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Studies on the role of insect hemolymph polypeptides: *Galleria mellonella* anionic peptide 2 and lysozyme

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ABSTRACT

The lysozymes are well known antimicrobial polypeptides exhibiting antibacterial and antifungal activities. Their antibacterial potential is related to muramidase activity and non-enzymatic activity resembling the mode of action of cationic defense peptides. However, the mechanisms responsible for fungistatic and/or fungicidal activity of lysozyme are still not clear. In the present study, the anti-*Candida albicans* activity of *Galleria mellonella* lysozyme and anionic peptide 2 (AP2), defense factors constitutively present in the hemolymph, was examined. The lysozyme inhibited *C. albicans* growth in a dose-dependent manner. The decrease in the *C. albicans* survival rate caused by the lysozyme was accompanied by a considerable reduction of the fungus metabolic activity, as revealed by LIVE/DEAD staining. In contrast, although AP2 reduced *C. albicans* metabolic activity, it did not influence its survival rate. Our results suggest fungicidal action of *G. mellonella* lysozyme and fungistatic activity of AP2 toward *C. albicans* cells. In the presence of AP2, the anti-*C. albicans* activity of *G. mellonella* lysozyme increased. Moreover, when the fungus was incubated with both defense factors, true hyphae were observed besides pseudohyphae and yeast-like *C. albicans* cells. Atomic force microscopy analysis of the cells exposed to the lysozyme and/or AP2 revealed alterations in the cell surface topography and properties in comparison with the control cells. The results indicate synergistic action of *G. mellonella* AP2 and lysozyme toward *C. albicans*. The presence of both factors in the hemolymph of naive larvae suggests their important role in the early stages of immune response against fungi in *G. mellonella*.

1. Introduction

The defense peptides constitute a crucial component of innate immunity in animals. A vast majority of these peptides are small, cationic, amphipathic molecules classified into three large classes: (i) linear α-helical peptides without cysteine, (ii) peptides with a structure stabilized with disulfide bridges, (iii) peptides with overrepresentation of one amino acid, e.g. proline or glycine. The positive net charge facilitates targeting of the anionic microbial membrane, while amphipathicity promotes their interaction with membrane phospholipids leading to compromising of membrane integrity and cell death [7,26,36,43,51]. In addition to the cationic antimicrobial peptides, anionic defense peptides comprising a relatively small and diverse group have been described. These peptides appear to have evolved to counteract pathogens which possess inherent resistance to cationic peptides or can develop such resistance. Ovine pulmonary surfactant peptides, the first characterized anionic antimicrobial peptides, are small molecules (720–825 Da) containing homopolymeric regions of 5–7 aspartic acid residues. Anionic α-helical defense peptides, e.g. enkelytin, dermicidin, and maximin 5, have been also described [6,18,56]. Some examples of insect anionic defense peptides have been reported, e.g. defensin-like peptides from *Bombyx mori* and *Spodoptera litoralis* [55,59]. Two anionic peptides, named anionic peptide 1 and 2 (AP1 and AP2), were purified and characterized from the hemolymph of the greater wax moth *Galleria mellonella* [14]. *G. mellonella* AP2, the unique 7 kDa peptide (p*I* 4.79) constitutively present in hemolymph in the concentration of 10–12 μM, did not influence bacterial growth;

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however, it exhibited low anti-*Pichia* activity in vitro (MIC 86.6 μM) [14,45].

Lysozymes are well known antimicrobial polypeptides exhibiting antibacterial and antifungal activities. Their antibacterial potential is related to enzymatic muramidase activity as well as non-enzymatic activity resembling the mode of action of cationic defense peptides [20,33,34,46]. However, the mechanism of lysozyme fungistatic and/or fungicidal activity is not clear. Lysozyme antifungal properties have been ascribed, among others, to enzymatic hydrolysis of the N-glycosidic bonds between polysaccharides and structural proteins as well as the β-1,4 linkages in chito-oligosascharides of fungal cell walls, direct binding of cationic lysozyme and yeast cell wall mannans leading to a decrease in viability, activation or deregulation of autolytic enzymes [41,52,57,61,62]. Lysozymes have been described in many insect species, including representatives of Lepidoptera, Diptera, Coleoptera, and Hymenoptera [1,44,48,49,58,63]. Due to similarities in the amino acid sequence and properties to the egg white lysozyme (EWL), most of the insect lysozymes belong to the c-type family of lysozymes [33]. The lysozymes present in insect hemolymph constitute an important component of the humoral immune response against invading pathogens, while those occurring in insect gut can serve as digestive enzymes, e.g. in *Drosophila melanogaster* and *Musca domestica* [35,53].

The *G. mellonella* lysozyme of a predicted molecular weight 14,027 Da and isoelectric point p*I* 9.28 contains 121 amino acids (SWISS-PROT data bank: locus LYC_GALME, accession P82174). In the hemolymph of naive *G. mellonella* larvae, lysozyme is present constitutively in the concentration of 0.76 μM (±0.17). In response to immune challenge its concentration increases reaching 4.41 μM (±1.47) in the hemolymph of *Escherichia coli*-immunized larvae [45]. The high anti-Gram-positive bacteria activity of *G. mellonella* lysozyme related to its muramidase activity has been well documented. Interestingly, *G. mellonella* lysozyme exhibits activity, albeit relatively low, also against selected Gram-negative bacteria, e.g. *E. coli*, *Salmonella paratyphi*, *S. choleraesuis* [63,64]. A significant in vitro increase in antibacterial activity of *G. mellonella* lysozyme in the presence of certain *G. mellonella* defense peptides and proteins, e.g. AP2 and apolipophorin III (apoLp-III), against Gram-negative as well as Gram-positive bacteria has been reported [64,66]. Our previous study revealed that AP2 acts synergistically with lysozyme toward Gram-negative bacteria, potentiating bacterial membrane perforating properties of lysozyme [64]. These findings, indicating synergistic antibacterial action of *G. mellonella* defense factors, confirmed earlier reports which demonstrated increased EWL activity against *Micrococcus luteus* and *E. coli*, respectively, in the presence of apoLp-III and antimicrobial peptides (attacins, cecropins, insect defensins) [10,15,21,25]. Antifungal activity of *G. mellonella* lysozyme toward *Saccharomyces cerevisiae* has been reported [57].

Recently, *G. mellonella* has emerged as a powerful alternative model host for studying pathogenesis and virulence factors of different human pathogens, including *Candida albicans* [2,11–13,19,22,23,50]. In response to immune challenge with fungal immunogens like zymosan or fungal cells (yeast *S. cerevisiae*, *Beauveria bassiana* blastospores), a remarkable increase in lysozyme activity in *G. mellonella* hemolymph has been reported [57,60]. In contrast, immunization of *G. mellonella* larvae with *C. albicans* caused only a minute increase in the hemolymph lysozyme concentration 24 h post-challenge, resembling a change induced by *M. luteus* injection [45]. As *G. mellonella* lysozyme exhibits high anti-Gram-positive bacteria activity, one can postulate that the presence of lysozyme in hemolymph in ca. 0.5 μM concentration is sufficient for *M. luteus* elimination. Accordingly, it could also be speculated that a similar level of lysozyme might be sufficient for effective *C. albicans* eradication by the insect immune system. To test this hypothesis, we examined anti-*C. albicans* activity of purified *G. mellonella* lysozyme using concentrations reflecting these reported in the hemolymph of the naive and immune-challenged larvae. Moreover, the effect of *G. mellonella* AP2, a defense peptide constitutively present in hemolymph, on lysozyme antifungal activity was investigated. Alterations in *C. albicans* metabolic activity as well as in cell surface topography and nanomechanical properties caused by both compounds were analyzed using confocal laser scanning microscopy and atomic force microscopy.

2. Materials and methods

2.1. Microorganisms

The yeast *C. albicans* (wild-type; Department of Industrial Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland) was grown in a liquid YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 37 °C. Bacteria *M. luteus* ATCC 10240 and *E. coli* D31 were grown in 2.5% Luria–Bertani (LB) medium at 28 °C and 37 °C, respectively.

2.2. Insect immune challenge, collection of hemolymph, and preparation of methanolic extracts

The larvae of *G. mellonella* (Lepidoptera: Pyralidae) were reared on honeybee nest debris (a natural diet) at 30 °C in the dark. Last instar larvae (250–300 mg in weight) were used in the study. The larvae were immune-challenged by puncturing with a needle dipped into a pellet containing live *E. coli* D31 and *M. luteus* cells and the hemolymph was collected 24 h after the immunization. The methanolic extracts containing antimicrobial peptides and proteins below 30 kDa were prepared from the hemocyte-free hemolymph as described earlier [14]. After removal of lipids, the extracts were freeze-dried and stored at −80 °C until used.

2.3. Purification of G. mellonella anionic peptide 2 and lysozyme

G. mellonella AP2 and lysozyme were purified from the immune hemolymph extract, in accordance with our previous study [64]. In brief, the freeze-dried extract was dissolved in 0.1% trifluoroacetic acid (TFA) and subjected to HPLC chromatography using a Supelcosil LC-18-DB 4.6 mm × 250 mm column (Sigma–Aldrich-Fluka-Supelco Company, St. Louis, MO, USA) and a two-buffer set, A: 0.1% TFA (v/v), B: 0.07% TFA, 80% acetonitrile (v/v). A linear gradient from 36 to 68% of buffer B over 30 min and 1 mL/min flow rate was applied. The peptides in the collected fractions were identified by N-terminal amino acid sequencing on Procise 491 (Applied Biosystems, Foster City, CA, USA) after SDS-PAGE, electrotransfer onto a polyvinylidene difluoride (PVDF) membrane, and staining with Coomassie Brillant Blue R-250. The anionic peptide 2 was obtained in a pure form, while lysozyme was additionally purified by gel filtration chromatography on a Superdex Peptide 10/300 GL column (Pharmacia Biotech, Uppsala, Sweden), using 0.1% TFA supplemented with 40% acetonitrile (v/v) as a mobile phase and a 0.4 mL/min flow rate. The purified peptides were quantitated by amino acid analysis as described elsewhere [3], lyophilized, and stored at −80 °C. For the experiments, they were dissolved in sterile deionized water.

2.4. C. albicans viability assay

The effect of *G. mellonella* AP2 and lysozyme on the *C. albicans* survival rate was determined using a colony counting assay. The log-phase *C. albicans* cells (12 μL; OD_{600} = 0.002) suspended in diluted YPD medium (0.1% yeast extract, 0.2% peptone, 0.2% dextrose) were incubated without (control) or in the presence of different concentrations of lysozyme and/or AP2 at 37 °C for 24 h.