based on X-ray crystallography and site-directed mutagenesis of PliI, Leysen *et al.* (2011) suggested that the molecular interaction between PliI and Vp-iLys proceeds by insertion of a loop of PliI into the active site of Vp-iLys. This loop contains an SG(x)xY domain that is conserved across the PliI, PliG and MliC/PliC families of inhibitors, and in which the serine 104 and tyrosine 107 residues (numbering for PliI from *Aeromonas hydrophila*) are critical for successful interaction. The same authors also constructed a molecular interaction model for PliI and Vp-Lys using *in silico* docking, but this should be corroborated by experimental elucidation of the structure of the complex.

Analogous to the contribution of c-type lysozyme inhibitors to bacterial c-type lysozyme tolerance also, PliI and PliG were shown to contribute to i-type lysozyme (Vp-ilys) tolerance of *A. hydrophila* and g-type lysozyme (Salmon g-type lysozyme, SalG) (Kyomuhendo *et al.* 2007) tolerance of *E. coli* respectively in case of outer membrane permeabilisation (Vp-ilys and SalG cannot autonomously cross the outer membrane of Gram-negative bacteria). These and previous findings on c-type lysozyme inhibitors suggest that bacterial lysozyme inhibitors may have an important function, for example, in bacteria–host interactions. Validating this assumption, however, remains a challenging task.

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MS received 24 November 2011; accepted 07 March 2012

Corresponding editor: Stuart A Newman