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Przeciwgrzybowe właściwości lizozymu i peptydu anionowego 2 *Galleria mellonella*

(Antifungal properties of *Galleria mellonella* lysozyme and anionic peptide 2)

Summary

Lysozyme and anionic peptide 2 (AP2) are constitutively present in *Galleria mellonella* hemolymph and constitute a part of the first line of defense during infections caused by different microorganisms. The high anti-Gram-positive bacteria activity of *G. mellonella* lysozyme, related to its muramidase activity, has been well documented (Yu K. H., Kim K. N., Lee J. H., Lee H. S., Kim S. H., Cho K. Y., Nam M. H., Lee I. H., 2002. Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella*, *Bombyx mori*, *Agrius convolvuli*. *Dev Comp Immunol* 26: 707-713). Interestingly, this protein exhibits non-enzymatic activity against *Escherichia coli*, Gram-negative bacterium (Zdybicka-Barabas A., Mak P., Klys A., Skrzypiec K., Mendyk E., Fiołka M. J., Cytryńska M., 2012. Synergistic action of *Galleria mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria. *Biochim Biophys Acta* 1818: 2623-2635). Antifungal activity of *G. mellonella* lysozyme toward *Saccharomyces cerevisiae* has been reported (Vilcinskas A., Matha V., 1997. Antimycotic activity of lysozyme and its contribution to antifungal humoral defence reactions in *Galleria mellonella*. *Anim Biol* 6: 19-29). However, in contrast to a well known mechanism of antibacterial action, antifungal activity of the lysozyme has not been fully elucidated yet.

G. mellonella AP2 exhibits low anti-Gram-positive bacteria and anti-*Pichia* activity *in vitro* (Cytryńska M., Mak P., Zdybicka-Barabas A., Suder P., Jakubowicz T., 2007. Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph. *Peptides* 28: 533-546). Moreover, AP2 enhances the non-enzymatic activity of the lysozyme against Gram-negative bacteria by increasing the bacterial membrane permeabilizing activity of the lysozyme (Zdybicka-Barabas A., Mak P., Klys A., Skrzypiec K., Mendyk E., Fiołka M. J., Cytryńska M., 2012. Synergistic action of *Galleria mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria. *Biochim Biophys Acta* 1818: 2623-2635). It could be postulated that these two defense factors play a significant role in antibacterial as well as antifungal response of *G. mellonella*.

The aim of this study was to explore the effect of *G. mellonella* lysozyme and anionic peptide 2 on *Candida albicans* cells and to explain the mechanism of antifungal action of *G. mellonella* lysozyme, which – similarly to its human counterpart – belongs to c-type family of lysozymes. *C. albicans*, being a part of the normal microflora, exists in the human gastrointestinal tract and women's reproductive tract. It is usually tolerated by the host organism, but in immunocompromised individuals it can cause infections, called candidiases. Recently, *G. mellonella* larvae have been widely used as alternative model hosts for studying pathogenesis and virulence factors of different pathogenic bacteria and fungi, including *C. albicans.*

Antifungal activity of *G. mellonella* lysozyme and AP2 was determined by using a colony counting method on solid media plates, and the metabolic activity of *C. albicans* cells after incubation with these agents was tested by a LIVE/DEAD staining method. Time-dependent alterations in *C. albicans* cell surface topography and nanomechanical properties of a cell surface as well as in ultrastructure of the fungal cells caused by both compounds were analyzed by atomic force microscopy (AFM) and transmission electron microscopy (TEM). The interaction of the *G. mellonella* lysozyme with *C. albicans* cell wall and cell membrane as well as translocation of the lysozyme into the cells was evaluated using FITC-labeled lysozyme. Examination of enzymatic activity of the lysozyme was performed by β-glucanase and chitinase activity assay. Furthermore, two fluorescent dyes, Congo Red and Calcofluor White, specifically binding to β-glucans and chitin, respectively, were used for imaging the alterations of these components in *C. albicans* cell wall. Induction of *C. albicans* apoptosis as a possible mode of *G. mellonella* lysozyme antifungal action was evaluated by staining of lysozyme-treated *C. albicans* with FITC-conjugated Annexin V, JC-1 and Hoechst dyes. An analysis of ionic balance disruption in *C. albicans* cells after treatment with *G. mellonella*

lysozyme using potassium channel inhibitors – tetraethylammonium ions (TEA) was also performed.

The results of study revealed that *G. mellonella* lysozyme at a relatively low concentration (0.5 μM), corresponding to its constitutive concentration in hemolymph, effectively limited the growth of *C. albicans*. AP2 also reduced the viability of the fungal cells. Moreover, the metabolic activity of *C. albicans* cells was reduced under the influence of lysozyme and AP2. The morphology as well as topography and properties of the surface of *C. albicans* cells were also changed. Further research showed that *G. mellonella* lysozyme interacted with the surface of *C. albicans* cells and translocated to the interior of the cells. However, the lysozyme did not hydrolyse polysaccharide components of *C. albicans* cell wall, thus its antifungal activity is probably based on a mechanism other than the enzymatic one. The final stage of the study revealed that antifungal activity of *G. mellonella* lysozyme was connected with disfunction of potassium channels in the *C. albicans* cell membrane. Furthermore, *C. albicans* cells treated with *G. mellonella* lysozyme exhibited features characteristic for cells undergoing programmed cell death, i. e. phosphatidylserine externalization in the outer membrane layer, loss of activity of mitochondria and altered morphology of nuclei. In conclusion, it can be postulated that antifungal activity of *G. mellonella* lysozyme against *C. albicans* involves induction of apoptosis.

Keywords: lysozyme, anionic peptide 2, *Galleria mellonella*, *Candida albicans*, apoptosis, atomic force microscopy, transmission electron microscopy