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LYTIC ACTION OF LYSOZYME ON CANDIDA ALBICANS

by

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ABSTRACT

Yeast cells of *Candida albicans* in lysozyme glucose solution were incubated in a 37° C water bath for 6 hours, spread on the surface of a Sabouraud's agar plate and incubated at 37° C for 18—24 hours. Scattered small colonies were seen on the agar surface compared with the thick full growth of the control culture incubated without lysozyme. Twenty-one strains of 6 standard Candida species of human isolation other than *Candida albicans*; *C. stellatoidea*, *C. tropicalis*, *C. pseudotropicalis*, *C. krusei*, *C. parapsillosis*, *C. guilliermondii*, showed essentially the same results as *Candida albicans*. A constant quantity of lysozyme caused destruction of Candida cells to an equal degree, regardless of varied concentrations of glucose. Dilution of lysozyme greater than 100 times the original (5 mg/ml) showed the same degree of candicidal activity, however, was dependent on the presence of minute amounts of glucose. The presence of NaCl prevented the lysis of Candida by lysozyme in various solutions. Candida cells with lysozyme in glucose solution was incubated for 6 hours in a 37° C water bath. Microscopic observations revealed drastic changes in cell morphology. Most of the cells were swollen, degenerated and some completely destroyed. The gram-positive characteristics of Candida cells changed to gram-negative. The combined activity of lysozyme with complement and antibody may play an important role in the protection against Candidiasis in vivo.

INTRODUCTION

In 1922 FLEMING found that saprophytic air cocci, and *Micrococcus lysodeikticus* were lysed by tears, nasal secretions, sputum, and other body fluids. The substance common to these fluids was enzymatic and was given the name 'lysozyme'.

FLEMING claimed that not only saprophytic *Micrococcus lysodeikticus*, but that other bacteria of human pathogenicity were also influenced by lysozyme. 16 of 22 strains of fecal streptococci were readily lysed by tears, and some strains of staphylococci and hemolytic streptococci were sensitive to lysis by tears and nasal secretion.

Since that time, the sensitivity of numerous species of organisms to the lytic action of lysozyme has been tested by various investi-

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gators. RIDLEY (1928) found that tears had an antibacterial effect on staphylococci. THOMPSON & GALLARDO (1940) described that 4 of 8 strains of staphylococcus aureus and 12 of 13 strains of staph. albus were lysed in human tears diluted from 1:10 to 1:160.

Although organisms belonging to the same genus show wide variations in the extent of lysis, there is a general agreement about the susceptibility of bacteria to lysozyme thus supporting several broad conclusions (SALTON 1957). The bacteria most vulnerable to lysis by lysozyme were micrococcus, sarcina, staphylococcus and bacillus (THOMPSON 1960). Gram negative bacteria, unless specifically pretreated, were most resistant to lysis by lysozyme (AMANO et al., 1954) (REPASKE 1956) (HOOK et al. 1960).

In the present study, the influence of lysozyme on 7 species of the genus Candida of human isolation was observed. Lysozyme was found to cause lysis and inhibition of growth of Candida species.

MATERIALS AND METHOD

Materials

25 standard strains of human isolation of Candida species were obtained from the National Institute of Health, Bethesda, Maryland, U.S.A., The Communicable Disease Center, Atlanta, Georgia, U.S.A., The Department of Mycology, McGill University, Montreal, Canada, and Temple University, Philadelphia, U.S.A.

A suspension of *Candida* cells was standardized as follows: The surface of a 19—24 hours (37° C) culture of *Candida* species in Sabouraud's agar was gently scraped using a wire loop. The material was suspended in 10 ml of distilled water in a tube* with a screw or rubber cap. The mixture was agitated to make an even suspension and its turbidity was measured using a Junior Coleman spectrophotometer at 320 mμ. Each suspension of Candida was adjusted to 50 % transmittance, either by further dilution with distilled water, or by further addition of Candida culture. A blank of 10 ml of distilled water was used to represent 100 % transmittance.

Method

Various dilutions of lysozyme** were prepared using diluents of glucose distilled water, physiologic saline and distilled water.

Using a pasteur pipette, 3 drops of standardized Candida suspension were mixed with the prepared solution of lysozyme and placed in a 37° C water bath for 5—6 hours, then inoculated on the surface of Sabouraud's agar and incubated for 24 hours at 37° C. Controls were prepared in exactly the same manner, omitting lysozyme. The 18—24 hours growth of Candida species in Sabouraud's agar was observed. Details of the method are as follows:

* B-D vacutainer (no additive, 165 × 16 mm or 100 × 16 mm) was found to be satisfactory.

** Crystalline product isolated from egg white was obtained from Nutritional Biochemicals, U.S.A. Lysozyme egg white crystalline 3 ×. Lysozyme crystalline 3 ×.

I. Incubation

3 drops of standardized Candida cell suspension were added to various dilutions of dextrose solution (5 %, 2.5 %, 1 %, 0.1 %) containing varying concentrations of lysozyme (5.0 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.62 mg/ml, 0.31 mg/ml, 0.16 mg/ml, 0.08 mg/ml, 0.04 mg/ml) and were incubated in a 37° C water bath for 6 hours.

The same dilutions of lysozyme were prepared substituting physiologic saline and distilled water for dextrose solution. The inhibitory effect of lysozyme upon Candida species in the three solutions were compared.

II. Inoculation on Sabouraud's Agar

Following six hours incubation in the waterbath, the tubes were shaken and using a Pasteur pipette, 2 drops were placed on the surface of a Sabouraud's agar plate (150 mm × 10 mm), then spread on the upper half using a wire loop.

As a control, two drops of standardized Candida cell suspension incubated without lysozyme were spread on the other half of the plate. The plate was incubated at 37° C for 8—24 hours, and the growth of Candida culture treated with lysozyme was compared with the growth of the untreated control of Candida culture.

Observations

I. Agar surface growth of *Candida albicans* pretreated with lysozyme and without lysozyme was compared

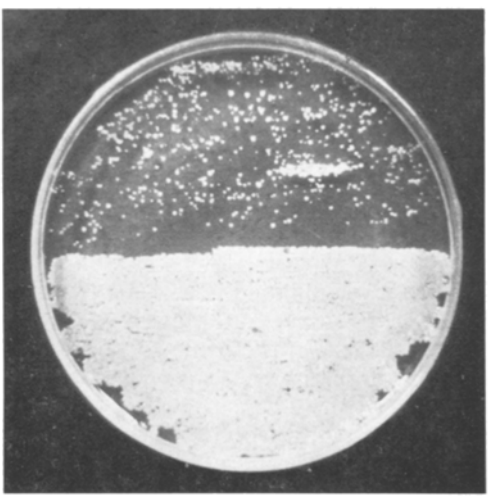

Fig. 1. Photograph of Sabouraud's agar plate.
Upper half – growth of *Candida albicans* pretreated with lysozyme glucose solution.
Lower half – same numbers of *Candida albicans* without lysozyme.

TABLE I.

(A) Growth of *Candida albicans* pretreated with Lysozyme and without Lysozyme

Candida albicans strains → / Treatment ↓	National Communicable Disease Center Strain	National Institute of Health Strain	McGill University Strain	Temple University Strain	Smith Strain Isolated at our own Laboratory
Lysozyme in Glucose solution *)	Slight growth **) (scattered small colonies)	No Growth	No Growth	Slight Growth	Slight Growth (Multiple faint colonies)
Control Glucose solution alone	Heavy growth ***)	Heavy growth	Heavy growth	Heavy growth	Heavy growth

*) Lysozyme in Glucose solution — Lysozyme (5 mg/ml)
— Glucose solution (1 % Glucose distilled water solution).

**) Slight growth — Scattered small colonies, covering 0—10 % of entire agar surface; see Fig. 1, upper half.

***) Heavy growth — Colony covering almost entire agar surface; see Fig. 1, lower half.

(B) Growth of Human Candida Species (Other than *Candida albicans*) Treated with Lysozyme and without Lysozyme (McGill University Strains)

Candida species → / Pretreatment with ↓	*Candida stellatoidea*	*Candida tropicalis*	*Candida pseudo-tropicalis*	*Candida krusei*	*Candida para-psillosis*	*Candida guillier-mondii*
With Lysozyme in glucose solution *)	No growth	Slight growth	No growth	Slight growth	No growth	No growth
Control without Lysozyme. Glucose solution alone	Heavy growth	Heavy growth	Heavy growth	Heavy growth	Heavy growth	Heavy growth

*) Lysozyme in Glucose solution — Lysozyme (5 mg/ml)
— Glucose solution (1 % glucose distilled water solution).

Results obtained are tabulated (Table I (A)) and illustrated (Fig. 1).

The above results showed that when *Candida albicans* was pretreated with lysozyme in glucose solution, only scattered small colonies of growth on Sabouraud's agar were observed following 18—24 hours incubation. On the contrary, *Candida albicans* treated under identical conditions but with omission of lysozyme had a full heavy growth.

Candida species of human isolation other than *Candida albicans* showed a similar pattern of results. 21 strains of 6 standard Candida species of human isolation other than *Candida albicans* (*C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsillosis, C. guilliermondii*) were treated with lysozyme as previously described. All organisms were sensitive to the action of lysozyme, re-

sulting in inhibition of growth compared with the full heavy growth of the control suspension. (Table I (B)).

II. Influence of Varying Concentrations of Lysozyme in Constant Glucose Concentration

In this experiment various concentrations of lysozyme in 0.1 % glucose were used.

Results showed that dilutions of lysozyme greater than 100 times the original dilution (0.04 mg/ml) caused inhibition of growth to the same degree as the more concentrated solutions of lysozyme (5 mg/ml). (Table II)

TABLE II

The Influence of Various Concentrations of Lysozyme Upon *Candida albicans* When Glucose Concentrations Were Kept Constant (0.1 %)

Tube Number	Lysozyme Concentration In Distilled Water	Glucose Concentration	Inoculation of Lysozyme treated Candida Cells on Sabouraud's agar	Results Growth of Candida Cells in Sabouraud's agar
1	5 mg/ml	0.1 %	2 drops of Pasteur pipette on upper half of the plate	Almost total inhibition. Close observation revealed scattered, faint colonies
2	2.5 mg/ml	0.1 %	,,	Inhibition - Same degree as No. 1
3	1.25 mg/ml	0.1 %	,,	,,
4	0.62 mg/ml	0.1 %	,,	,,
5	0.31 mg/ml	0.1 %	,,	,,
6	0.16 mg/ml	0.1 %	,,	,,
7	0.08 mg/ml	0.1 %	,,	,,
8	0.04 mg/ml	0.1 %	,,	,,
Control	No Lysozyme	0.1 %	2 drops of Candida cells (without treatment of Lysozyme) were spread on lower half of the plate	Full heavy growth

III. The Influence of a Constant Concentration of Lysozyme in Varying Concentrations of Glucose solution

In this experiment 5 mg/ml of lysozyme was prepared in varying concentrations of glucose solution (5 %, 2.5 %, 1 %, 0.1 %, 0.01 %).

The results obtained showed that a constant concentration of lysozyme caused growth inhibition to the same degree in 5 % glucose and in 0.01 % glucose solution. Thus the concentrations of glucose used in the diluent did not appear to be critical at this level.

IV. The Inhibition of Candicidal Action of Lysozyme by NaCl (Table III)

The candicidal action of lysozyme in 0.1 % glucose saline solution (Table III, Tube 1) and lysozyme in 0.1 % glucose distilled water

TABLE III
Inhibitory Influence of NaCl Upon The Candicidal Action of Lysozyme

Tube Number	Solution	Lysozyme	Amount of Candida Suspension	Growth Results in Sabouraud's agar
1	0.1 % glucose in physiologic saline	5 mg/ml	3 drops into 1 ml of solution	Moderate to heavy growth
2	0.1 % glucose in physiologic saline	None	,,	Same as above
3	0.1 % glucose in distilled water	5 mg/ml	,,	Almost complete suppression of growth
4	0.1 % glucose in distilled water	None	,,	Heavy growth
5	Physiologic saline	5 mg/ml	,,	Moderate growth
6	Physiologic saline	None	,,	Moderate growth (same as No. 5).
7	Distilled water	None	,,	Heavy growth

solution (No. 3) was compared. Results obtained indicated that the presence of NaCl (0.1 % glucose in saline) strongly interfered with the candicidal activity of lysozyme.

Although physiologic saline alone showed slight inhibitory activity to Candida growth (Table III, Tubes No. 2 and No. 6), the presence of NaCl in lysozyme saline solution or in lysozyme glucose saline solution did not enhance the destructive activity of lysozyme; on the contrary, NaCl acted as a protective to *Candida albicans* cells.

V. Changes in Morphological and Staining Characteristics of Candida albicans by the Action of Lysozyme

I. Loss of gram-positivity and degeneration of Candida cells.

Candida suspension in lysozyme glucose solution was incubated in a 37° C water bath for six hours. The tube was shaken and a drop of solution was spread on a glass slide, dried by air, and stained with Gram's stain.

Microscopic observations revealed that the cells were fewer in number than found in the control (simple glucose solution without lysozyme). The cells were fuzzy in outline and had a swollen appearance. The blue staining of Gram positive characteristics of most of the cells were lost, leaving a faint pinkish colour. Some cells were degenerated showing onlytheliberated pinkish cytoplasmic component, others became transparent leaving empty ghost cells. Occasionally quite distinct tiny deep blue granules were noted within the degenerated cells.

After prolonged incubation, the cells seemed to gradually lyse, leaving only a faint pinkish amorphous cell, with no morphological characteristics. When lytic action was completed no Candida cells were seen.

II. Comparison in morphology of Candida cells incubated in lysozyme distilled water, lysozyme physiologic saline and lysozyme glucose solution.

Morphology of the cell was best retained in solutions of lysozyme distilled water, relatively well preserved in lysozyme physiologic saline, however, the most dramatic morphological changes were seen in solutions of lysozyme glucose.

Gram positivity of the cell appeared to run parallel to preservation of its morphological detail. Solutions of lysozyme in distilled water caused the least change in the staining reaction and lysozyme in glucose solution caused the most dramatic change with complete loss of Gram positive characteristics.

Discussion

From the present experiment it appears that the growth of Candida of human isolation is inhibited or destroyed in vitro by the action of lysozyme. The phenomenon of growth inhibition was enhanced by the presence of small amounts of glucose.

Lysozyme, an aminopolysaccharidase was discovered by Fleming in 1922. Subsequent workers agreed that lysozyme had bacteriostatic and bactericidal action, and was present in various tissues and body fluids.

Lysozyme acts on the cell wall and causes lysis of a few species of bacteria, particularly the gram-positive bacteria. A major component of the cell wall of most gram positive bacteria is mucocomplex which is dissolved by lysozyme. Lysozyme possesses a specific ability to hydrolyze the mucocomplex substances to subunits of relatively low molecular weights. Considering the gram-positive characteristics of the Candida species, it is reasonable to expect that lysozyme would cause lysis of its cell wall.

To quote Dubos (1946) – 'The discovery that certain bacterium is susceptible to lysozyme can consequently be used as evidence that this organism possesses as an essential component of its structure the substrate probably an acetyl amino polysaccharide which is hydrolyzed by the lysozyme.' From the present experiment, this statement could be applied to Candida species of human isolation.

It is a well known biological fact that cells exhibit high vulnerability during the active dividing process. In the present experiment, the addition of minute amounts of glucose in solution appeared to accelerate the action of lysozyme on Candida species, possibly because Candida cells are highly vulnerable during their budding process with high metabolic activity in glucose solution. Lysozyme in distilled water, on the contrary, did not kill the Candida cells at concentrations used by the present study, and the starvation or nutrient depletion in simple distilled water did not appear to result in much autolysis or cell death. The mechanism that shut off possible intracellular mureinase during starvation making the Candida cell-wall resistant to the action of lysozyme is not fully understood.

The presence of small quantities of salts in glucose solution caused the action of lysozyme on *Candida albicans* to be greatly inhibited and appeared to prevent morphological degeneration and subsequent death of the cell. This interference of enzyme action due to the presence of salts or buffers was observed by SCHLENK & DAINKO (1965). They described that the presence of minute amounts of NaCl interfered with the action of ribonuclease upon the yeast cells of *Candida utilis* and *Saccharomyces cerevisiae*. ROTHSTEIN (1963) described the binding of cations by the yeast cell surface, and explained the competitive action of the ions with the binding of metabolites, and their transfer through the cell membrane. He showed that cations, especially Ca^{++} and Mg^{++} are also inhibitory and probably occupied sites on the cell membrane where enzyme has to be attached for action. KOZINN, CAROLINE & TASCHDJIAN (1964) exposed *Candida albicans* to lysozyme (1 mg/ml) in phosphate buffer for 1 hour at 37° C and found no inhibitory action of lysozyme on Candida cells.

The fact that gram-positive bacteria treated with lysozyme becomes gram negative has been known for some time. In 1948, WEBB reported that the action of lysozyme on heat-killed cells of *Clostridium welchii* and *Staphylococcus albus* causes these organisms to become gram negative. This fact was confirmed in 1961 by SALTON. The present experiment showed that the gram-positive characteristics of Candida species, although irregular, changed to gram-negative by the action of lysozyme in glucose solution. At the same time, degeneration and loss of cell morphology was observed. Lysozyme is known to affect the cellular components essential for maintenance of the cell's morphological structure (EPSTEIN & CHAIN 1940). BOASSEN (1938) concluded that the lytic action of lysozyme is due to the increased cellular permeability which permits cell material to diffuse into the medium. SALTON (1953) using crystalline lysozyme from egg white showed that lysozyme causes dissolution of the rigid cell wall structure of *Micrococcus lysodeikticus*. In other experiments (GERHARDT et al., 1956) (SALTON, 1961), it was demonstrated that crushed protoplast from *B. megaterium* that had been Gram stained prior to wall removal by lysozyme could be decolorized. The present study showed that the structural integrity of yeast cells of Candida species appears to be the most important factor for the preservation of gram positivity.

AMANO et al. (1954), HOOK, CAREY & MUSCHEL (1960) and GLYNN (1968) observed that lysozyme accelerates the destruction and lysis of some gram-negative bacteria exposed to complement and antibody. The candicidal action of lysozyme in phagocytic monocytes and in various tissues and body fluids (WEISS et al., 1966) combined with the activity of complement and antibody leads one to assume that lysozyme plays an important role in the prevention of Candidiasis in vivo.

In early studies of lysozyme, FLEMING, (1922), (1929), FLEMING & ALLISON (1922) compared the amount of lysozyme found in extracts

of various human organs. Extracts of kidney, brain and skin contained the least amount of lysozyme compared to extracts of other organs. It is a well known fact that kidney and brain, and with certain reservations skin, are the most susceptible to infection by *Candida albicans* both clinically and experimentally. (LOURIA et al. 1960, HASENCLEVER & MITCHEL, 1961, LOURIA et al. 1963).

In experiments involving pseudo-germtube production in various organ extracts, the percentage of cells forming pseudo-germ tubes and their subsequent growth was greatest in extracts from kidney, brain, and the skin (MACKENZIE, 1965).

The lysozyme content in tears and nasal secretions is much higher than in saliva (FLEMING 1922) (1929), (FLEMING & ALLISON 1922) or vaginal washings (THOMPSON 1940). Mouth and vaginal candidiasis is much more common than Candida infection of the eye or nose, which usually only occur in special conditions of the host (CHICK & CONANT 1962).

Although it is unlikely that a single factor is involved, the correlation between the incidence of Candida infection, and the lysozyme activity of tears, nasal secretions, saliva, vaginal washings and various human organ extracts, appears to be too close to be fortuitous, and may be worth further investigation.

Zusammenfassung

Candida albicans-Zellen sind in Lysozyme-glukose-Lösung bei 37° C in Wasserbad für 6 Stunden bebrütet worden; sie sind dann an der Oberfläche von Sabouraud's Agarplatten ausgestrichen und bei 37° C für 18—24 Std. bebrütet worden. Zerstreute, kleine Kolonien sind an der Agarfläche erschienen, im Vergleich mit dem dicken, vollen Wachstum der Kontrolkultur, die ohne Lysozyme bebrütet worden ist. Einundzwanzig Stämme von sechs Standard-Candida Arten aus menschlichen Quellen außer *C. albicans:* d.h. *C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsillosis, C. guilliermondii,* zeigten im wesentlichen dasselbe Ergebnis wie *C. albicans*. Eine konstante Quantität von Lysozyme bewirkte die Zerstörung der Candida-Zellen zu gleichem Grade ohne Rücksicht auf die wechselnde Konzentration der Glukose. Eine großere Verdünnung von Lysozyme als die hundertfache des Originals (5mg/ml) zeigte denselben Grad der candicidalen Aktivität , jedoch war sie von der Gegenwart einer kleinsten Menge von Glukose abhängig. Die Gegenwart von NaCl hat die Lyse von Candida durch Lysozyme in verschiedenen Lösungen verhindert. Candida-Zellen waren mit Lysozyme in Glukoselösung für 6 Std. in Wasserbad bei 37° C bebrütet. Mikroskopische Beobachtung hat einen großen Wechsel in der Zellmorphologie enthüllt. Die meisten Zellen waren geschwollen, degeneriert, und manche völlig zerstört. Die grampositive Eigenart der Candida-Zellen wechselte in die gram-negative. Die vereinigte Aktivität von Lysozyme mit Komplement und Antikörper mag eine wichtige Schutzrolle gegen Candidiasis in vivo spielen.

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