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Lysozymes and lysozyme-like proteins from the fall armyworm, *Spodoptera frugiperda*

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ABSTRACT

Lysozyme is an important component of the insect non-specific immune response against bacteria that is characterized by its ability to break down bacterial cell-walls. By searching an EST database from the fall armyworm, *Spodoptera frugiperda* (Negre et al., 2006), we identified five sequences encoding proteins of the lysozyme family. The deduced protein sequences corresponded to three classical c-type lysozymes *Sf*-Lys1, *Sf*-Lys2 and *Sf*-Lys3, and two lysozyme-like proteins, *Sf*-LLP1 and *Sf*-LLP2. *Sf*-Lys1 was purified from the hemolymph of *Escherichia coli*-challenged *S. frugiperda* larvae. The mature protein had a molecular mass of 13.975 Da with an isoelectric point of 8.77 and showed 98.3% and 96.7% identity with lysozymes from *Spodoptera litura* and *Spodoptera exigua*, respectively. As the other insect lysozymes, *Sf*-Lys1 was active against Gram positive bacteria such as *Micrococcus luteus* but also induced a slight permeabilization of the inner membrane of *E. coli*. Genes encoding these five *Sf*-Lys or *Sf*-LLPs were differentially up-regulated in three immune-competent tissues (hemocytes, fat body and gut) after challenges with non-pathogenic bacteria, *E. coli* and *M. luteus*, or entomopathogenic bacterium, *Photorhabdus luminescens*. *Sf*-Lys1 and *Sf*-Lys2 were mainly induced in fat body in the presence of *E. coli* or *P. luminescens*. *Sf*-Lys3, which had an acidic isoelectric point, was found to be the most up-regulated of all five *Sf*-Lys or *Sf*-LLPs in hemocytes and gut after challenge with *P. luminescens*. More molecular data are now available to investigate differences in physiological functions of these different members of the lysozyme superfamily.

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1. Introduction

Since its discovery (Fleming, 1922), more than 20,000 papers have been published on lysozyme from all animal and plant kingdoms. When restricted to insects, this tremendous literature still contains 281 publications. However, even though this molecule is one of the most intensively studied, our knowledge on lysozyme remains incomplete and new interesting findings on this molecule are published every year.

Lysozyme (EC 3.2.1.17) is a well known enzyme which has the ability to cleave the β-1,4 glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycans, one component of bacterial cell-wall (Jolles and Jolles, 1984). It participates in immune defense and is constitutively present in immune-competent tissues even though its secretion is induced after bacterial challenge (Engstrom et al., 1993). It has bacteriolytic and/or bacteriostatic activities towards Gram positive bacteria by itself (Yu et al., 2002) and Gram negative bacteria on whose it may act synergistically with other antimicrobial peptides such as cecropins (Cytrynska et al., 2001). Besides its antimicrobial activity, lysozyme was shown to have many other functions. Among them, it has been reported that members of the lysozyme superfamily might be recruited for a digestive role in mammals (Irwin and Wilson, 1989) as well as in invertebrates (Kopacek et al., 1999; Kylsten et al., 1992). In addition to the enzyme activity, lysozyme may associate with nucleic acids (Steinrauf et al., 1999). Recently, kinetics and affinities of the bindings involved in the interaction of lysozyme with DNA have been characterized (Lin et al., 2008). Conformational changes of both lysozyme and DNA occur, suggesting that the interaction of lysozyme with DNA molecules may interfere with DNA replication, modulate gene expression, and block bacterial and viral infections. Therefore, lysozyme may represent part of the innate immune system with a very broad protective spectrum.

In invertebrates, more than 90 different sequences encoding lysozymes could be found in protein databases. This ubiquitous

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enzyme has been reported from the main insect orders, *Coleoptera* (Altincicek et al., 2008; Anselme et al., 2008), *Diptera* (Daffre et al., 1994; Ursic Bedoya et al., 2005), *Hemiptera* (Araujo et al., 2006; Ursic-Bedoya et al., 2008), *Hymenoptera* (Evans et al., 2006), *Isoptera* (Fujita et al., 2002) and *Lepidoptera* (Bae and Kim, 2003; Lee and Brey, 1995; Liu et al., 2004; Mulnix and Dunn, 1994). They are expressed in different tissues involved in immune response such as hemocytes (Lavine et al., 2005) and fat body (Fujimoto et al., 2001; Mulnix and Dunn, 1994), or tissues from the digestive tract such as salivary glands (Fujita et al., 2002; Kylsten et al., 1992; Liu et al., 2004) and gut (Daffre et al., 1994; Kollien et al., 2003). Most of them are similar in molecular characteristics to chicken lysozyme (Hultmark, 1996) with a LYZ1 domain containing a lysozyme catalytic cleft composed of a catalytic site and a calcium binding domain. However, some insect lysozymes lacking one or both of the catalytic glutamate and aspartate residues necessary for the muramidase activity have been reported (Gandhe et al., 2007; Tanaka et al., 2008) and were named lysozyme-like proteins (LLP).

Lysozyme is an inducible antimicrobial peptide which presents a κB-like motif in its promoter (Sun et al., 1991). Its induction after bacterial challenge or injection of fungal or bacterial cell-wall components (β-1,3-glycans or LPS) has been reported for lysozymes involved in immune defense (Bae and Kim, 2003; Gandhe et al., 2007; Lee and Brey, 1995; Sun et al., 1991; Zhang et al., 2009). Up-regulation of digestive lysozymes from blood-sucking arthropods has also been described after feeding (Fujita et al., 2002; Kollien et al., 2003; Kopacek et al., 1999; Ursic Bedoya et al., 2005). On the other hand, *Heliothis virescens* lysozyme has been shown to be down-regulated by polyDNA virus such as CsPDV from *Campoletis sonorensis* (Shelby et al., 1998). Recently, we have shown that a lysozyme from *Spodoptera frugiperda* has lower transcript levels in hemocytes 24 h after injection of *Hyposoter didymator* polyDNA virus (HdIV) compare to saline. Conversely, *S. frugiperda* lysozyme mRNA levels are higher 24 h after injection of heat-inactivated HdIV, with higher fold changes in the fat body than in the hemocytes (Barat-Houari et al., 2006).

A c-type lysozyme has been characterized from the hemolymph of the beet armyworm, *Spodoptera exigua* (Bae and Kim, 2003), a crop pest insect closely related to *S. frugiperda*. It is a 14,313.83 kDa protein with a basic isoelectric point of 8.59 encoded by a single copy gene. *S. exigua* lysozyme was mainly expressed in the fat body and then released into the hemolymph. Its basal level of activity in hemolymph was increased by the injection of different microbial cell-wall components, with lipopolysaccharides from the entomopathogenic bacteria, *Xenorhabdus nematophila*, found to be the best inducer.

In the fall armyworm, *S. frugiperda*, we found by searching the Spodobase cDNA database (Negre et al., 2006) that the genome of this insect contains at least 5 genes encoding members of the lysozyme family. An analysis of their deduced amino acid sequences combined with a phylogenetic study indicated that three of these proteins were classical c-type lysozymes and were named *Sf*-Lys1, *Sf*-Lys2 and *Sf*-Lys3. The two others belonged to the LLP family since they were lacking one or both Glu and Asp residues necessary for muramidase activity and were named *Sf*-LLP1 and *Sf*-LLP2. We have then successfully purified a c-type lysozyme which was found to be *Sf*-Lys1. This purified *S. frugiperda* lysozyme was partially characterized for its activity towards Gram positive and Gram negative bacteria. Finally, we showed by quantitative RT-PCR that the genes encoding these five proteins were differentially up-regulated after bacterial challenge with non-pathogenic (*Escherichia coli*, *Micrococcus luteus*) or entomopathogenic (*Photorhabdus luminescens* wild-type TT01) bacteria. The gene expression patterns were found to be dependent on the type of bacterial strain injected and on the tissue analyzed.

2. Materials and methods

2.1. Bacteria

E. coli CIP 54.8 (Collection de l'Institut Pasteur, France) was used to challenge *S. frugiperda* larvae. Culture of *E. coli* was carried out with Luria–Bertani (LB) medium in a 37 °C shaking incubator (250 rpm). Prior to use in experimental challenge assays, 100 μl of the overnight culture were inoculated into 5 ml of fresh LB medium and the culture was kept, under shaking, until OD_{540} was 0.8.

2.2. Insects and immune challenges

The Lepidoptera *S. frugiperda* were reared on artificial diet at 24 °C with a photoperiod of 16 h/8 h (light/dark). One-day-old sixth instar larvae of *S. frugiperda* were used in the experiments.

Infections were performed by injecting 20 μl of a PBS (phosphate-buffered saline, DIFCO) washed *E. coli* CIP 54.8 solution (5×10^6 bacteria/ml) per larvae. Hemolymph from 10 individuals was collected, on ice, 8 h post-infection, in an eppendorf tube containing 1 ml of anticoagulant buffer (van Sambeek and Wiesner, 1999). Then, hemocytes were removed by a short centrifugation (1 min at 800 × *g*) and *S. frugiperda* plasma was stored at −20 °C until use. A total of 7.5 ml of plasma, corresponding to about 65 mg of proteins, was collected from 150 individuals. In order to check the presence of antibacterial activities in crude extracts, hemolymph from naive or *E. coli*-injected larvae was directly collected in eppendorf tubes containing few crystals of phenylthiourea, an inhibitor of phenoloxidase, and then centrifuged for 1 min at 800 × *g*.

2.3. Purification protocol

A four-step purification procedure was used. Plasma from immunized larvae was acid precipitated by the addition of HCl until pH was 2.0 and stirred overnight at 4 °C. After centrifugation at 10,000 × *g* for 30 min, the supernatant was loaded on a Sep-Pak Vac 20cc C_{18} cartridge (Waters) previously activated with 70% ethanol according to the manufacturer's instructions and then equilibrated in 0.1% trifluoroacetic acid (TFA). Unbound proteins were removed by washing the cartridge with 40 ml of 0.1% TFA. The elution of bound proteins was achieved by the use of 10% steps of acetonitrile (ACN) from 10% to 80% in 0.1% TFA. After concentration by freeze-drying using a Cryonext equipment (Cryo-Rivoire, France), the eluted fractions were tested for antibacterial activity with a growth inhibition assay (see below). Fraction eluted with 40% ACN was loaded on a Nova-Pak C_{18} reverse phase high pressure liquid chromatography (HPLC) column (60 Å, 4 μm, 3.9 mm × 150 mm, Waters) previously equilibrated with 0.1% TFA. The elution was performed using a continuous 1%/min linear gradient of ACN in 0.1% TFA. Eluted protein peaks were collected manually, concentrated and tested for antibacterial activity as indicated above. Fractions containing antimicrobial activity against *M. luteus* were further purified on a symmetry C_{18} reverse-phase HPLC column (100 Å, 3.5 μm, 4.5 mm × 75 mm, Waters) with a two-step linear gradient of ACN (6%/min for 10 min followed by 0.6%/min for 17 min). Purified active fractions were concentrated by freeze-drying and used for further characterization.

2.4. Antibacterial assays

2.4.1. Growth inhibition assay

Antibacterial activity was assayed by measuring diameters of zones of bacterial growth inhibition in 1-mm thin agar plates con-

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