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RESEARCH ARTICLE

Exploring the antioxidant potential of hen egg white lysozyme N-acetylmuramide glycan hydrolase in full fat mozzarella cheese

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ABSTRACT

To explore the antioxidant potential of hen egg white lysozyme N-acetylmuramide Glycan hydrolase in full fat Mozzarella cheese, an experiment was conducted where control sample was brined without the addition of lysozyme; while four samples were brined along with 0.02%;w/v (T1), 0.04%;w/v (T2), 0.06%;w/v (T3) and 0.08%;w/v (T4) lysozyme. The antioxidant potential of hen egg white lysozyme in full fat Mozzarella cheese samples were measured through several methods, including 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid assay (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, superoxide radical scavenging activity (PMS-NADH system), free fatty acid analysis and lipid peroxidation inhibition assay in a linoleic acid emulsion system. Intensity scale was used to evaluate the sensory characteristics of cheese. The results showed the percentage inhibition for DPPH, ABTS and superoxide anion activity in control sample increased up to 30 days; but decreased afterwards till 40 days of storage However in case of lysozyme treated samples % inhibition increased throughout storage. Free fatty acid % in full fat Mozzarella cheese was affected by lysozyme and storage days. Lipid peroxidation inhibitory activity in α-linoleic acid model system of lysozyme added samples showed lower absorbance at 500 nm showing higher lipid peroxidation inhibition. Furthermore, it was observed that absorbance was decreased till 30 days of storage which increased further upto 40 days storage period. The study showed that addition of lysozyme improved the shelf life and sensory characteristics of full fat Mozzarella cheese.

Keywords: Lysozyme; Antioxidants; N-acetylmuramide glycan hydrolase; Full fat mozzarella cheese

INTRODUCTION

Control of lipid oxidation in natural cheeses is very critical to protect their flavor and keeping qualities. When the reactive oxygen species (ROS) surpassed the natural antioxidant capacity of the cheeses, the oxidation conditions develop. The products of lipid oxidation/ ROS produced during cheese production, storage and cookery have become a challenging and concerning issue for human health. Upon ingestion, fat rich products after multiple digestion phases are hydrolyzed and oxidized and produce a pool of compounds in gastrointestinal tract. These oxidation products initiate inflammation of gut and subsequently carcinogenic processes (Estévez et al., 2017). The fat content of cheeses ranges between 3%–30%. However, some fresh cheeses are highly prone to oxidation reactions if not packed or stored at suitable conditions (Fox et al., 2017; Fedele and Bergamo, 2001) that lowers their shelf-life. Buffalo cheese contains higher fat content than in bovine milk cheese (Addeo et al., 1977).Therefore, due to the conditions during the process of cheese manufacturing, which include heat-treatment and sufficient exposure to oxygen, greater amounts of lipo-peroxides are produce along with greater consumption of alpha-tocopherol in any product manufactured from buffalo's milk (Balestrieri et al., 2002). Oxidative stability of cheese is affected by storage conditions. Light as well as modified atmosphere caused undesirable changes in cheese such as off flavors

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and oxidation products. Lipid peroxidation in fresh cheese is successfully controlled by protective systems (Soto-Cantú et al*.,* 2008; Bandyopadhyay et al*.,* 2008).

Oxidative damage can be reduced by taking antioxidants through diet (Halliwell, 1996; Halliwell, 2012; Sies, 2019; Valko et al., 2007). The internal chemical and physical balance is maintained if the cellular components is protected from harmful ROS. To prevent the oxidation of food and pharmacological products, synthetic antioxidant are used now a days such as propyl gallate (PG), butylated hydroxy anisole (BHA) and butylated hydroxy toluene (Gülçin, 2012). However, the use of BHT and BHA is prohibited due to their possible harmful and oncogenic effects (Vandghanooni et al., 2013; Williams et al., 1999). Therefore, the consumer's attention for the use of natural antioxidants swift the scientists to discover new dietary antioxidant sources (Fox et al., 2017; Gülçin, 2012; Halliwell et al., 1995; Samaranayaka and Li-Chan, 2011). Even though egg is familiar for its excellent nutritional quality but it is not being used as antioxidant. It has many proteins in egg white including ovalbumin, Ovo-transferrin and lysozyme. The egg yolk is rich with phosvitin, carotenoids and free aromatic amino acids.

All organisms contained an appreciable amount of lysozyme in their body. About 0.3–0.4g lysozyme is present in one egg (Mine and Kovacs-Nolan, 2004). Lysozyme is a host defensive protein which contains a domain of 18-amino acid. It binds ROS producing agents such as advanced glycation end products (AGE) that increases oxidative stress. In the presence of polyethylene glycol 400(PEG400), palatable zein films were produced by Wei et al. (2018) containing ascorbic acid (AA) and lysozyme (LY). The combined effect of ascorbic acid and lysozyme on the properties of the created films was evaluated in detail. The result indicated that the developed zein films have good antioxidant and enhanced antimicrobial properties. Similarly, Mehyar et al., (2018) analyzed the impact of chitosan coating of halloumi cheese, with or without natamycin or lysozyme, on its shelf life, sensory properties and microbiological quality held at 38 °C or 25°C in 5%, 10% or 15% (w/v) NaCl solution. The coatings used in cheese enhanced the shelf life of cheeses which were brined with 5% and 10% NaCl at 38°C up to 5 days.

Liu et al. 2006) reported that some enzymes such as lysozyme defends our body from severe oxidative damage. It is also important for reduction of oxidation stress, cellular ROS and genes. To our knowledge, very few studies have been published relating to the antioxidant behavior of lysozyme in dairy products especially fat rich products like full fat Mozzarella cheese. Therefore, this research plan was specially designed to explore the antioxidant potential of hen egg white lysozyme in full fat Mozzarella cheese during the storage period of forty days.

MATERIALS AND METHODS

Starter culture, coagulant and lysozyme

Starter thermophilic culture (*Lactobacillus helveticus and Streptococcus thermophilus*) and Lysozyme and rennet (coagulant) were purchased from Sigma-Aldrich China Inc. Shanghai, China and Chr. Hansen (Melbourne, *Victoria, Australia*) respectively.

Production of mozzarella cheese

Buffalo milk was used for the preparation of Mozzarella cheese and it was purchased from B-Block, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki-Pakistan.

Five batches of Mozzarella cheese were manufactured with 10L of milk for each batch (Zisu & Shah, 2007). First batch was manufactured without the addition of lysozyme and used as control. Pasteurized milk (65 ^{0}C) was cooled to 37 ^{0}C for starter culture inoculation (3.6%; w/v). The milk was coagulated with rennet that was added at the rate of 20ppm. After curd formation, the coagulum was cut into short cubes and cooked in its whey to a temperature of 42 °C until 6.2 pH reached. The whey was separated and kept the curd warm at 37 ^{0}C that result the compactness of curd. This compact curd mass was progressively piled and turned for cheddaring. The curd looked smooth and elastic at 4.9 pH then it was milled and stretched in hot water at 92 ^{0}C. The Mozzarella cheeses was brined (3%; w/v) for the period of 8 hours along with 0.02% w/v, 0.04% w/v, 0.06% w/v and 0.08% w/v lysozyme and designated as T1, T2, T3 and T4 respectively. Control sample (T0) were prepared without the addition of lysozyme. The cheese samples were refrigerated at 4 ± 1°C and chemical analysis were performed at different intervals 0, 10, 20, 30 and 40 days of storage.

Physicochemical analysis of milk

According to the method of (AOAC, 2011) fat%, protein%, moisture content%, pH, acidity% and total solid were performed.

Physicochemical analysis of Mozzarella cheese

Moisture in cheese samples was determined using oven dry method at 105 ± 5°C until constant weight obtained (AOAC, 2011). Fat was measured following the method of (Bligh and Dyer, 1959). Nitrogen content was measured with the help of Kjeldahl method and it was multiplied with 6.38 factor to convert it into protein. pH, acidity, total solids and ash contents were also measured following the

methods described in (AOAC, 2011). All analysis were performed in triplicate at 0, 10, 20, 30 and 40 day intervals.

Amino acid analysis of lysozyme

The amino acid composition of lysozyme was performed using an amino acid analyzer (Biochrom 30 +, Biochrom Limited. Cambridge, UK). Briefly, 500 micron powder sample was used. To protect the methionine and cysteine, powder sample was oxidized with performic acid. The oxidation results in the conversion of amino acid into met-methionine sulfone and cys-cystic acid. Thereafter, sample was hydrolyzed for the period of 24 h with 6 M hydrochloric acid/phenol. During hydrolysis pH was adjusted at 2.2. The sample was filtered and poured in sample vials for amino acid contents determination with the help Biochrom 30 + AA analyzer using ion-exchange chromatography Ullah et al. (2017).

Antioxidant properties

Preparation of water-soluble extracts (WSE) of Mozzarella cheese

For the preparation of water-soluble cheese extract, took 20g of grated cheese sample and 50 ml distilled water in tube for each sample. After the sonication of mixture and followed the centrifuged for 10 minutes at 14000 rpm. After centrifugation upper layer containing fat was discarded and the remaining aqueous phase was filtered using Whatman filter paper no. 01. The 4.6 pH of extract was adjusted using 1 N HCl. To remove further impurities the extracts were further filtered using syringe filters 0.22-micrometer size and were stored at 20 ^{0}C for further analysis (Kariyawasam et al., 2019)

2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assays of Mozzarella cheese samples were carried out using stock solution of ABTS (7mM) with potassium persulfate (2.45Mm) to produce ABTS cations and this solution was placed in dark for 12-16 hours. After that phosphate buffer saline 5mM having pH (7.4) was used to dilution of this solution. Then 1ml of diluted ABTS solution was added along with 10 microliters of sample or Trolox standard in phosphate buffer saline. The absorbance was measured using Spectrophotometer for every 10 minutes. Solvent blanks were run and the values were recorded as a mean of duplicates. The percentage inhibition was calculated at 734 nm. These values were used and plotted for the antioxidant concentration and trolox for the reference value (Gupta et al., 2009).

2, 2-diphenyl-1-picrylhydrazyl (DPPH) Assay

In order to assess DPPH scavenging activity 5 ml sample was mixed with 1ml of 1mM fresh DPPH solution and keep it room temperature for 30 minutes. Double beam spectrophotometer was used for absorbamnce measurement of samples and blank (1 M phosphate buffer) at 517nm wavelenght (Shimada et al., 1992). The results were calculated as follows:

$$\% \text{ inhibition} = \frac{A1 \text{ X } 100}{A0}$$

Where as, A1 and A_0 represent the absorbance of sample and blank respectively.

PMS-NADH superoxide radical scavenging activity

Tris HCl buffer having pH 8 was used to generate super oxide radicals. Buffer also contained 50 micro moles of lM nitro-blue tetrazolium (NBT), 78 micro moles of reduced NADH, 10 micro moles of phenazin methosulfate (PMS) and different concentration of water soluble extracts. This mixture was incubated for 5 minutes at 25 ^{0}C. The change in colour with the reaction of superoxide radicals and nitro-blue tetrazolium was estimated against blank using spectrophotometer at 560nm. Trolox was used as standard. The inhibition of super oxide ions was estimated through given formula:

$$\% \text{ Inhibition} = \frac{A0\text{-}A1 \text{ x } 100}{A0}$$

Where as, A1 and A_0 represent the absorbance of sample and blank respectively (Shi et al., 2019)

Free fatty acid (FFA) analysis of full fat mozzarella cheese samples

For the neutration of ethanol, 50 ml ethanol was taken in a titration flask and phenolphthalein 1% solution was used as a indicator. Titrate the ethanol against NaOH 0.1N until light pink colour appeared. Cheese samples (10g) was grated and added into the ethanol flask. It was heated for 2-3 minutes at boiling temperature. If the pink colour persists after 2 minutes, there are no free fatty acids. If the pink colour disappears it means free fatty acids are present. After the disappearance of colour the flask containing cheese and ethanol was titrated against 0.1N NaOH. The final volume of 0.1N NaOH used was noted and free fatty acid% can be calculated by using this formula (AOAC, 2019)

$$\text{FFA\%} = \frac{\text{volume of NaOH used x Normality of NaOH x282}}{\text{1000 x weight of sample taken}} \text{X } 100$$

Lipid peroxidation inhibition assay

According to the method of Osawa and Namiki (1985) lipid peroxidation inhibition activity was monitor by Linoleic

acid model system in cheese samples. Mozzarella cheese sample (1.3mg) was dissolved in 10 ml of phosphate buffer (50mM) with pH 7.0 and transfer it into solution having linoleic acid 0.13 ml and 10 ml ethanol (99.5%). After that 25 ml distilled water was added and it was placed in a screw caped bottle and keep it in dark at 40 ± 1 °C. For the degree of linoleic acid oxidation, ferric thiocyanate value was measured by double beam spectrophotometer at 500 nm. In this process, resultant solution 0.1 ml mixed with 30% ml ammonium thiocyanate (0.1ml), 20 mM ferrous chloride solution (0.1ml) in 3.5% Hal and 75% ethanol 4.7 ml (Batool et al., 2018)

Sensory analysis

The sensory analysis was performed using an intensity scale graduated from 0 to 10; where 0 means the sensory characteristics is too low to be perceived by the sensory panel and 10 means the characteristics is existing at higher rate. The scores for sensory characteristics were given within an intensity scale of 1–10 by the sensory panel. Each cheese sample was evaluated three times and scores were given to appearance, taste, color and flavor characteristics within a total of 10 (Gulzar et al., 2020).

Statistical Analysis:

Each treatment was replicated and analyzed three times (n=3×5; ±SD n=3×5). The data was organized and analyzed statistically using SASS software, two-way analysis of variance (ANOVA) technique. The Duncan Multiple Range Test was used to compare means (David et al., 1961).

RESULTS AND DISCUSSIONS

Physicochemical analysis of cheese

The physicochemical analysis of control and lysozyme added cheese samples are depicted in table 1.

Analysis of variance showed that treatment had significant ($p<0.05$) while storage had non- significant effect on the protein concentration of Mozzarella cheese ($p>0.05$). Protein% of Mozzarella cheese increased with higher concentration of lysozyme, due to the protein nature of lysozyme (Carrillo et al., 2016). Fat % of mozzarella cheese did not vary with treatments as well as storage days. However, during storage, the mean value of fat % of control sample was less than the lysozyme treated sample, it may be due to the ability of lysozyme to prevent lipolysis that results in the reduction of free fatty acids (Doosh and Abdul-Rahman, 2014). Lysozyme concentration and storage had non-significant influence ($p>0.05$) on ash% of Mozzarella cheese. pH value significantly ($P<0.05$) decreased with storage period however lysozyme had no effect on the pH value of cheese. The acidity significantly ($P<0.05$) increased through the storage period both in control and lysozyme treated samples. However, it was observed that the progression of acidity was faster in control sample contrary to lysozyme added cheese samples. It is mainly due to the antibacterial nature of *lysozyme* that slowed down the activity of lactic acid bacteria (Luo et al., 2019).

Mean squares indicated that moisture content in cheese samples changes significantly ($p<0.05$) with storage while treatments had non-significant ($p>0.05$) influence on the moisture percentage of all cheese samples. Mean values of moisture percentage of control at 0 and 40 days were 49.6±1.41 and 45.1±1.81 respectively. While mean values of T4 were 49.21±1.21 and 45.1±1.12 at 0 and 40 days of storage period respectively. Total solids percentage in Mozzarella cheese samples varied significantly ($p<0.05$) with treatment and storage days. Mean values for total solid content of control at 0 and 40 days were 50.30±1.44 and 54.9±1.83 respectively. The loss in moisture and increase in total solids was mainly due to moisture loss during storage (El-Sayed and El-Sayed, 2020).

Amino acid analysis of lysozyme

The amino acid contents in lysozyme are depicted in Table 2. The results showed that lysine amino acid is present in high concentration while alanine and ornithine are not detected in lysozyme sample.

Antioxidant properties of full fat Mozzarella cheese

Analysis of variance regarding DPPH, ABTS radical and superoxide radical scavenging activity indicated that lysozyme and storage significantly ($p<0.05$) influenced the percent inhibtion in full fat Mozzarella cheese samples Table 3. Mean values for DPPH scavenging activity of control sample was 2.52±0.01 while in case of T4 it was increased to 9.12±0.11 at 0 day of storage. Mean values for ABTS radical scavenging activity of control and T4 were 1.53±0.05 and 5.90±0.09 respectively at 0 day of storage period. Mean values of Supper oxide anion scavenging activity of control and T4 at 0 day of storage period were 6.23±0.01 and 21.11±0.11 respectively. It was observed that %inhibition for DPPH, ABTS and superoxide anion activity in control sample increased up to 30 days; but after 40 days of storage %inhibition was decreased. The increased % inhibition in control sample during early storage might be due to primary proteolysis occurring in cheese. Gupta et al., (2009) also stated that DPPH scavenging activity in cheddar cheese were dependent upon degree of proteolysis and type of starter culture.

In case of lysozyme treated samples (T1, T2, T3 and T4) percentage inhibition increased from lower to higher concentration of added lysozyme throughout storage

Table 1: Effect of lysozyme on chemical composition of full fat Mozzarella cheese

Parameters	Treat.	0 day	10 days	20 days	30 days	40 days
Protein (%)	T0	25.0±0.05^{o}	24.91±0.01^{q}	24.88±0.01^{s}	24.89±0.01^{r}	24.92±0.01^{p}
	T1	26.13±0.01^{k}	26.11±0.01^{m}	26.12±0.05^{l}	26.13±0.01^{k}	26.10±0.01^{n}
	T2	26.20±0.01^{g}	26.19±0.01^{h}	26.16±0.01^{j}	26.19±0.01^{H}	26.18±0.01^{i}
	T3	26.24±0.01^{d}	26.22±0.57^{e}	26.21±0.01^{f}	26.21±0.57^{f}	26.24±0.01^{d}
	T4	26.29±0.01^{b}	26.30±0.01^{a}	26.30±0.01^{a}	26.28±0.01^{c}	26.29±0.01^{b}
Fat (%)	T0	18.51±0.01	18.48±0.01	18.47±0.01	18.46±0.14	18.46±0.01
	T1	18.52±0.01	18.50±0.01	18.51±0.01	18.49±0.01	18.50±0.07
	T2	18.51±0.01	18.50±0.01	18.49±0.01	18.48±0.01	18.50±0.01
	T3	18.51±0.01	18.49±0.01	18.50±0.01	18.48±0.01	18.51±0.02
	T4	18.51±0.01	18.48±0.01	18.49±0.01	18.50±0.02	18.51±0.11
Ash (%)	T0	1.29±0.15^{x}	1.27±0.23^{v}	1.16±0.21^{x}	1.28±0.23^{o}	1.26±0.17^{w}
	T1	1.90±0.19^{f}	1.87±0.23^{j}	1.36±0.22^{s}	1.49±0.20^{q}	1.33±0.18^{t}
	T2	2.03±0.23^{g}	2.0±0.17^{h}	1.50±0.18^{p}	1.57±0.20^{q}	1.47±0.11^{r}
	T3	2.15±0.12^{d}	2.10±0.23^{f}	1.63±0.20^{m}	1.69±0.21^{l}	1.58±0.18^{n}
	T4	2.27±0.16^{a}	2.24±0.25^{b}	2.12±0.20^{e}	2.18±0.23^{c}	1.86±0.19^{k}
Total solids (%)	T0	50.75±1.54^{t}	51.5±1.49^{q}	52.3±1.73^{x}	53.7±1.85^{o}	54.9±1.83^{w}
	T1	50.75±1.54^{t}	51.5±1.49^{q}	52.3±1.73^{s}	53.7±1.40^{q}	54.2±1.45^{t}
	T2	51.23±1.20^{s}	51.40±1.23^{r}	52.60±2.03^{p}	53.10±1.72^{q}	54.80±1.7^{r}
	T3	51.67±1.16^{p}	51.9±1.21^{n}	52.6±1.94^{m}	53.1±1.45^{l}	54.5±1.41^{n}
	T4	52.03±1.19^{m}	51.7±1.26^{o}	52.80±2.03^{e}	53.2±1.43^{c}	54.7±1.61^{k}
pH	T0	4.96±0.12^{a}	4.89±0.11^{d}	4.83±0.13^{h}	4.75±0.10^{m}	4.68±0.09^{0}
	T1	4.92±0.12c	4.86±0.13^{f}	4.79±0.14^{k}	4.72±0.11^{o}	4.65±0.12^{r}
	T2	4.88±0.10^{e}	4.81±0.10^{j}	4.73±0.13^{m}	4.69±0.09^{p}	4.61±0.13^{t}
	T3	4.94±0.09^{b}	4.89±0.08^{d}	4.84±0.16^{g}	4.76±0.12^{l}	4.69±0.11^{p}
	T4	4.89±0.10^{d}	4.82±0.10^{j}	4.75±0.12^{m}	3.69±0.09^{p}	4.63±0.09^{s}
Acidity (%)	T0	0.05±0.11^{a}	0.06±0.41^{a}	0.09±0.20^{a}	0.11±0.10^{a}	0.14±0.22^{a}
	T1	0.051±0.22^{a}	0.057±0.11^{a}	0.06±0.29^{b}	0.08±0.16^{a}	0.10±0.07^{a}
	T2	0.053±0.07^{a}	0.04±0.04^{a}	0.04±0.33^{a}	0.07±0.27^{b}	0.09±0.12^{a}
	T3	0.052±0.10^{a}	0.03±0.05^{a}	0.05±0.11^{a}	0.08±0.01^{a}	0.08±0.01^{a}
	T4	0.053±0.23^{a}	0.05±0.21^{a}	0.05±0.21^{a}	0.08±0.11^{a}	0.08±0.07^{ab}
Moisture (%)	T0	49.6±1.41^{a}	48.7±1.38^{c}	47.3±2.30^{n}	46.9±1.83^{p}	45.1±1.81^{v}
	T1	49.2±1.53^{d}	48.5±1.45^{h}	47.7±1.72^{k}	46.3±1.38^{r}	45.8±1.44^{s}
	T2	49.3±1.20^{d}	48.60±1.21^{g}	47.4±1.24^{m}	46.9±1.71^{p}	45.2±1.69^{u}
	T3	49.1±1.15^{e}	48.1±1.21^{j}	47.6±1.95^{l}	46.9±1.44^{p}	45.3±1.40^{t}
	T4	49.21±1.21^{c}	48.3±1.28^{i}	47.2±2.05^{o}	46.8±1.61^{o}	45.1±1.12^{w}

The means standard deviations followed by the same letter in the same column indicate that the differences are not significant (P>0.05). T0: Mozzarella cheese without addition of lysozyme (Control), T1: Mozzarella cheese with addition of lysozyme (0.02%), T2: Mozzarella cheese with addition of lysozyme (0.04%), T3: Mozzarella cheese with addition of lysozyme (0.06%), T4: Mozzarella cheese with addition of lysozyme (0.08%).

(40 days). This is due to the production of water soluble peptides and their correlated effect with added lysozyme (Hernández-Ledesma et al., 2014). Furthermore, it is due to the specific composition and sequence of amino acids present in lysozyme (Benedé and Molina, 2020). Lysozyme also has higher concentration of amino acid (lysine) (Table 2) that showed antioxidant properties in Mozzarella cheese (Hernández-Ledesma et al., 2014).

Free fatty acids analysis

Free fatty acid % in full fat Mozzarella cheese samples significantly ($p<0.05$) affected by the added lysozyme and storage days Table 4. Mean values of control and T4 (0.08% lysozyme concentration) at 0 day of storage were 3.80±0.12 and 1.88±0.11 respectively. Free fatty acid % of control were higher than all lysozyme added treatments. Although free fatty acids produced in lysozyme treated samples but the amount produced was less as compared to control sample. It is mainly due to the ability of hen egg white lysozyme to suppress reactive oxygen species generation that leads to the formation of free fatty acids (Liu et al., 2006). It was observed that free fatty acid % increased throughout the storage period. This is most probably due to inappropriate packing and storage conditions (Gulzar et al., 2019).

Lipid peroxidation inhibitory activity 279 in α-linoleic acid model system

It is indicated in Fig. 1 that, all lysozyme treated samples showed lipid peroxidation inhibitory activity in α-linoleic acid model system. Lipid peroxidation inhibition of the control and lysozyme treated sample (T4) was 4.50% ±1.59 and 25.4% ±0.12 at 0 day of storage respectively (Fig. 1).

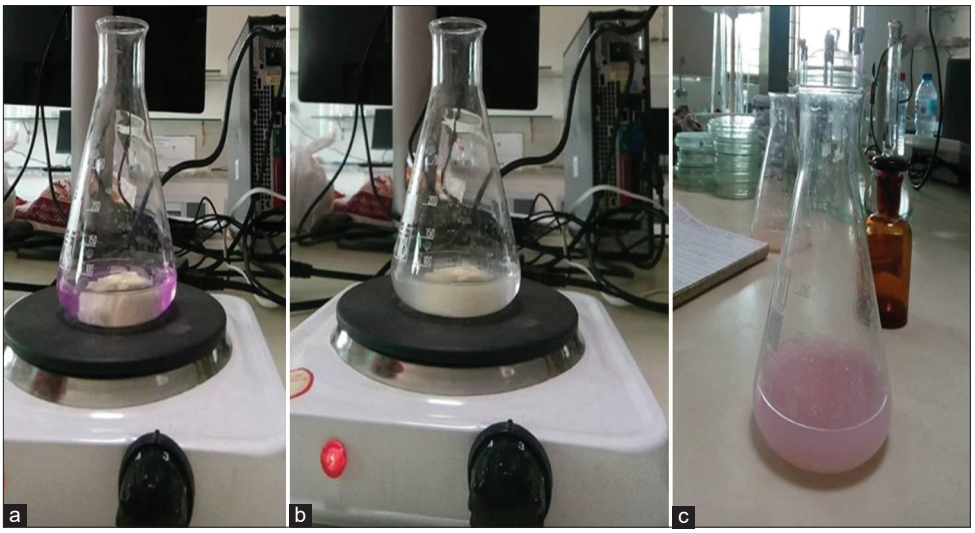

Fig 1. Pictorial Representation of Free fatty acids% at 40 days of storage period (a) Heating of cheese in ethanol containing phenolphthalein indicator (b) color of phenolphthalein disappears upon heating indicates presence of free fatty acids (c) After titration with 0.1 N NaOH pink color appears again

Table 2: Amino acid profile of lysozyme

Sr. No.	Name of amino acid	DM basis (%)
1	Cysteine	6.45±0.11
2	Methionine	0.80±0.20
3	Aspartic Acid + Asparagine	3.23±0.24
4	Threonine	4.82±0.13
5	Serine	4.81±0.15
6	Glutamic Acid + Glutamine	4.75±0.23
7	Glycine	4.79±0.33
8	Alanine	-
9	Valine	4.00±0.15
10	Isoleucine	6.24±0.16
11	Leucine	9.70±0.18
12	Phenylalanine	3.90±0.19
13	Histidine	2.38±0.16
14	Lysine	9.63±0.16
15	Tyrosine	3.05±0.16
16	Arginine	0.72±0.15
17	Proline	1.47±0.12
18	Ornithine	-

Each value is the mean of three replicates.

Furthermore, the results indicated that as the storage increased the absorbance decreased up to 30 days but after that increase in absorbance at 500 nm was observed. Lower absorbance at 500 nm indicated higher lipid peroxidation inhibition Fig. 1. Therefore, the Mozzarella cheese made with 0.08% lysozyme had a greater inhibition on lipid peroxidation than the others. Greater inhibition is most probably due to presence of antioxidant peptides in lysozyme. Memarpoor-Yazdi et al., (2012) Identified NTDGSTDYGILQINSR peptide in the lysozyme during hydrolysis with papain, trypsin or a combination of the two enzymes. They investigated that this peptide is responsible for the antioxidant activity of hydrolysate.

Sensory analysis of full fat Mozzarella cheese

Results regarding the sensory characteristics; appearance, taste, color and flavor showed significant relationship with treatment and storage days ($p<0.05$) Table 5. Mean values showed that at 0 day of storage lysozyme did not influence flavor scores. However, these are increased with the increase in lysozyme concentration during storage. The sensory panel gives 1.0±0.20 and 1.4±0.05flavor score to T0 and T4 respectively at 0 day of storage. While, scores increased to 5.9±0.05 and 9.0±0.11 for T1 and T4 at 40 days of storage respectively (Table 5). Scores for appearance increased with the advancement in storage time and added lysozyme concentration. Mean values for taste showed better scores to the treatments containing lysozyme than control. Also, taste scores increased with passage of storage period. Color of cheese samples did not influence with lysozyme and storage days.

Sensory evaluation of cheese illustrated that lysozyme did not impart any undesirable effect to cheese sensory characteristics (Carrillo et al., 2016). The improvement of appearance, taste and flavor of cheese with the addition of lysozyme was observed that was definitely due to the fact that lysozyme destroyed psychrophilic bacteria (Mehyar et al., 2018) that have the ability to produce lipases and proteases during storage (Yuan et al., 2018). These lipases degrade the fats into fatty acids and higher concentration of free fatty acids, aldehydes and ketones that produce rancidity and off flavors. Likewise, proteases also break proteins into low molecular weight peptides that cause bitter flavors (Stoeckel et al., 2016).

Table 3: Effect of Lysozyme on DPPH, ABTS and Super oxide anion scavenging activity content of full fat Mozzarella cheese during storage

Parameters	Treat.	Storage Days				
		0-days	10-days	20-days	30-days	40 days
DPPH	T0	2.52±0.01^{x}	4.01±0.01^{v}	4.98±0.01^{u}	5.15±0.01^{t}	2.98±0.01^{x}
	T1	3.12±0.01^{w}	6.96±0.01^{r}	10.02±0.01^{m}	13.21±0.01^{j}	19.08±0.01^{g}
	T2	6.21±0.01^{s}	7.12±0.01^{q}	13.03±0.01^{k}	17.41±0.01^{h}	21.92±0.01^{d}
	T3	7.28±0.01^{p}	8.12±0.11^{o}	17.09±0.01^{i}	21.69±0.01^{e}	24.89±0.01^{b}
	T4	9.12±0.11^{m}	10.67±0.06^{l}	21.21±0.05^{f}	23.73±0.01^{c}	26.1±0.11^{a}
ABTS	T0	1.53±0.05^{x}	2.39±0.05^{v}	4.28±0.1^{r}	5.15±0.01^{p}	3.08±0.05^{u}
	T1	2.09±0.11^{w}	3.84±0.09^{t}	7.35±0.04^{l}	14.43±0.11^{h}	15.56±0.04^{f}
	T2	3.08±0.01^{u}	5.76±0.06^{o}	8.34±0.11^{k}	15.12±0.05^{g}	15.81±0.1^{e}
	T3	4.12±0.13^{s}	6.21±0.03^{n}	9.13±0.05^{j}	15.91±0.01^{d}	16.02±0.22^{c}
	T4	5.09±0.09^{q}	6.92±0.01^{m}	9.74±0.11^{i}	16.05±0.13^{b}	16.82±0.13^{a}
Super oxide anion scavenging activity	T0	6.23±0.01^{t}	5.42±0.11^{u}	5.35±0.11^{v}	4.67±0.01^{w}	3.24±0.05^{x}
	T1	18.98±0.11^{g}	18.21±0.01^{k}	17.96±0.12^{l}	16.43±0.08^{p}	15.21±0.10^{s}
	T2	19.46±0.05^{f}	18.87±0.11^{h}	17.78±0.01^{m}	16.43±0.11^{p}	15.97±0.11^{q}
	T3	20.23±0.11^{c}	19.67±0.07^{d}	18.28±0.01^{i}	17.62±0.12^{o}	15.91±0.11^{r}
	T4	21.11±0.01^{a}	20.85±0.5^{b}	19.55±0.13^{e}	18.23±0.11^{J}	17.63±0.01^{n}

The means standard deviations followed by the same letter in the same column indicate that the differences are not significant (P>0.05). T0: Mozzarella cheese without addition of lysozyme (Control), T1: Mozzarella cheese with addition of lysozyme (0.02%), T2: Mozzarella cheese with addition of lysozyme (0.04%), T3: Mozzarella cheese with addition of lysozyme (0.06%), T4: Mozzarella cheese with addition of lysozyme (0.08%).

Table 4: Free fatty acid percentage

Parameters	Storage Days				
Treatments	0-days	10-days	20-days	30-days	40 days
T0	3.80±0.12^{s}	4.23±0.5^{p}	7.80±0.11^{k}	9.87±0.07^{p}	11.2±0.12^{a}
T1	3.20±0.05^{v}	4.01±0.17^{o}	7.5±0.11^{l}	9.37±0.10^{g}	10.91±0.01^{b}
T2	2.80±0.10^{w}	3.85±0.05^{r}	7.32±0.12^{m}	8.93±0.07^{h}	10.34±0.11^{c}
T3	2.10±0.07^{x}	3.7±0.31^{t}	7.15±0.05^{n}	8.51±0.12^{t}	9.8±0.13^{i}
T4	1.88±0.11^{y}	3.66±0.07^{u}	7.05±0.10^{o}	8.17±0.32^{j}	9.58±0.11^{f}

The means standard deviations followed by the same letter in the same column indicate that the differences are not significant (P>0.05). T0: Mozzarella cheese without addition of lysozyme (Control), T1: Mozzarella cheese with addition of lysozyme (0.02%), T2: Mozzarella cheese with addition of lysozyme (0.04%), T3: Mozzarella cheese with addition of lysozyme (0.06%), T4: Mozzarella cheese with addition of lysozyme (0.08%).

Table 5: Sensory characteristics of full fat Mozzarella cheese

Parameters		Storage Days				
Treatments		0-days	10-days	20-days	30-days	40 days
T0	Appearance	2.0±0.11^{e}	3.3±0.05^{d}	3.9±0.21^{c}	4.9±0.07^{b}	5.2±0.18^{a}
	Taste	1.0±0.11^{e}	2.6±0.09^{d}	2.9±0.1^{c}	3.5±0.15^{b}	4.1±0.03^{a}
	Color	8.0±0.10	7.1±0.03	8.3±0.20	8.1±0.29	8.0±0.31
	Flavor	1.0±0.20^{e}	2.3±0.25^{d}	3.5±0.19^{c}	4.1±0.11^{b}	5.2±0.10^{a}
T1	Appearance	3.3±0.13^{e}	4.1±0.20^{d}	5.1±0.11^{c}	5.5±0.10^{b}	6.5±0.22^{a}
	Taste	1.3±0.05^{e}	3.1±0.10^{d}	3.1±0.04^{c}	4.1±0.21^{b}	5.6±0.11^{a}
	Color	8.1±0.11	8.0±0.17	8.1±0.21	8.2±0.16	8.0±0.09
	Flavor	1.2±0.17^{e}	3.2±0.11^{d}	4.7±0.03^{c}	5.0±0.14^{b}	6.5±0.05^{a}
T2	Appearance	4.5±0.05^{e}	5.4±0.13^{d}	6.4±0.10^{c}	6.8±0.13^{b}	7.3±0.11^{a}
	Taste	1.5±0.07^{d}	3.5±0.01^{d}	4.2±0.11^{c}	4.8±0.05^{b}	5.3±0.10^{a}
	Color	8.3±0.5	7.2±0.02	5.6±0.11	1.7±0.10	1.5±0.01
	Flavor	1.4±0.34^{e}	4.3±0.28^{d}	5.5±0.15^{c}	6.2±0.11^{b}	7.4±0.01^{b}
T3	Appearance	5.6±0.10^{e}	6.7±0.32^{d}	7.3±0.04^{c}	7.5±0.11^{b}	8.1±0.09^{a}
	Taste	1.5±0.10^{e}	4.7±0.16^{d}	5.5±0.21^{c}	5.8±0.11^{b}	6.6±0.06^{a}
	Color	8.0±0.07	8.5±0.12	8.2±0.03	8.1±0.14	8.9±0.53
	Flavor	1.5±0.12^{e}	5.5±0.18^{d}	6.2±0.06^{c}	7.3±0.01^{b}	8.5±0.02^{a}
T4	Appearance	5.9±0.07^{e}	7.8±0.28^{d}	8.7±0.20^{c}	8.3±0.06^{b}	8.6±0.08^{a}
	Taste	1.6±0.12^{e}	5.8±0.21^{d}	6.8±0.19^{c}	6.8±0.05^{b}	7.0±0.11^{a}
	Color	8.3±0.24	8.6±0.18	8.7±0.21	8.2±0.05	8.5±0.11
	Flavor	1.4±0.05^{d}	5.8±0.26^{c}	6.5±0.17^{c}	7.5±0.03^{b}	9.0±0.11^{a}

The means standard deviations followed by the same letter in the same column indicate that the differences are not significant (P>0.05). T0: Mozzarella cheese without addition of lysozyme (Control), T1: Mozzarella cheese with addition of lysozyme (0.02%), T2: Mozzarella cheese with addition of lysozyme (0.04%), T3: Mozzarella cheese with addition of lysozyme (0.06%), T4: Mozzarella cheese with addition of lysozyme (0.08%).

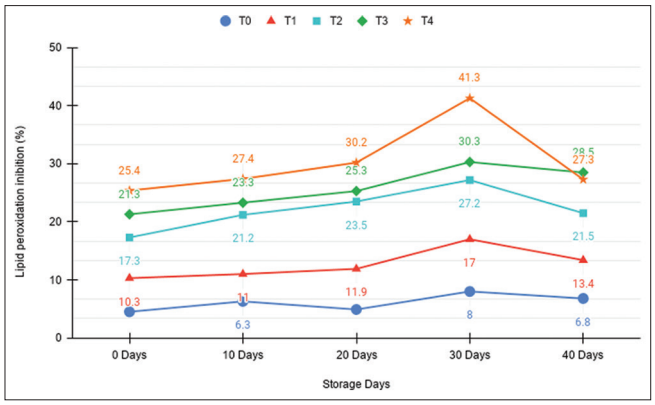

Fig 2. Changes in lipid peroxidation Inhibition (%) of full fat Mozzarella cheese during 293 40 days of storage. Values are presented as mean of three values. The level of significance 294 is presented at P<0.05 compared to control at the same storage period.

CONCLUSION

The results of this study confirmed the antioxidant potential of lysozyme which improved the shelf life by limiting ROS and sensory characteristics of full fat Mozzarella cheese. A dose rate of 0.06 % lysozyme is recommended for full fat Mozzarella cheese.

Author contributions

Iqra Muqadas Saleem did data analysis and interpretation. Nabila Gulzar gave research concept and design. Saima Rafiq and Mubashrah Munir assisted in data analysis and inetrpretation. Ishtiaq Ahmad helped in writing of article and Muhammad Ajmal has critically reviewed the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest."

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