The Utilization of Milk Protein in Antimicrobial Coatings and Films Production

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Abstract: The inhibitory effect of casein–based film and coating emulsions supplemented with nisin, propolis, zeolite and potassium sorbet as antimicrobial substances at different concentrations(0.1,0.2 and 0.3%) against some pathogenic and spoilage bacteria was examined .The casein- based film and coating emulsions supplemented with 0.3% nisin or propolis showed the highest inhibition effect toward the indicator bacteria. The rheological, mechanical and thermal properties of the selected antimicrobial coating and films were determined. The thickness, Tensile strength, elongation, elasticity and yield of casein-based film supplemented with propolis was higher than those of casein-based film supplemented with nisin. Also, the results revealed that the same film showed the highest Tg value. Therefore, casein films incorporated with propolis or nisin have the potential to provide a safe packaging system to decrease microbial growth in foods.
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Key words: casein, film, coating,, inhibitory effect, nisin, propolis, rheological ,mechanical and thermal properties

1. Introduction

A variety of techniques have been developed to maintain the quality and microbial safety of foods, being food packaging one of these methods. The first packaging materials based on cellulose were developed in 1856, and in 1907 phenol-formaldehyde (bakelite) resins were synthesized. This was the starting point of a series of developments and innovations giving birth to a great diversity of packaging materials which nowadays are employed [**Miller and Krochta**, **1997**].

Packaging systems are intended to protect the food from its surroundings acting as physical/mechanical, chemical and microbiological barrier to maintain quality, safety, and to prolong the packaged food shelf- life [**Floros *et al.*, 1997**]. Food quality and its average shelf-life are decreased when the foodstuff interacts with its environment gaining or losing moisture and aroma, or taking oxygen leading to oxidative rancidity. Alternatively, microbial contamination may produce food spoilage, or even food poisoning. In multi component foods the quality and shelf life are reduced when moisture, aroma or lipids migrate from one food component to another. Food packaging also provides important information to the consumer (nutrition facts, ingredients, expiration date, etc.), and makes the food available for a long period of time [**Krochta** and **De Mulder-Johnston, 1997**]. Initially, food packaging contributed to easy handling of food products to manufacturers, distributors and consumers. However, this has changed due to a growing consumer demand for minimally processed and easy preparation foods, where natural food additives are favored over their synthetic counterparts [**Ouattara** et *al.*, **2000** and **Branen & Davidson, 2004**].

Petrochemical based plastics have been widely used because of their availability in large quantities at low cost and favorable mechanical and barrier properties to oxygen, and heat seal ability [**Tharanathan, 2003**]. Nowadays the use of synthetic packaging materials has considerably raised with a concomitant increase in environmental pollution, since they are recalcitrant. Plastic materials may be degraded by naturally occurring microorganisms in the environment, but the process may take about 150 years (low density polyethylene), while paper can be naturally biodegraded in about one year [**Regaldo *et al.*, 2006**.].

There has been a growing interest for edible films and coatings made from renewable and natural polymer, such as protein, polysaccharide and lipids, In recent years trying to reduce the amount of wastes, capable of protecting the food once the primary packaging is open, and because of public concerns about environmental protection [**Cha and Chinnan, 2004**]. Natural polymers or polymers derived from natural products, like food protein, offer the greatest opportunities since their biodegradability and environmental compatibility are assured **(Krochta and De Mulder-Johnston, 1997).** In addition, films made from protein can supplement the nutritional value of the food **(Gennadios and Weller, 1990).** The mechanical properties of protein-based edible films

are also better than those of polysaccharide and fat based films because proteins have a unique structure films because proteins have a unique structure (based on 20 different monomers) which confers a wider range of functional properties, especially a high intermolecular binding potential **(Cuq *et al*., 1995).** Protein-based edible films can form bonds at different positions and offer high potential for forming numerous linkages (**Ou *et al*., 2005**).

Milk protein based edible films are excellent oxygen, lipid, and aroma Barriers (**Sydim and Sarikus, 2006**). Antimicrobial edible films and coatings have received attention as an innovative means to control surface contamination of foods with pathogens (**Oussalah *et al*., 2004)**. The major potential food application of antimicrobial coatings includes fish, poultry, bakery goods, cheese, fruits, and vegetables **(Labuza and Breene 1988).**

The objectives of this research were to (1) study the antimicrobial activities of casein coating emulsions supplemented with nisin, proplis, potassium sorbate and zeolite at different concentrations (0.1,0.2,0.3%) against some pathogenic and spoilage bacteria and (2) assess the rheological, mechanical and thermal properties of the best selected antibacterial films and coatings.

2. Material and Methods

Material

Casein was obtained from the New Zealand Dairy Board, UK.

Glycerol and Potassium sorbet were purchased from El-gomhoria Company, Egypt.

Butyl hydroxy toloune (BHT) was obtained from across-organies company Newgersey U.S.A.

Nisin powder was obtained from Zhejiang Sliver elephant Bio-Engineering Co., LTD, China.

Carboxy methyl cellulose (CMC), Zeolite and potassium sorbet were obtained from Sigma – Aldrich Canada LTd, Canada

Propolis extract was prepared according to (Boryana *et al.*, 2007). 2g of propolis obtained from Fayoum governorator were put in the extraction solvent (70% ethanol). After extraction, the sample was filtered and the filtrate diluted to 100 ml. with 70% ethanol in a volumetric flask.

Organisms and preparation of culture

Escherichia coli ATCC 25922 was obtained from American Type Cultural Collection, USA. *Escherichia coli* O157 and *Listeria monocytogenes* 1/2b were obtained from Dr. Hommer, institute FÜR hygiene, Kiel, Germany, while the spoilage bacteria, *Enterococcus durans* DC 6-1, *Enterococcus faecium* DC4-2 and *Enterococcus faecalis* RC8-2, were obtained from Dairy Department, Faculty of Agriculture, Azhar University.

All strains maintained on nutrient agar slants at 4 ºC and sub- cultured at 37 ºC for 24h in nutrient broth.

-Preparation of antimicrobial casein – based film and coating emulsions:

The antimicrobial casein – based film and coating emulsions were prepared according to **(Letendrs *et al.,* 2002).**). Casein (90% w/v protein) was solubilized at room temperature for 1h. in distilled water containing 5.0% (w/v) glycerol and 0.25% (w/v) of Carboxy methyl cellulose (CMC) for a total protein concentration of 5.0 % (w/v) in the film – forming solution . CMC was added as protein stabilizer and glycerol as plasticizer **(Ressouany *et al*., 1998).**1ml of oleic acid was added. The antimicrobial substances, nisin, propolis, zeolite or potassium sorbet at concentration 0.1, 0.2 and 0.3%, was added. The control treatment (without antimicrobial substances) also was prepared. Each solution was adjusted to pH 7.0 with drop-wise addition of 2M NaOH. The film – forming solutions were homogenized at room temperature for 10 min using mechanical stirrer and treated by thermal treatment (90°C for 30min). Sample of each film – forming solution was taken to determine the inhibitory activity .For each treatment, 35ml of film – forming solution was poured onto glass plate after filtered through cheese cloth to remove foam and undissolved impurities and dried for 24h at room temperature . Films were peeled from the plates and stored at 25°C.

-Test for inhibitory activity:

The inhibitory activity of casein emulsions containing different antimicrobial substances (nisin, propolis, zeolite or potassium sorbet at different concentrations (1,2and3%) and control against the indicator bacteria, pathogenic and spoilage bacteria, was determined by well assay method **(Benkerroum *et al*., 2000).** Appropriate cell dilutions were prepared in 0.85 saline to obtain counts 10^2 and 10^3 per ml. An appropriate number of wells, each 7 mm in diameter, were made in the agar inoculated with individual indicator bacteria. The test was performed for pathogenic bacteria on selective media, Violet Red Bile Lactose Agar for *E. coli* **(Marshal, 1993)** and Listeria selective agar base for *Listeria monocytogenes* **(VAN NETTEN *et al.* 1989).** while, nutrient agar was used for spoilage bacteria, *enterococci*. The casein emulsions containing different antimicrobial substances at different concentrations were dropped in the wells. The assay for each culture was carried out duplicate. The plates were pre incubated at 4 ºC for 3hrs for the diffusion of tested milks, followed by incubation at 37 ºC for 20hrs after incubation; the plates were examined for zones of inhibition around the wells.

The treatments with the highest inhibitory activity will be selected to study the rheological, mechanical and thermal properties.

-Rheological measurements:

Rheological parameters (shear rate and shear stress) of the selected antimicrobial casein-based coating emulsions and control were measured using a Brookfield Engineering labs DV- III Rheometer at 30°C .The samples were placed in a small sample adapter and a constant temperature water bath was used to maintain the desired temperature. The viscometer was operated between 10 and 60 r.p.m. The sc4-25 spindle was selected for the measurement.

-Film thickness:

The thickness of the selected antimicrobial films and control was measured using a digital micrometer (mitutoyo digimatic indicator corporation, model: pk-1012 E, Japan). Film strips were placed between the micrometer jaws and gap and was slowly reduced until the first contact was noted. The measurements were taken at average 4 different locations on each natural film as reported by **(Tien *et al.*, 2000).**

-Mechanical properties of selected antimicrobial films:

Tensile strength, elongation, elasticity and yield

Tensile strength, elongation , elasticity and yield were measured by machine model (zwick 4201) .To evaluate the tensile strength and percentage of elongation at break. The films were cut into strips 1.5cm wide. The instron grip separation was set at 15 cm. These were gripped at each end by a jaw and then the jaws were moved a part at the controlled speed until and young's modulus was automatically recorded according **(Hernandez, 2004).** The ultimate tensile strength is the amount of force necessary to break the strip and was **calculated as follows:**

TS=L/c.s.a,

TS=L/W×T

Where: **TS**: tensile strength (Newton /mm2)

L: load (Newton)

CSA: capacity of surface area (mm2)

W: the sample width (mm)

T: the material thickness (mm)

Elongation is the percent increase in length of the sample at the instance of break and was calculated **as follows:**

Elongation = E/c.d ×100

Where: **E**= extension,

Cd=controlled distance.

-Thermal analysis:

Glass transition temperature:

The glass transition was determined according to **Hsu *et al.*(2003).** The glass transition temperature of the selected films, control and raw material (casein) was measured using differential scanning calorimeter (DSC 50) Japan. Thermal analysis system equipped with a liquid nitrogen cooling accessory during the measurement, dry helium gas was purged into the sample holder at a flow rate of 20 ml/min to obtain uniform heat-transfer characteristics. Desiccant was placed in the dry box and the dry box was flushed with dry nitrogen at a flow rate of 2000 ml/min. A sealed 20ml volatile sample pan and lid were used as the reference sample. Calibration was performed using n-Decane (mp=29C°) and indium (mp=156C°, DHF=28.45j/g). The differential scanning calorimeter sensitivity was 0.02 C° and 0.01 mw.

Thermo graphometeric analysis(TGA):

The effect of range of temperature (0-200°C) on the weight loss of the two selected films, control and raw material (casein) was studied using thermo graphometeric analyzer (shimadzu, 50) Japan, according to **Ogale *et al.* (2000).** The estimation was carried out in the micro analytical center, Faculty of Science, Cairo University.

3. Results and Disscussion

1-Inhibitory action of casein – based coating emulsions supplemented with different antimicrobial substances against some pathogenic and spoilage bacteria.

The inhibitory action of casein – based coating emulsions supplemented with different antimicrobial substances against some pathogenic bacteria is shown in Table (1).It was obvious from the obtained data that the tested pathogenic strains revealed different response to the examined antimicrobial substances. It was evident to notice that all tested antimicrobial substances were able to either suppress or retard growth of either *E.coli* ATCC 25922 or *E.coli* O157, while their actions toward *L.monocytogenes*1/2b were variable. Also, as could be expected wide inhibition zone increased as antimicrobial substances concentration increased.

Among these antimicrobial substances, nisin with different concentrations (0.1-0.3) showed strongly inhibited activity against *L. monocytogenes* since the zone of the inhibition varied from 12-29mm .

It was of interest to notice that, no antimicrobial activity toward *L.monocytogenes* strain detected by sorbet at different tested concentrations and Propolis at 0.1&0.2%. Moreover, *E.coli* O157 strain was more sensitive toward propolis preservative at the tested concentrations. In contrast, zeolite attained moderate inhibition effect against all the target pathogenic bacteria.

From the results, the Nisin and propolis at 0.3% showed the highest inhibition zones for all tested pathogenic bacteria.

The inhibitory effect of the casein-based emulsions supplemented with different antimicrobial substances against spoilage bacteria, enterococci, is

shown in Table (2). It could be observed that all antimicrobial substances used had the dampening effect of the enterococci growth. The nisin and propolis at 0.3% showed the highest inhibitory activity toward all tested strains and *Enterococcus faecium* DC4-2 was the highest sensitive strain (inhibition zone 25.5mm) . The Zeolite had the less inhibitory effect against all tested strains at different concentrations

Table (1): Inhibitory action of coatings supplemented with different antimicrobial substances against some pathogens

Antimicrobial substances conc.(%)	*E.coli* ATCC 25922	*E.coli* O157	. *Listeria monocytogenes*1/2b
Nisin			
0.1	15.00	13.50	12.00
0.2	20.00	17.50	22.50
0.3	26.50	23.50	29.00
Propolis			
0.1	15.00	17.500	ND
0.2	20.00	21.50	ND
0.3	30.00	29.00	20.00
Potassium sorbet			
0.1	20.00	16.00	ND
0.2	22.50	22.70	ND
0.3	30.00	27.00	ND
Zeolite			
0.1	17.50	12.20	12.00
0.2	20.00	15.60	15.00
0.3	21.50	19.00	18.00

ND : not detected * Initial diameter of the agar well=8mm

Table (2): Inhibitory action of casein-based coatings emulsions supplemented with different antimicrobial substances against *Enteococcus* spp

Antimicrobial substances conc. %	*Enterococcus durans* DC6-1	*Enterococcus faecium* DC4-2	*Enterococcus faecalis* RC8-2
	Diameter of inhibition zone (mm)		
Nisin			
0.1	18.50	20.00	19.00
0.2	20.00	24.00	19.50
0.3	22.50	25.50	22.00
Propolis			
0.1	17.50	18.00	18.00
0.2	22.00	24.50	19.10
0.3	21.00	25.50	25.00
Potassium sorbet			
0.1	18.00	16.50	15.00
0.2	20.50	22.50	16.00
0.3	21.00	25.00	19.00
Zeolite			
0.1	17.50	15.00	17.00
0.2	19.00	19.00	18.50
0.3	20.00	20.50	20.50

* Initial diameter of the agar well=8mm

These outcomes are similar to that reported by **Teerakaran *et al*., 2002** who stated that the nisin is effective in inhibiting growth of many Gram-positive, food-borne pathogenic bacteria and their spores. **Janes *et al*., 2002** reported that the nisin has antimicrobial activity against *L.monocytogenes.* Nisin also can inhibit growth of some Gram-negative bacteria, *E.coli*, **(Ray, 1992).** Also, **Ivancajic *et al*.(2010)**which showed that propolis had a significant anti-bacterial activity against *E.coli* , while the weakest effect was on *Enterococcus faecalis*.

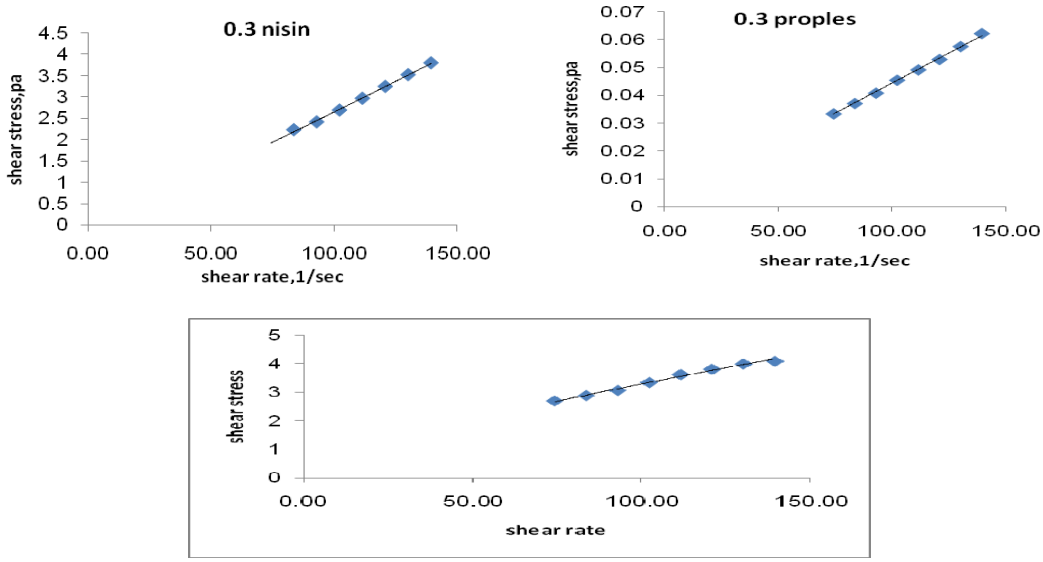
In conclusion,Casein – based coating and films supplemented with 0.3% nisin or propolis were selected to the rheological, mechanical and thermal properties.

2- Rheological properties of selected antimicrobial casein – based film and coating emulsions:

The relation between shear rate and shear stress of selected casein – based film and coating emulsions and control is shown in Fig.(1) and Table (3).The results show that the samples exhibited non-Newtonian pseudoplastic behavior and fits well to the following equation.

$$\tau = k\gamma^n \rightarrow (1)$$

Where: τ: shear stress, pa **γ:** shear rate 1/sec
k: consistency index **n:** flow behavior index



control

Figure (1): Relation between Shear rate and Shear Stress of nisin, propolis at 0.3% and control.

Table (3) presents the parameters, consistency index (k) and flow behavior index (N), obtained by fitting to the power law model for control, nisin and propolis.

Table (3): Relation between consistency index (k) and flow behavior index(n) for nisin , propolis at 0.3%.

Treatments	K	N
Control	2.52	0.69
Nisin 0.3	0.018	1.07
Propolis 0.3	0.014	0.98

3-The thickness and mechanical properties of the selected antimicrobial films:

Mechanical properties of edible films are important to ensure that the film has adequate mechanical strength and integrity during transportation, handling and storage of foods coated with edible films

The thickness, tensile strength, elongation, elasticity and yield, of the selected antimicrobial films (casein – based films supplemented with 0.3% nisin or propolis) and control are shown in Table (4).

Table (4) The mechanical properties of the selected antimicrobial films:

Treat-ments	Thickness um	Tensile strength ($N.M.M^2$)	Elongation (%)	Elasticity ($N.M.M^2$)	Yield N
Control	39	182.8	154	6.3	4.7
Propolis	35	574.8	197	8.4	6.2
Nisin	30	405.8	177	7.6	5.4

It can be observed that the control had the highest thickness value (39 um). The thickness of film supplemented with 0.3%nisin was lower than that supplemented with 0.3%propolis.From the results; it can be observed that the highest value of tensile strength (574.8 $N.M.M^2$), elongation (197%), elasticity (8.4 $N.M.M^{2)}$ and yield (6.2 N) was recorded for the casein –based film supplemented with 0.3% propolis. Also, 0.3% nisin film showed higher tensile strength (405.5 $N.M.M^2$), elongation (177%), elasticity (7.6 $N.M.M^{2)}$ and yield (5.4 N) than those of control film. High film elongation is always a desirable characteristic if the film is to be used for food applications **(Chen, 1995).**Similar results were reported by **(Hotchkiss, 1995**) who pointed out that incorporation of additives such as antimicrobial agents into edible film formulations could affect film's mechanical properties.

4-glass transition temperature (Tg) and Thermo graphometeric analysis (TGA).

The glass transition behavior of food plays a main role in the quality and storage stability of the product.The Thermo graphometeric analysis (TGA) indicate the thermal degradation temperature of tested protein film**(Ogale *et al.*,2000).The** glass transition temperatureTg **and** Thermo graphometeric analysis (TGA) of two selected antimicrobial films, control and raw material (casein) is presented in Table (5) and Figures (2:5). From the results, it could be indicated that the glass transition temperature of casein (raw material) was (89. 40 ºC) .The casein- based film supplemented with 0.3% propolis had a higher Tg.(38.46ºC) than that supplemented with 0.3% nisin (36.14 ºC) or control 29.88 ºC). Below(89.40 ºC),

(29.88 ºC), (38.46ºC) and (36.14 ºC) for raw material, control, propolis film and nisin film films ,respectively, these are in glassy state and above these temperatures the raw material and films are in rubbery state .Similar observations were found by **(Cuq et al.,1997)** who reported that the heating of thermoplastic polymer above Tg produces rubbery state and cooling to room temperaturecan convert rubbery products to glassy state. Also, It could be noticed that from the results of thermogravimetric analysis(TGA) the total weight loss occurred for casein film when heated at temperature of (25-100 ºC) the minimum loss(7.4%) and maximum loss (10.93%) were observed for raw material(casein) and nisin film,respectively. These results may be useful in choosing the proper temperature for processing edible films.

Table (5): Glass transition temperature (Tg) and Thermo gravimeteric analysis (TGA) of raw material and films

Treatments	Tg	TGA		
		Frest stages	second stages	Total stages
Casein(raw material)	89.40	2.61	4.79	7.4
Control	29.88	10.58	One stage	10.58
Propolis	38.46	2.91	6.74	9.65
Nisin	36.14	2.89	8.04	10.93

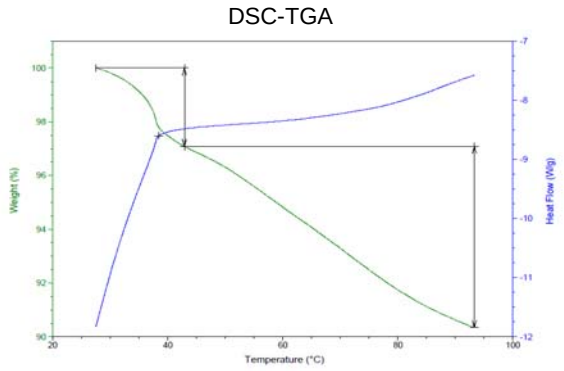


Fig.(2):The relation between glass transition temperature and thermogravimetric analysis (TGA) for 0.3% nisin.

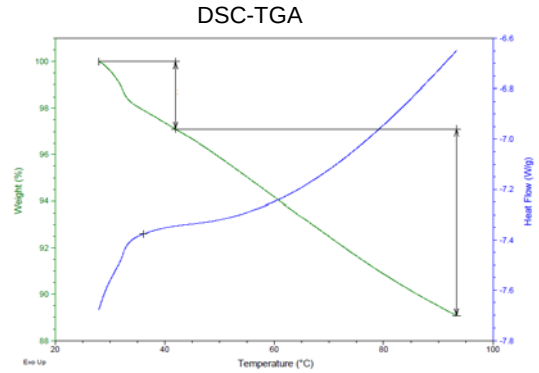


Fig.(3):The relation between glass transition temperature and thermogravimetric analysis (TGA) for 0.3% propolis.

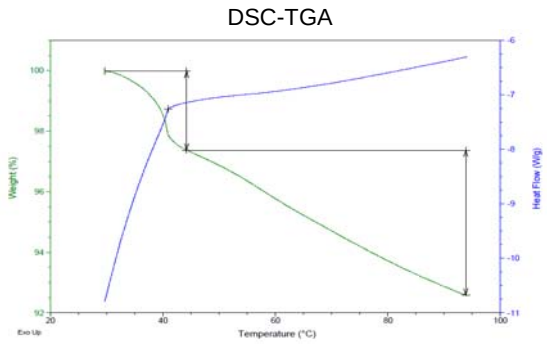


Fig.(4):The relation between glass transition(TG) temperature and thermogravimetric analysis (TGA) for casein.

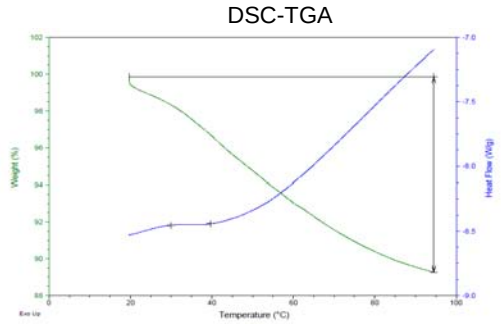


Fig.(5):The relation between glass transition(TG) temperature and thermogravimetric analysis (TGA) for control.

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