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Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay

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The aim of this comparable study was to evaluate two different methods for the assay of lysozyme activity. Lysozyme activity was assayed by turbidimetric and fluorescence-based methods with hen egg white lysozyme as standard. The standard activity of each assay was calibrated in *units of activity per mg* under the experimental conditions (37 °C) so that direct comparison between these two assays could be made. The turbidimetric assay was performed using a 0.36 mg/ml *Micrococcus lysodeikticus* suspension and a microtiter plate reader capable of analyzing enzyme kinetics at 450 nm, and the linearity, in the range of 2.3–23.8 units/ml, presented a correlation coefficient (R^2) as high as 0.9967. The fluorescence-based assay was performed with EnzChek® kit using a suspension of *Micrococcus lysodeikticus* labeled with fluorescein and a fluorescence microplate reader. The linearity in the range of 0.47–5.28 units/ml, presented a correlation coefficient (R^2) as high as 0.9881. Thus, the turbidimetric assay provides a simple rapid accurate and specific method for the determination of lysozyme activity but with relatively low sensitivity in comparison with the fluorescence-based method which was demonstrated to be a high sensitivity assay, and hence a reliable applicable technique to determine lysozyme activity at low levels e.g. in cell culture systems.

1. Introduction

Lysozyme (or muramidase) (EC 3.2.1.17) is a ubiquitous enzyme present in a wide range of biological fluids and tissues within animal and plant kingdoms, such as human serum, urine, tears, seminal fluid and milk. It receives attention as a model protein for structural, physicochemical, crystallographic, enzymatic, immunological, and evolutionary studies. It is well-known for its antibacterial properties by catalyzing the hydrolysis of β-(1 → 4)-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of microorganisms. Lysozyme also exhibits antiviral, antitumor and immune modulatory activities (Vidal et al. 2005). The determination of the lysozyme concentration in body fluids is helpful in the diagnosis of several diseases. Some of these diagnostic uses are given in the table.

Since lysozyme is widely applied in medicine and immunohistochemistry, and it is used also in gene and cell engineering, many strategies have been developed to determine it. Quantitative lysozyme determinations are performed on two principles:

1. Lysozyme assay methods relying on the protein itself, such as the electrophoretic, chromatographic, immunoenzymatic, spectrophotofluorometric, and fluorescence polarization techniques. For example, the enzyme-linked immunosorbent assay (ELISA) has high sensitivity, high specificity and convenience, especially for analysis of a large number of samples (Vidal et al. 2005). More recent is the resonance light-scattering technique with functionalized CdTe (cadmium telluride) nanoparticles with sensitivity at the nanogram level (Li et al. 2007). Another new analytical procedure for assay of lysozyme as a protein at ng pro ml–level by using immobilized reagents in flow injection chemiluminescence (CL) systems was reported. These reagents, including luminol and periodate, were immobilized on the anion-exchange column in the flow injection system. Through water injection, luminol and periodate were eluted from the anion-exchange column to generate chemiluminescence, which was inhibited in the presence of lysozyme. By measuring the decrease of CL intensity, lysozyme could be quantitatively determined (Song and Hou 2003).

2. Lysozyme assay methods relying on the lysozyme lytic activity against the cell wall of *Micrococcus lysodeikticus* as a substrate. The reaction course is most often followed turbidimetrically, nephelometrically or fluorometrically in the liquid phase. The method can also be applied to the solid phase with agarose gel as reaction medium. The catalytic activity of lysozyme is dependent on the pH, ionic strength, substrate concentration, and sodium and potas-

Table: Some diagnostic uses of lysozyme

Body fluid	Diagnostic use of measurement
Serum lysozyme levels	Peritoneal tuberculosis (Velayati et al. 1994) Intrathoracic lymphadenopathy (Lodhat and Mir 1980) Leprosy (Near and Lefford 1992) Ocular sarcoidosis (Baarsma et al. 1987) Non-tubercular respiratory tract diseases (Marino et al. 1982) Pancytopenia (Iavorkovskii et al. 1978) Differential diagnosis of acute leukaemia (Jaworkowsky et al. 1972) Sepsis (Lawton 2006)
Cerebrospinal fluid lysozyme levels	Neurological disease (Firth 1985) Central-nervous-system tumours (Di Lorenzo and Palma 1976) Tuberculous meningitis in children (Mishra et al. 2003) Ventriculitis (Schroeder et al. 2000) Differential diagnosis of bacterial and viral meningitis (Babich et al. 1992)
Urinary lysozyme levels	Rejection of the transplanted kidney (Bastecka et al. 1979; Ellis et al. 1978) Differential diagnosis of chronic glomerulonephritis and chronic pyelonephritis (Tofilo and Antonov 1988)
Tears lysozyme levels	Facial paralysis (Hara et al. 1987) Dry eye (Rong 1984)
Sperm lysozyme levels	Male infertility (Kuz'min et al. 1991)
Faecal lysozyme levels	Chronic inflammatory bowel disease (Dick 1982)

sium concentrations of the reaction mixture. The effect of these factors is interdependent, so that it is difficult to evaluate properly the effects of each parameter separately. Albumin enhances the reaction rate, and probably there are also more complicated interactions due to other proteins (Mörsky 1983). In the agar diffusion method lysozyme catalyzes the hydrolysis of *Micrococcus lysodeikticus* while incubating together, resulting in a transparent ring around it. This method is simple, but time-consuming needs sometimes more than 10 h and is easily interfered by other proteins. Besides, huge relative error exists because it is hard to control the thickness of agar, definition of the penumbra, and measuring the diameter is not very accurate. Another method using agarose is agarose rocket electrophoresis, in which the bacteria can be resolved when the lysozyme moves in the electric field and reacts with the substrates in the gel, forming a transparent peak of which the value was proportional to the logarithm of lysozyme concentration. The turbidimetric method is simple and quick but with a low sensitivity and narrow linear ranges. Lee and Yang improved this assay by using a microplate format. Resonance scattering spectral (RSS) assay is also a new method for the determination of lysozyme activity with high sensitivity, good selectivity, based on the catalytic effect of lysozyme on the hydrolysis of *Micrococcus lysodeikticus* and its resonance scattering effect (Jiang and Huang 2007).

As mentioned above, some lysozyme assay techniques based on the nature of the enzyme molecule as a protein have been described and investigated. However, these methods suffer from drawbacks of precision, because the lysozyme effects as a therapeutic agent depend mostly on its activity as enzyme.

Thus, one of our objectives in this study was to evaluate a fluorescence-based assay method which measures levels of lysozyme activity in solution. This assay measures lysozyme activity on *Micrococcus lysodeikticus* cell walls, which are labelled with the fluorophore fluorescein to such a degree that the fluorescence is quenched. Lysozyme action can relieve this quenching; resulting in a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase can be measured with any spectrofluorometer, mini-fluorometer or fluorescence microplate reader that can detect fluorescein. This assay was compared in this study with the classical photometric assay of lysozyme activity, which detects changes in the turbidity of a bacterial suspension, *Micrococcus lysodeikticus*, caused by the enzymatic activity of lysozyme (Moreira-Ludewig and Healy 1992).

In this study, calibrated chicken egg white lysozyme standards were used in each assay. The activity of these chicken egg white lysozyme standards was measured in 'units of activity' per mg under our conditions, so that direct comparison between these two assays, in units, could be made.

2. Investigations and results

2.1. Standard calibration

It was necessary for the chicken egg white lysozyme standards, which were used in both the fluorescence-based and the turbidimetric lysozyme assay, to be calibrated according to the laboratory conditions.

One unit (1 U) of lysozyme activity was defined by the manufacturer as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 25 °C, in a 2.6 ml reaction mixture (1 cm light path). In our study, we have defined the unit of lysozyme activity under the used conditions as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* suspension as substrate, under the conditions of pH 6.24 and 37 °C, in a 250 µl reaction mixture using 96 well microtiter plate.

2.2. Fluorescence-based lysozyme assay using EnzChek®

A straight line diagram with a correlation coefficient of 0.9935 was obtained with the EnzChek® lysozyme standard calibration spectrophotometrically under our conditions (Fig. 1).

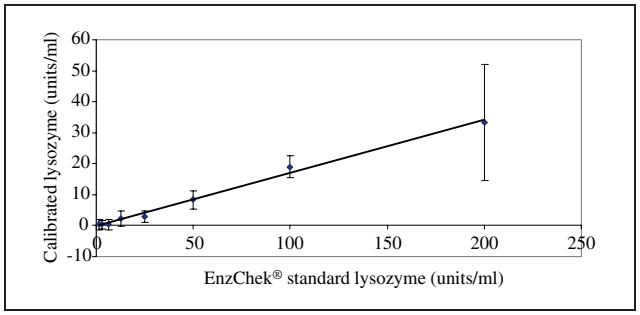

Fig. 1: Calibration diagram of chicken egg white lysozyme standard of EnzChek® on spectrophotometer

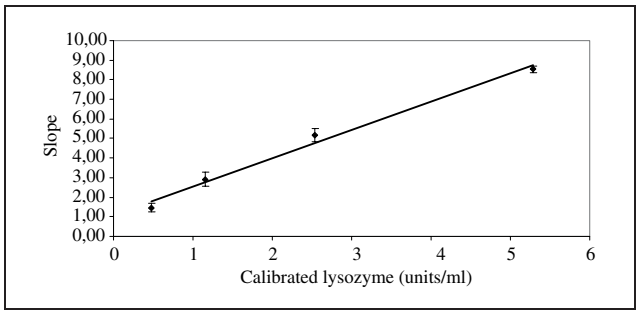

Fig. 2: Chicken egg white lysozyme standard curve using the EnzChek® Lysozyme Assay Kit. All points were measured in triplicate in at least 3 independent experiments

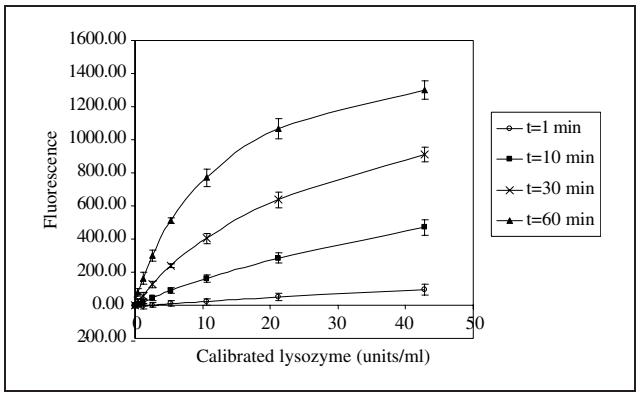

Fig. 3: Detection of lysozyme activity using the EnzChek® Lysozyme Assay Kit. Increasing concentrations of lysozyme were incubated with the DQ lysozyme substrate for different time periods at 37 °C. The fluorescence was measured in a fluorescence microplate reader using excitation/emission of 485/530 nm. The background fluorescence was subtracted from each value. All points were measured in triplicate in at least 3 independent experiments

The rate of lysis induced by lysozyme represented in the slope values of the kinetic curves of the lysozyme concentrations was linear in the range of 0.47–5.28 units/ml (Fig. 2).

The linear regression equation and the correlation coefficient of this standard curve were: $y = 1.4389x + 1.1312$ and $R^2 = 0.9881$, respectively. This fluorescence-based assay was able to detect lysozyme activity down to 0.13 unit/ml with good linearity ($R^2 = 0.9892$) if a high concentration of the lysozyme substrate (150 µg/ml) was used.

2.3. Turbidimetric assay

A straight line diagram with a correlation coefficient of 0.9933 was obtained with the Sigma lysozyme standard

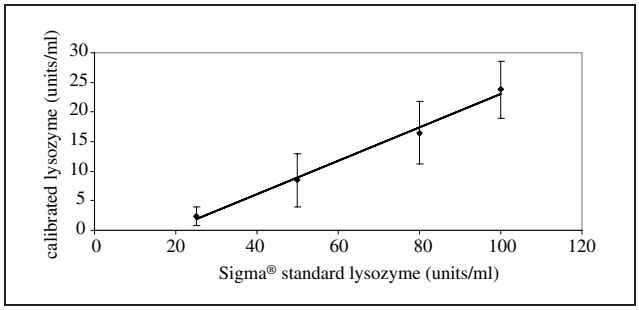

Fig. 4: Calibration diagram of chicken egg white lysozyme standard from Sigma

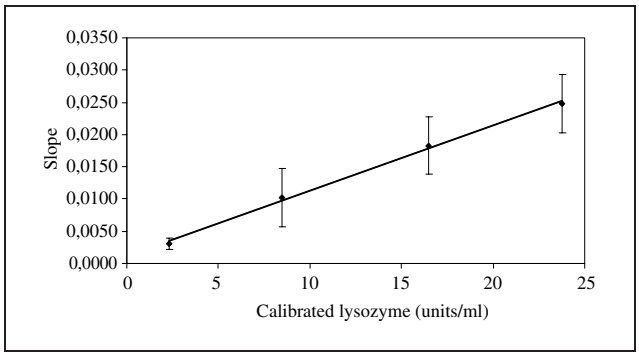

Fig. 5: Chicken egg white lysozyme standard curve using the microtiter plate turbidity assay. All points were measured in triplicate, and the curve is representative of three separate experiments

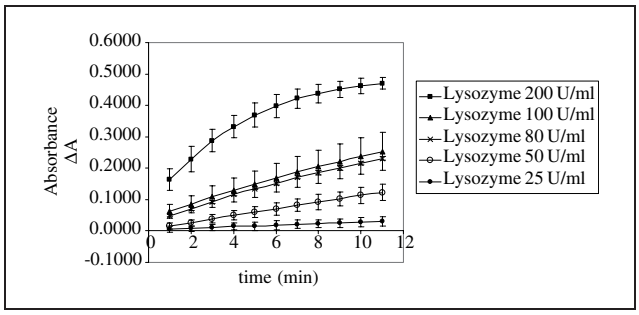

Fig. 6: Chicken egg white lysozyme standard kinetic curve using the microtiter plate turbidity assay. Increasing concentrations of lysozyme were incubated with 0.36 mg/ml *Micrococcus lysodeikticus* suspension for 10 min at 37 °C. The absorbance was measured using microplate reader with a 450 nm filter. A background absorbance was subtracted from each value. All points were measured in triplicate in at least 3 independent experiments

calibration spectrophotometrically under our conditions (Fig. 4). Sigma egg white lysozyme standard was stated to have an activity of 45200 units/mg (pH 6.24, 25 °C, 2.6 ml reaction mixture, 1 cm light path). In comparison to our calibration diagram, however, an activity of approximately 12530 units/mg was recorded under our conditions (pH 6.24, 37 °C, 250 µl reaction mixture). The rate of lysis induced by lysozyme, represented in the slop values of the kinetic curves of the used lysozyme concentrations, was linear in the range of 2.3–23.8 units/ml (0.5–2.2 µg/ml), according to our unit definition (Fig. 5).

The linear regression equation and the correlation coefficient of this standard curve were: $y = 0.001x + 0.0012$ and $R^2 = 0.9967$, respectively.

The enzyme activity of lysozyme can be blocked by the specific competitive inhibitor, *N*,*N*′,*N*″-triacetylglucosamine, also referred to as *N*,*N*′,*N*″-triacetylchitotriose (TAC) or chitotriose (Mink et al. 2004). The IC_{50} of TAC

was determined with 0.8 mg/ml (1.28 mM) in the turbidimetric assay detecting the accuracy of the used method.

3. Discussion

In the present study, we have described two simple, rapid and versatile lysozyme activity assay methods. The calibration of the lysozyme standards into units of activity under our used conditions was essential for the accurate comparison between these two assay methods.

The EnzChek® fluorescence-based lysozyme activity assay has been proved to be good in regard to sensitivity and linearity, basing on the catalytic effect of lysozyme to labelled *Micrococcus lysodeikticus* cells and the increase in fluorescence that is proportional to lysozyme activity. This method has a detection limit of lysozyme concentration down to 0.13 unit/ml with good linearity. On the other hand, the turbidimetric microplate lysozyme assay has been proved to have good linearity but less sensitivity in comparison with the EnzChek® fluorescence-based assay. It has a detection limit down to 2.3 units/ml. However, this detection limit of the turbidimetric microplate lysozyme assay is sufficient to provide a simple, accurate and inexpensive method to determine lysozyme levels in tissues or body fluids such as saliva and tears which contain high lysozyme levels, whereas the EnzChek® fluorescence-based assay can provide a high-sensitivity method for detecting lysozyme at low levels in cell culture systems.

4. Experimental

4.1. Materials and reagents

EnzChek® Lysozyme Assay Kit was purchased from Molecular Probes™ (Invitrogen Detection Technologies). Lysozyme from chicken egg white (45200 Units/mg solid, 50800 Units/mg proteins) in the form of lysozyme chloride was purchased from Sigma (Germany). One unit (1 U) of lysozyme activity was defined by the manufacturer as decreasing a 0.001 absorption value at 450 nm per min for catalytic hydrolysis of *Micrococcus lysodeikticus* under the conditions of pH 6.2 and 25 °C. 4000 U/ml lysozyme stock solution was prepared with a phosphate buffer (0.1 M; pH 6.24) immediately before use. The working solutions were obtained by diluting the stock solution to the appropriate concentrations with the phosphate buffer (0.1 M; pH 6.24). Lyophilized *Micrococcus lysodeikticus* cells were purchased from Sigma. A 0.36 mg/ml *Micrococcus lysodeikticus* working suspension was prepared with a phosphate buffer (0.1 M; pH 6.24) immediately before use, and mixed for 30 min in vortex mixer. *N*,*N*′,*N*″-triacetylglucosamine was supplied from Sigma, a 10 mg/ml stock solution was prepared in phosphate buffer (0.1 M; pH 6.24).

4.2. Apparatus

The fluorescence was measured using a fluorescence microplate reader with fluorescein filter (Tecan Austria GmbH). The absorption was measured using a microplate reader with a 450 nm filter (Tecan Austria GmbH). Results were compiled and analyzed using Tecan XFluor software via a Microsoft® Excel™ interface from a personal computer linked directly to the plate reader.

4.3. Fluorescence-based lysozyme assay using EnzChek® kit

DQ Lysozyme substrate stock suspension (1.0 mg/ml) and the 1000 U/ml lysozyme stock solution were prepared according to the manufacturer. Preparation of a lysozyme standard curve: 8 wells were filled with 50 µl of reaction buffer, 50 µl of the 1000 U/ml stock solution of lysozyme were added to the first well, mixed by pipetting, then 50 µl were transferred to the second well. This process was repeated from one well to the next, except 50 µl from the mixture in the seventh well was discarded and nothing were added to the eighth well. Thus, the lysozyme concentration will range from 500 U/ml to 0 U/ml in the 50 µl volumes, for a range of 250 U/ml to 0 U/ml in the final 100 µl volumes (units according to the conditions stated from the manufacturer). The DQ lysozyme substrate working suspension was prepared by diluting the 1 mg/ml stock suspension 20-fold in reaction buffer A. 50 µl volume was used for each reaction. The final concentration of the DQ lysozyme working suspension was two-fold lower in the final reaction buffer. Starting the reaction 50 µl of the DQ lysozyme substrate working suspension was added to each microplate well containing the standard curve samples. Fluorescence was measured every 5 min to follow the kinetics of the reaction at 37 °C for 60 min. Digestion products from the DQ lysozyme substrate showed an absorption maximum at 494 nm and a fluorescence emission maximum at 518 nm. The fluorescence values derived from the controls without enzyme were subtracted (Fig. 3). The chicken egg white lysozyme standard (EnzChek®) was calibrated spectrophotometrically by measuring the decrease in absorption per minute, using the same dilutions and conditions used in the turbidimetric assay described below.

4.4. Turbidimetric lysozyme assay

Preparation of the lysozyme standard curve: 8 wells were filled with 50 µl phosphate buffer (0.1 M; pH 6.24). 50 µl of the 4000 U/ml stock solution of lysozyme were added to the first well, mixed by pipetting, then 50 µl were transferred to the second well. This process was repeated from one well to the next, except for the seventh well where 50 µl from the mixture were discarded and nothing was added to the eighth well. Thus the lysozyme concentration ranged from 2000 U/ml to 0 U/ml in 50 µl volumes, for a range of 400 U/ml to 0 U/ml in the final 250 µl volumes. An additional well was filled with 50 µl of 400 U/ml lysozyme solution to achieve an additional final lysozyme concentration of 80 U/ml in the final 250 µl volume (units according to the conditions stated by Sigma). The reaction was started by addition of 200 µl of the 0.36 mg/ml *Micrococcus lysodeikticus* working suspension to each microplate well containing the standard curve samples. The absorption was read at a wavelength of 450 nm every minute to follow the kinetics of the reaction for 10 min at 37 °C. The absorbance values derived from controls without enzyme were subtracted (Fig. 6). The chicken egg white lysozyme standard (Sigma) was calibrated by measuring the decrease in absorption per minute under the conditions described before.

References

Baarsma GS, La Hey E, Glasius E, de Vries J, Kijlstra A (1987) The predictive value of serum angiotensin converting enzyme and lysozyme levels in the diagnosis of ocular sarcoidosis. Am J Ophthalmol 104: 211–217.

Babich EM, Derkach SA, Vasil'eva IB, Eliseeva IV, Makarenko KK, Valiukh SV (1992) The rapid differential diagnosis of bacterial and viral meningitis by using the lysozyme test. Zh Mikrobiol Epidemiol Immunobiol 5–6: 25–27.

Bastecka D, Kavan L, Erben J, Rozsival V, Prochazkova J, Navratil P, Bratova M (1979) Diagnosis of rejection of the transplanted kidney with respect to urine values of sodium, beta-glucuronidase, lysozyme and proteinuria. Vnitr Lek 25: 982–988.

Dick W (1982) Lysozyme: basic facts and diagnostic importance. Fortschr Med 100: 1230–1234.

Di Lorenzo N, Palma L (1976) Spinal-fluid lysozyme in diagnosis of central-nervous-system tumours. Lancet 307: 1077.

Ellis L, McSwiney RR, Tucker SM (1978) Urinary excretion of lysozyme and N-acetyl-beta-D-glucosaminidase in the diagnosis of renal allograft rejection. Ann Clin Biochem 15: 253–260.

Firth G, Rees J, McKeran RO (1985) The value of the measurement of cerebrospinal fluid levels of lysozyme in the diagnosis of neurological disease. J Neurol Neurosurg Psychiatry 48: 709–712.

Hara A, Kusakari J, Kawamoto K, Hara S (1987) Topographic diagnosis of facial paralysis by the assay of lysozyme concentration in tears. Acta Otolaryngol Suppl 435: 130–135.

Iavorkovskii LI, Solovei DIa, Grant KhIa, Riauzova LIu (1978) Lysozyme content in the blood as a criterion for the differential diagnosis of pancytopenic states. Probl Gematol Pereliv Krovi 23: 24–27.

Jaworkowsky L, Bersin W, Grant H, Rudens J (1972) Serum lysozyme in the differential diagnosis of acute leukemias. Folia Haematol Int Mag Klin Morphol Blutforsch 97: 265–278.

Jiang ZL, Huang GX (2007) Resonance scattering spectra of micrococcus lysodeikticus and its application to assay of lysozyme activity. Clin Chim Acta 376: 136–141.

Kuz'min MD, Luda AP, Mikhailova EA, Bukharin OV (1991) The diagnosis of male infertility based on the lysozyme level in sperm. Lab Delo 7: 39–41.

Lawton NJ (2006) Development of a rapid immunoassay for human pathogenic markers. PhD Thesis, Cranfield University, United Kingdom, p. 152.

Li J, He XW, Wu YL, Li WY, Zhang YK (2007) Determination of lysozyme at the nanogram level by a resonance light-scattering technique with functionalized CdTe nanoparticles. Analytical Sciences 23: 331–335.

Lodhat SC, Mir MA (1980) Usefulness of serum lysozyme measurement in diagnosis of intrathoracic lymphadenopathy. Tubercle 61: 81–85.

Marino C, Tesi A, De Luca Cardillo C, Tiezzi G (1982) Blood lysozyme in the diagnosis of non-tubercular respiratory tract diseases. Arch De Vecchi Anat Patol 65: 53–67.

Mink SN, Jacobs H, Duke K, Bose D, Cheng Z, Light RB (2004) N,N′,N″-triacetylglucosamine, an inhibitor of lysozyme, prevents myocardial depression in *Escherichia coli* sepsis in dogs. Crit Care Med 32: 184–193.

Mishra OP, Batra P, Ali Z, Anupurba S, Das BK (2003) Cerebrospinal fluid lysozyme level for the diagnosis of tuberculous meningitis in children. J Trop Pediatr 49: 13–6.

Moreira-Ludewig R, Healy CT (1992) A rapid microtiter plate method for the detection of lysozyme release from human neutrophils. J Pharmacol Toxicol Methods 27: 95–100.

Mörsky P (1983) Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. Anal Biochem 128: 77–85.

Near KA, Lefford MJ (1992) Use of serum antibody and lysozyme levels for diagnosis of leprosy and tuberculosis. J Clin Microbiol 30: 1105–1110.

Rong BL (1984) Quantitative determination of lysozyme in tears and diagnosis of dry eye. Zhonghua Yan Ke Za Zhi 20: 228–231.

Schroeder S, Stuerenburg HJ, Escherich F, Pfeiffer G (2000) Lysozyme in ventriculitis: a marker for diagnosis and disease progression. J Neurol Neurosurg Psychiatry 69: 696–697.

Song ZH, Hou S (2003) A new analytical procedure for assay of lysozyme in human tear and saliva with immobilized reagents in flow injection chemiluminescence system. Anal Sci 19: 347–352.

Tofilo AP, Antonov MP (1988) Study of electrophoretic mobility of urinary lysozyme in the differential diagnosis of chronic glomerulonephritis and chronic pyelonephritis. Ter Arkh 60: 40–42.

Velayati AA, Masjedi MR, Keyhani M, Mojibian M, Hajipour R (1994) Measurement of serum and peritoneal ADA and lysozyme levels for the diagnosis of peritoneal tuberculosis. Tubercle Lung Dis 75: 49.

Vidal ML, Gautron J, Nys Y (2005) Development of an ELISA for quantifying lysozyme in hen egg white. J Agric Food Chem 53: 2379–2385.