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Stimulation of lysozyme release by selected microbial preparations

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Lysozyme is an important factor of innate immunity and a unique enzybiotic in that it exerts not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer, and immunomodulatory activities. The purpose of the present study was to investigate whether *in vitro* exposure to microbial preparations can affect the release and production of lysozyme activity in human monocytic cell line THP-1. Lysozyme activity levels in cell culture fluids were measured using highly sensitive fluorescence-based lysozyme activity assay. Different preparations of bacteria and ascomycetes stimulated lysozyme release result in a higher lysozyme activity after one hour exposure. The demonstrated ability of selected microbial preparations to enhance the release of lysozyme activity can present a new mechanism contributing to explaining biological characteristics of microbial preparations, including their antibacterial and immune-stimulating properties.

1. Introduction

Human lysozyme (1,4-*N*-acetylmuramidase, E.C.3.2.1.17) is an ubiquitous low molecular–weight enzyme present in a wide range of biological fluids and tissues within the animal and plant kingdoms (Balboa et al. 2003). Since its first description in 1922 in tears and nasal secretions (Flemming 1922), a variety of literature has accumulated on its structure, function, genetics, biosynthesis, regulation, enzyme activity, and properties. Lysozyme is one of the most important factors of innate immunity, possessing antibacterial, antiviral, antitumor and immune modulatory activities (Vidal et al. 2005).

Among major cell types responsible for the production of lysozyme are monocytes/macrophages and neutrophilic granulocytes as a part of their surveillance functions in the immune system (Bandlow and Kiihne 1980). Pathogen-associated molecular patterns (PAMP) are molecules unique to microorganisms that are not associated with human cells. They include LPS, peptidoglycan, lipoteichoic acids, mannose, flagellin, pilin, bacterial DNA, and viral double-stranded RNA. Recognition of microorganisms and signaling are essential functions of the cells of innate immune system. A number of pattern recognition receptors involved in opsonization, complement activation, and phagocytosis have been described. Receptors expressed on the surface of cells specialized in the recognition of bacterial products, including bacterial lipoproteins and lipoteichoic acids are the Toll-like receptors (TLRs) (Medzhitov et al. 1997). The bacterial LPS, present in the outer membrane of gram-negative bacteria, activate the TLR-4 as the membrane receptor that triggers LPS signaling.

Monocytes express this receptor associated with increased TNF-alpha production (Witkamp and Monshouwer 2000). LPS is also able to stimulate the secretion of lysozyme by monocytes and other cells of the immune system (Timm et al. 1984). The purpose of the present study was to investigate whether *in vitro* exposure to various concentrations of selected preparations of microbial cell walls can induce the release of lysozyme activity in the human monocytic cell line THP-1 in comparison to LPS.

2. Investigations, results and discussion

The table shows that selected preparations of microorganisms (bacteria, yeast and fungi) are able to stimulate the release of lysozyme in the human monocytic cell line THP-1. In general, a tendency appears that substances derived from microorganisms enhance the secretion of lysozyme in the investigated cell system. As well as preparations from Gram-positive bacteria (genus *Bacillus* and *Mycobacterium*) and Gram-negative bacteria (*Brucella*) were active in the test system. At the indicated concentrations the microbial preparations did not induce any cytotoxicity as proven by a viability test (data not shown).

In accordance with reports in the literature (Wiernik et al. 1986) LPS prepared from *Salmonella* was able to enhance the lysozyme release. Also macrophages and monocytes respond to LPS by an enhanced lysozyme production (Goethe and Loc 1994). This effect demonstrated the suitability of the THP1-test system for the investigation of different microbial preparations regarding their ability to stimulate lysozyme release.

Interestingly, preparation from the ascomycetous fungus *Penicillium chrysogenum* activated the monocytic cells to secrete lysozyme but preparations from *Mucor racemosus* and *M. mucedo* did not show that activity. The reason for that might be based in the different structure of the water-soluble cell components yielded after cell mill treatment (procedure I) between these two species.

Because of the presence of a variety of microbial cell wall components able to activate the innate immune systems via a recognition of pathogen-associated molecular patterns (PAMP) the activity of the microbial preparations in the used monocytic cell system is not surprising. Not only parts of the cell walls or membranes bind to PAMP-receptors but also bacterial DNA is able to activate cells of the immune system via

Table: Secreted lysozyme activity of $1 \times 10^{+6}$ cells/ml THP-1 cells after treatment with preparations of different microorganisms

Sample / Strain	(batch-no.)	Protein content (%)	Concentration for testing (μg/ml)	Release of lysozyme (%)
Control		–	–	100 ± 16
LPS from *Salmonella enteriditis*		n.d.	10	143 ± 25*
Preparations according method I (water-soluble cell components)				
Candida albicans DSM 5197	(CA262/10068)	22.2	10	121 ± 57
Candida parapsilosis DSM 4884	(CP291/13129)	5.9	10	132 ± 32
Penicillium chrysogenum DSM 5753	(PN297/19030)	2.2	10	183 ± 50*
Penicillium glabrum DSM 5752	(PF293/03020)	0.7	10	104 ± 41
Penicillium roqueforti DSM 5504	(PR272/12118)	7.0	1	127 ± 33
Aspergillus niger DSM 6563	(AN224/28045)	2.3	1	123 ± 25
Mucor racemosus DSM 2845	(MR299/17040)	3.9	10	121 ± 40
Mucor mucedo (+) DSM 4886	(MM240/13066)	16.0	10	108 ± 25
Mucor mucedo (–) DSM 4885	(MM241/27066)	6.3	10	116 ± 26
Mycobacterium bovis BCG 125	(MB236/04046)	12.3	1	121 ± 21
Mycobacterium phlei DSM 4817	(MP246/31017)	21.8	10	122 ± 53
Preparations according method II (water-insoluble cell fragments)				
Bacillus cereus DSM 5194	(BC550/12011)	5.3	10	126 ± 17*
Bacillus firmus DSM 4816	(BF546/25015)	21.2	10	158 ± 56*
Bacillus subtilis DSM 5330	(BS543/03114)	3.3	1	147 ± 84
Mycobacterium bovis BCG125	(MB547/04046)	6.8	10	144 ± 11*
Mycobacterium phlei DSM4817	(MP548/31017)	15.1	1	188 ± 56*
Prepaprations according method III (cell parts after acidic extraction)				
Mycobacterium bovis BCG 125	(MB411/04046)	40.5	10	150 ± 44*
Brucella melitensis Rev-1	(BM423/09031)	27.2	10	139 ± 33*

Supernatant of control cultures without sample addition were considered to have 100% lysozyme activity. All points were measured in triplicate, and the values are representative of average value of at least three separate experiments (±SD). Values with asterisk are significantly different ($P < 0.05$, Mann- Whitney U test) from values of control cultures.

binding to these receptors (Dalpke and Heeg 2002; Griebel et al. 2005). Therefore whole cell preparations and extracts of bacteria seem to be able to stimulate lysozyme an release. Especially the water-insoluble cell fragments (procedure II) and the trichloroacetic-extracted cell parts (procedure III) showed significant effects indicating that rather substances with high molecular weight activated the monocytic cells presumably after phagocytosis.

Lysozyme is well-known as an important factor of innate immunity, since the principal function attributed to lysozyme in most animals is host defense (Jolles and Jolles 1984). Lysozyme is an unique enzybiotic in that it exerts not only antibacterial activity, but also antiviral, anti-inflammatory, anticancer, and immunomodulatory activities. It is able to control the growth of susceptible bacteria and to modulate host immunity against infections (Sava 1996). The production and release of lysozyme activity seems to be a common mechanism in stimulation the innate immune system (Keshav et al. 1991). Our results may contribute to explain the immunostimulant properties of microbial preparations, including their antibacterial, antiviral, anti-inflammatory or immune-stimulating activities. The stimulation of the immune system, through elevation of lysozyme activity levels, introduced these preparations as potential immunostimulant agents.

3. Experimental

3.1. Materials

The microbial preparations were made by Sanum-Kehlbeck GmbH & Co. KG (Hoya, Germany) with approved methods (GMP controlled production size methods). Different procedures were applied with different final products.

3.1.1. Procedure I:

Water-soluble cell components after procedures of fermentation, purification, cell mill treatment, sterilization (sterile filtration at 0.2 μm) and lyophilisation; regarding preparations of:

Candida albicans DSM 5197 (used in e.g. Albicansan^{R} D4 capsules and D5 drops),
Candida parapsilosis DSM 4884 (used in e.g. Pefrakehl^{R} D4 capsules and D5 drops),
Penicillium chrysogenum DSM 5753 (used in e.g. Notakehl^{R} D4 capsules, D5 drops and D5 tablets),
Penicillium glabrum DSM 5752 (used in e.g. Quentakehl^{R} D4 capsules and D5 drops),
Penicillium roquefortii DSM 5197 (used in e.g. Fortakehl^{R} D4 capsules, D5 drops and D5 tablets),
Aspergillus niger DSM 6563 (used in e.g. Nigersan^{R} D4 capsules, D5 drops and D5 tablets),
Mycobacterium bovis BCG 125 (used in e.g. Bovisan^{R} D4/D6 drops),
Mycobacterium phlei DSM 4817 (used in e.g. Utilin S^{R} D6 drops),
Mucor racemosus DSM 2845 (used in e.g. Mucokehl^{R} D4 capsules, D5 drops and D5 tablets)
Mucor mucedo (+) DSM 4886 and *M. mucedo (-)* DSM 4885 (used in e.g. Mucedokehl^{R} D4 capsules and D5 drops).

3.1.2. Procedure II:

Water-insoluble cell fragments after procedures of fermentation, purification, cell mill treatment, sterilization (121 °C, 25 min) and lyophilisation; regarding preparations of:
Bacillus cereus DSM 5194 (used in e.g. Latensin^{R} D4/D6 capsules),
Bacillus firmus DSM 4816 (used in e.g. Recarcin^{R} D4/D6 capsules),
Bacillus subtilis DSM 5330 (used in e.g. Utilin(H)^{R} (syn. Utilin phlei) D4/D5/D6 capsules),
Mycobacterium bovis BCG125 (used in e.g. Bovisan^{R} D5/D6 capsules),
Mycobacterium phlei DSM 4817 (used in e.g. Utilin S^{R} D4/D6 capsules).

3.1.3. Procedure III:

After procedures of fermentation, purification, cell mill treatment, extraction (aqueous trichloroacetic acid 10 % m/v), adjusting to pH 5–6, concentration of macromolecular substances > 10 kD by dialysis, sterilization (sterile filtration at 0.2 μm) and lyophilisation; regarding preparations of:
Mycobacterium bovis BCG 125 (used in e.g. Sanukehl Myc^{R} D6 drops)
Brucella melitensis Rev-1 (used in e.g. Sanukehl Brucel^{R} D6 drops).
See (Tab. 1). LPS from *Salmonella enteriditis* was purchased from Sigma.

3.2. Protein content

The content of protein is analysed by spectrophotometry. The method is based on precipitation of proteins by using a trichloroacetic acid (TCA) solution. The turbidity is measured at 600 nm. Bovine serum albumin (BSA) is used as reference standard.

3.3. Sample preparation

Sample (lyophilisate according to procedures A, B or C, respectively, 20–30 mg) are weighed in a 1.0 ml volumetric flask, diluted and dissolved with potassium phosphate buffer (0.1 M, pH 6.0).
Test Solution: Clear sample solution (200 µl) are mixed with 800 µl TCA solution (5 g/100 ml purified water).
Stock solution: 30 mg BSA are weighed into a 10.0 ml volumetric flask and dissolved with potassium phosphate buffer (0.1 M, pH 6.0).
11 References solutions (0 µg/ml to 600 µg/ml) were prepared by using different amounts of stock solution and buffer as well as mixing of 200 ml dilution with 800 ml TCA solution (5 g/100 ml purified water).
The evaluation of system suitability is carried out with reference solutions 1–11. The coefficient of correlation of the linear regression (concentration versus absorbance) is calculated. Acceptance criterium: $r \geq 0.999$.

3.4. Lysozym activity

Each examined microbial preparation was dissolved in PBS in a 1 mg/ml stock solution, and then diluted with culture medium to the final concentrations.

3.4.1. Cell lines and culture medium

Human monocytic leukemia cell line THP-1 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany. Sterile RPMI-1640 medium without phenol red, PBS, L-Glutamine, and fetal bovine serum were obtained from Biochrom, Germany. Aprotinin from bovine lung, obtained from Sigma, was dissolved in phosphate buffer (pH 7) in a 10 mg/ml (7.3 mM) stock solution, and then diluted with the culture medium to a final concentration of 2 µg/ml (0.3 µM).

3.4.2. Kits

EnzChek Lysozyme Assay Kit was purchased from Molecular Probes™ (Invitrogen Detection Technologies, USA). DQ lysozyme substrate (*Micrococcus lysodeikticus*) stock suspension (1.0 mg/ml) and 1000 units/ml lysozyme stock solution were prepared according to the manufacturer.

3.4.3. Cell culture

The human monocytic leukemia non-adherent cell line THP-1 was maintained in RPMI-1640 medium without phenol red, supplemented with 10% fetal bovine serum and L-glutamine. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cell cultures were passaged every 3–4 days. Passages from 5 to 30 were used for the experiments. Cell counts and viability were determined with Casy® cell counter (Schärfe System GmbH).

3.4.4. Incubation of cell cultures with microbial preparations

THP-1 cells were counted, viability determined and pelleted by centrifugation. Cells were seeded into 24-well plates at an initial concentration of 1.0×10^6 cells/ml. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 for 1 h in culture medium (control) or in preparations containing culture medium. Aprotinin was added to the incubation culture medium at a final concentration of 2 µg/ml (0.3 µM), in order to achieve best lysozyme determination results, as proved by previous experimental results (Helal and Melzig 2010). Supernatants of the examined and control cell cultures were collected after one hour incubation period, and used to determine lysozyme activity released into culture medium.

3.4.5. Determination of cell viability

In order to determine the cytotoxicity of the microbial preparations, viability of cells after exposure to each preparation for the incubation period was measured using CASY® cell analyzer system. Cell viability was assessed based on the integrity of plasma membrane.

3.4.6. Fluorescence-based lysozyme activity assay

Lysozyme was quantified according to a sensitive fluorescence-based method using an EnzChek® kit described previously (Helal and Melzig 2008). Briefly, using 96-well black microplate, 50 µl volume of cell culture supernatant was used for each reaction. Culture medium without cells was used as a control. Starting the reaction 50 µl of the DQ lysozyme substrate working suspension was added to each microplate well containing the experimental or the standard curve samples. Fluorescence intensity of each reaction was measured every 5 min to follow the kinetic of the reaction at 37 °C for 60 min, using fluorescence microplate reader with fluorescein filter (Tecan Austria GmbH). Digestion products from the DQ lysozyme substrate have an absorption maximum at 494 nm and a fluorescence emission maximum at 518 nm. Lysozyme activity levels of the experimental samples were determined from the standard curve. Lysozyme standard curve was linear with a correlation coefficient mean of $R^2 = 0.9962$. The rate of degradation of fluorescein labeled *Micrococcus lysodeikticus* suspension induced by lysozyme standard at different concentrations, represented in the slop values of the kinetic curve of each concentration, was linear in the range of 4–63 units/ml (data not shown).

3.5. Statistical analysis

Data in the Table are presented as mean ± SD. Differences between groups were assessed by the Mann-Whitney U test. A probability of $p < 0.05$ was considered significantly different.

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