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STUDY ON LYSOZYME LEVEL, DISTRIBUTION AND EFFECT OF HEAT TREATMENT IN BUFFALO AND COW MILK

[30]

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ABSTRACT

Lysozyme concentration was measured by a spectrophotometeric method. The recoveries of this method were 105.4±4.8 and 98.3±3.2 % for10 and 30 ng/ml lysozyme concentrations added to diluted milk sample respectively, therefore the method was suitable for assay lysozyme in milk. It was found that the average level of lysozyme in buffalo milk was 3.85±0.93 µg/ml and in cow milk was 1.67±0.65 µg/ml, the difference was highly significant (P<0.001). The distribution showed that the lysozyme in buffalo milk was mostly concentrated in skim milk (96.45 %) similar to that in cow milk (97.43 %) and the residual amount of lysozyme was associated with cream. The effect of the different pasteurization methods was studied. The results showed considerable decrease (P < 0.001) in lysozyme concentration in buffalo and cow milk at low temperature long time (65°C for 30 min.), while, at 75°C for 15 sec and 85°C for 1 sec. (high temperature short time) there were insignificant (P>0.05) and significant (P<0.05) increases in lysozyme contents respectively in both types of milk. The data obtained when heating at 100°C for 5 min. indicated that most lysozyme content was affected. In addition the seasonal variation showed insignificant differences (p > 0.05) in lysozyme levels in winter and summer seasons.

INTRODUCTION

Lysozymes (N-acetylmuramide glycanohydrolase EC: 3.2.1.17) are enzymes which widespread in most biological systems (**Klaeger *et al* 1999 and Wagstrom *et al* 2000**). The milk of several species of animals contains lysozyme. However, its enzymatic properties vary among species. Human and equine milks are very rich in lysozyme; while milk of many other species contains low concentrations (**Mckenzie & White, 1986 and Elagamy *et al* 1996**). Bovine milk lysozyme (BML), human milk lysozyme (HML) and camel milk lysozyme have been isolated in pure and homogeneous form (**Wang & Kloer, 1984; Duhiman, 1988 and White *et al* 1988**). Moreover the composition and sequence of amino acids of various lysozyme were determined by several authors (**Mckenzie *et al* 1985; Jolles *et al* 1990 and Hall *et al* 2001**).

Lysozyme is an important antimicrobial agent in milk, which kills bacteria by cleaving the *B*-1, 4- glycosidic bond between N-acetyl muramic acid and N-acetyl glucosaamine residues cell wall and used as food preservatives (**Elagamy, 1992; Chandan, 2001 and Priyadarshini & Kansal, 2002**). The level of lysozyme in milk changes during lactation with high level in colostrums (**Lewis-Jones & Reynolds, 1983 and Montagne**

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et al 2001). Lysozyme activity was affected by heat treatment and storage procedures (**Jauregui-adell, 1975; Evans *et al* 1978; Friend *et al* 1983; Griffiths, 1986 and Lawrence, 2001).**

Most methods for determination of lysozyme activity, in fact, involve measurement of the initial turbidity clearing rate in bacterial cell suspension containing the lysozyme sample. Concentrations of lysozyme in solution or mixtures can be estimated by one of several immunological or enzymatic methods (**Selsted & Martinez, 1980 and Stelzner *et al* 1982).** A simple and ultra sensitive enzymatic assay for the quantitative determination of lysozyme in the picogram range in complex biological mixture was reported by **Selsted & Martinez (1980).** Also, **Mckenzie & White (1986)** determined lysozyme activity at low levels with emphasis on the milk enzymes. Recently, lysozyme was determined by HPLC method and microparticle-enhanced nephelometric immunoassay (**Montagne *et al* 2000 and Keith *et al* 2001).**

Lysozyme activity increases with somatic cell count and mastitis (**Carlsson & Björck 1989 and Semba, 2000**), therefore, the determination of lysozyme activity in milk is useful for diagnosis of bovine mastitis. Moreover, the quantification of serum and urine lysozyme is useful diagnosis procedure for Leukemia disease (**Brierre *et al* 1974**). Lysozyme concentrations increase during abnormal cell behavior. Recently, **Vizoso *et al* (2001)** reported that lysozyme may be a new prognostic factor in patients with breast cancer.

Buffalo milk is relatively more resistant to microbial spoilage than bovine milk (**Priyadarshini & Kansal, 2002).** The information about lysozyme in buffalo milk is not fully investigated. Therefore the object of this study aimed to give information on lysozyme content, distribution, effect of heat treatment, and effect of season on buffalo milk lysozyme in comparison with cow milk.

MATERIALS AND METHODS

Milk samples

1- Buffalo milk was obtained from Cairo University, Faculty of Agriculture herd and Mahlat Mosa Station belongs to Animal Production Research Institutes.
2- Cow milk was obtained from Cairo University Faculty of Agriculture herd and Qrada Station.

Milk samples were fractionated by the method which was described previously by **El-Gazzar *et al* (1999).** Several pilot experiments for dilutions were carried out to reach suitable dilution to give the best recovery results. So the samples of raw milk and their fractions (cream, skim milk and butter milk) were diluted at rat of 1:500 with distilled water.

Heat treatment

Fresh raw whole milk was heat treated at different conditions for pasteurization (65± for 30 min,, at 75± for 15 Sec., and at 85°C for 1 Sec.) and boiling for 5 min., then lysozyme activity was determined at room temperature

Seasonal variation

The samples of bulk buffalo milk from Mahlat Mosa Station was used for this study through winter (Dec. – Mars) and summer (Apr. – Sep.) season.

Lysozyme (Enzyme)

Chicken egg-white lysozyme (EC: 3.2.1.17) was obtained from Sigma Company.

Preparation of standard solution

A stock solution of egg-white lysozyme 1mg/ml was prepared in 0.05M buffer pH 7.4 and stored at 2°C, the lysozyme concentration was determined by measuring of the absorbance at 280 nm, taking $A_{1cm}^{1\%}$= 27.3 according to **Mckenzie and White, 1986.** Subsequent dilutions were made into buffer containing a final concentration of 1mg/ml of crystalline bovine serum albumin (BSA, Sigma). BSA was incubated in all incubations for protein stabilizing. In absence of BSA, the determinations of lower enzyme concentrations (less than 10 µg/ml) deviated from linearity and duplicated determination were much more variable **(Selsted & Martinez, 1980).**

Substrate

Micrococcus lysodeikticus was obtained from Sigma Chemical Company. It was used as substrate which lyses by lysozyme. The suspension of *M. Lysodeikticus* was prepared by mixing

3.5mg/L with buffer pH 7.4 (The cell was suspended by gentle swirling).

Lysozyme assay

The reaction mixture (as described in **Priyadarshini & Kansal, 2002**) contained 2.1 ml cell suspension, 0.3 ml bovine serum albumin (1g/L), 0.3 ml sod.azide, a source of lysozyme and 0.05 M potassium buffer pH 7.4 to a final volume 3 ml. The mixture mixed very well. The test tubes assay was incubated at 37°C for 18 hr. according to **Selsted & Martinez (1980).** The absorbance of suspension was measured at 450 nm by using Shumadzu UV-Visible Recording Spectrophotometer UV- 240 .The averaged reduction in absorbance relative to control incubated without enzyme was plotted against the lysozyme concentrations. Change of absorbance was linear with respect to enzyme concentrations from5 – 70 ng /ml (0.5 – 7 ng/ml in the reaction mixture) as shown in **Fig. (1)**

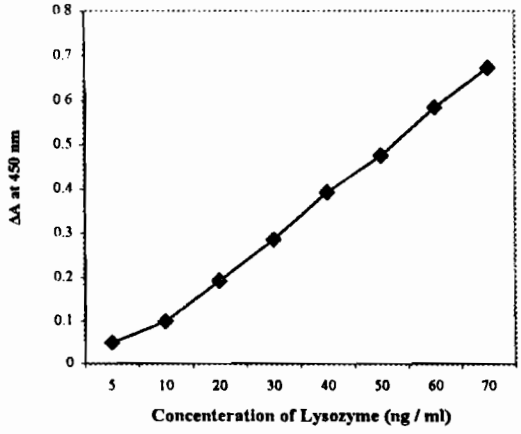

Fig. 1. Standard Curve for determination of Lysozyme Concn.

The linear equation was calculated to facilitate the calibration of lysozyme concentration in the present study

$$Y = 0.042 + 0.0668\,X$$

Where **Y** represent the change in absorbance at 450 nm (ΔA at 450 nm) and **X** the concentration of lysozyme (ng/ml) in the reaction mixture. In assay mixture 0.5 ng/ml equivalent to5 ng/ml for 0.3 ml diluted milk sample (1:500) equivalent to 2.5µg/ml for original sample.

The method of determination of lysozyme concentration in milk in this study is based essentially on the procedure of **Selsted and Martinez (1980).**The spectrophotometer rate–analysis assay is a commonly used technique because of its speed and sensitivity – with the aid of recording double-beam spectrophotometer, lysozyme levels as low as 0.02 µg/ml can be measured in matter of minutes, **(Selsted & Martinez, 1980).** Since the linear range of this assay method is quite limited and sample dilution is required. Our limit of detection in the reaction mixture corresponding to 0.5 ng/ml in assay mixture equivalent to 5 ng/ml for diluted sample, and this value represent to substantial sensitivity. Previously, the limit of detection for bovine milk lysozyme was 0.1ng/ml reaction mixture, equivalent to 6 ng/ml milk sample as given by **Mckenzie and White (1986).**

Statistical Analysis

The statistical analysis of results was determined according to **Bailey (1995).**

RESULTS AND DISCUSSION

Recovery study was performed to check the validity of the procedure **(Table 1).** Standard lysozyme at concentrations 10 and 30 ng/ml are added to diluted buffalo milk (1:500), then subjected to lysozyme determination . The recoveries of lysozyme were 105.4 ▯ 4.8 and 98.3 ▯ 3.2 % for concentrations at 10 and 30 ng/ml respectively as shown in **Table (1).**Thus, there was no indication that the buffalo milk constituents interfere with the expression of cell lytic activity. These results is in agreement with the result which was performed by adding lysozyme standard to cow milk **(Mckenzie and White, 1986).**

Table 1. Recovery percent of lysozyme added to milk sample

Lysozyme added ng/ml	Original lysozyme in milk sample ng/ml	Lysozyme recovered ng/ml	Recovery of added lysozyme (%)
10	8.23	19.24 ±0.9	105.4±4.8
30	8.23	37.58±1.27	98.3± 3.2

Lysozyme content in buffalo and cow milk

Lysozyme is an antimicrobial agent in milk; therefore the evaluation of lysozyme concentrations in buffalo and cow milk is very important. The average concentration of lysozyme in all raw buffalo milk samples (n = 15) was found 3.85± 0.93 µg/ml with a range from 2.55 to 5.54 µg/ml as shown in **Table (2).** While, the average lysozyme concentration in cow milk (n = 11) was found 1.67 ±0.65 µg/ml with a range from 0.6–2.69 µg/ml as shown in **Table (2).** This range of concentration was similar to the level found by **Chandan *et al* (1968)** in bovine milk < 0.3 mg/100 ml (i.e.<3 µg/ml). Also **Göze *et al* (1977)** reported an average lysozyme from healthy udder. It was 0.73 µg /ml and this result was in the range of the data obtained. **Table (2)** showed that the differences in concentration of lysozyme between cow and buffalo milk were highly significant ($P < 0.001$). The data showed that the concentration of lysozyme in buffalo milk was double the value observed in cow milk. These results agree with that obtained by **Priyadarshini and Kansal (2002).** They found that the lysozyme activity in buffalo milk was double the value observed in bovine milk. In general the data obtained indicated that the range value of lysozyme in buffalo milk was higher than in cow milk. These results may be the cause that buffalo milk is relatively more resistant to microbial spoilage than bovine milk.

Table 2. Different in lysozyme levels in milk from buffalo and cow

Type of milk	Lysozyme concentrations (µg/ml)	
	Range	Mean±SD
Buffalo	2.55 – 5.54	3.85 ±0.93 (n=15)
Cow	0.60 – 2.69	1.67±0.65 (n=11)

P < 0.001

Mckenzie *et al* 1985 they found that lysozyme content of milk of 20 Friesian and Jersey cows was 0–1.120 µg/ml (mean = 0.400 µg/ml) and reported that lysozyme content was usually higher in p.m than in a.m milking.

Lysozyme distribution between raw milk and their fractions

The lysozyme distribution in buffalo and cow milk was represented in **Tables (3 & 4).** It was clear that lysozyme content in buffalo milk mostly located in skim milk (96.45%) and the residual amount was associated with cream (4.20%). Similar data was abtained in case of cow milk about 97.43 % of lysozyme found in skim milk and 3.21 % in cream. **Kinsella and Whitehead (1989)** reported that lysozyme was involved in the structure of whey proteins. However, the released lysozyme from cream into butter milk during churning were 54.55 and 93.75 % for buffalo and cow milk respectively.

Table 3. Distribution of lysozyme between raw buffalo milk and its fractions

Fraction	Lysozyme content. (µg/ml)	Vol. (ml)	Total content of lysozyme (µg/ml)	Recovery (%)
Whole milk	5.5	4000	22000	100
Skim milk	5.9	3580	21127	96.45
Cream	2.2	420	924	4.20
Butter milk	2.8	180	504	54.55

Table 4. Distribution of lysozyme between raw cow milk and its fractions

Fraction	Lysozyme content. (µg/ml)	Vol. (ml)	Total content of lysozyme (µg.ml)	Recovery (%)
Whole milk	2.4	4900	12470	100
Skim milk	2.7	4500	12150	97.43
Cream	1.00	400	400	3.21
Butter milk	2.5	150	375	93.75

The effect of heat treatments on the lysozyme concentrations in buffalo and cow milk

The present study investigated how lysozyme content in buffalo and cow milk was affected by different heat treatments. Raw and pasteurized milk samples treated by heating at 75°C for 15 sec., 85°C for 1 sec. and 65° for 30°C min.) were analyzed and the data obtained were represented in **Tables (5 & 6)**. There were highly significant differences (P<0.001) in concentrations in cow and buffalo milk at 65°C for 30°C min. with mean values 0.71±0.22 and 2.05± 0.52, µg/ml, respectively. The decrease in lysozyme concentration in cow milk was 54.19 % which was more than in buffalo milk 43.83%. This indicated that the lysozyme in buffalo milk was more stable than in cow milk with this treatment. **Evans *et al* (1978)** found that accurate pasteurization at 62.5°C for 30 min. produced a loss of 23.7 % in human milk. Pasteurization at 750C for 15 sec., the mean values of lysozyme concentrations in cow and buffo milk were 1.60± 0.64 and 3.83±0.31 µg/ml, respectively. The data showed insignificantly (P>0.05) increase in lysozyme concentrations in both types of milk. The increase in lysozyme concentrations were 3.23 and 4.93% for cow and buffalo milk respectively. While, lysozyme concentration was increased significantly (P<0.05) by heating treatment at 85°C for 1 sec. The lysozyme concentration increased by 25.48% in buffalo milk similar to cow milk 23.87 %

The data showed similar effect of the different pasteurization methods on the lysozyme contents in cow and buffalo milk. It was observed that lysozyme in milk was activated by high temperature short time pasteurization and inhibited at low temperature long time pasteurization. Data indicated that the time of heating was more effective than the degree of temperature. Previous study by **Friend *et al* (1983)** showed that bovine milk lysozyme and human milk lysozyme retain > 75 % of their activity in milk heated at 75°C for 15 min. However, **Goldblum *et al* (1984)** found that lysozyme concentration in human milk was increased significantly by heating at 72°C for 15 sec. and suggested that lysozyme may be largely sequestered in milk. This data is close similar to results obtained by heating at 75°C for 15 sec.

When two types of milk (cow and buffalo) subjected to 100°C for 5 min. lysozyme concentration totally reduced (> 90 %). This result was in agreement with data reported by **Ford *et al* (1977)** and **Weaver & Kroger (1978)**.

Table 5. Effect of different pasteurization methods on lysozyme concentration in cow milk (n = 5)

Lysozyme Conc. µg/ml	Raw milk	Pasteurization milk at: 65°C for 30 min	75°C for 15 sec	85°C for 1 sec
Range	0.90–1.95	0.45–1.05	0.87–2.00	1.07–2.44
Mean± SD	1.55± 0.57	0.71± 0.22	1.60± 0.64	1.92± 0.74
Increase %	--	--	3.23	23.87
Decrease %	--	5 4.19	--	--
Significant	--	P<0.001	P>0.05	P<0.05

Table 6. Effect of different pasteurization methods on lysozyme concentration in buffalo milk (n = 5)

Lysozyme Conc. µg/ml	Raw milk	Pasteurization milk at: 65°C for 30 min	75°C for 15 sec	85°C for 1 sec
Range	3.44 – 4.19	1.20 – 2.60	3.59 – 4.34	4.04 – 5.69
Mean± SD	3.65± 0.31	2.05± 0.52	3.83± 0.31	4.58± 0.74
Increase %	--	--	4.93	25.48
Decrease %	--	43.83	--	--
Significant	--	P<0.001	P>0.05	P<0.05

Effect of winter and summer season on the lysozyme content in buffalo milk

Table (7) showed the mean values of lysozyme concentrations in buffalo milk samples collected from the same farm through winter (Dec.–Mars) and summer (Apr.–Sep.). It was found that the concentration of lysozyme in milk

in winter season ranged from 3.1–5.09 µg/ ml with mean value 3.89±0.81 µg/ml. While, in summer season the range was 3.5 – 4.34 µg/ml and the average value was 3.91±0.42 µg/ml. According to *T-TEST* no significant differences were found (P>0.05) in the mean value of lysozyme concentration due to different season. This may indicate that the lysozyme level in milk independent on feed, but it needs more investigation.

Table 7. Lysozyme levels of buffalo milk through winter and summer season.

Seasons	Lysozyme concentrations (µg/ml)	
	Range	Mean±SD
Winter	3.1–5.09	3.89±0.81
Summer	3.5–4.34	3.91± 0.42

P < 0.001

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حوليات العلوم الزراعية
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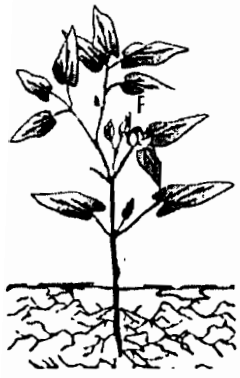
مجلد (٥١)، عدد (٢)، ٤٣٩-٤٤٦، ٢٠٠٦



دارسة على مستوى و توزيع الليزوزيم وتأثير المعاملة الحرارية في اللبن الجاموسى والبقرى

[٣٠]

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يعتبر الليزوزيم من الإنزيمات الهامة المضادة للبكتيريا. ويوجد فى اللبن بنسب متباينة باختلاف أنواعه.

ولذا تهدف هذه الدراسة إلى تقدير تركيز الليزوزيم في اللبن الجاموسى والبقرى و توزيعه فى اللبن ومشتقاته(اللبن الفرز – القشدة – اللبن الخض) ومدى تأثير المعاملة الحرارية و اختلاف الموسم على نشاطه.

وقد تم تقدير تركيز الليزوزيم فى اللبن بطريقة القياس الطيفي وهذه الطريقة مناسبة لتقدير الليزوزيم فى اللبن وبتقدير نسبة الاسترجاع وجد أن النسبة ١٠٥,٤±٤,٨ و٩٨,٣±٣,٢ لتركيزى الليزوزيم ١٠ و٣٠ نانو جرام/ مل على التوالي.

وقد كان متوسط تركيز الليزوزيم فى اللبن الجاموسى ٣,٨٥±٠,٩٣ ميكروجرام/ مل أعلى من متوسط الليزوزيم فى اللبن البقرى ١,٦٧± ٠,٦٥ ميكروجرام/ مل. وبد راسة توزيع الليزوزيم فى اللبن ومشتقاته أوضحت النتائج أن وجود الليزوزيم فى اللبن الفرز الجاموسى بنسبه ٩٦,٤٥ % مشابه لما وجد فى اللبن الفرز البقرى (٩٧,٤٣%) والكميه المتبقية مرتبطة بالقشدة.

كما أظهرت نتائج طرق البسترة المختلفة نقص معنوى كبير فى تركيز الليزوزيم عند البسترة البطيئة (٦٥ درجة لمدة ٣٠ دقيقة) بينما عند البسترة لحرارة مرتفعة ووقت قصير (٧٥ درجة مئوية لمدة ١٥ ثانية) و(٨٥ درجة مئوية لمدة ثانية واحدة) وجد أن هناك زيادة غير معنوية ومعنوية فى محتوى الليزوزيم في كل من اللبن الجاموسى والبقرى على الترتيب.

وعند تسخين اللبن على درجة ١٠٠ مئوية لمدة ٥ دقائق لوحظ فقد نشاطه بدرجة كبيرة.

وبدراسة تأثير اختلاف الموسم على مستوى الإنزيم فى اللبن الجاموسي وجد أن هناك اختلاف غير معنوي فى فصلى الشتاء و الصيف.

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