Research Article

Chitosan and Cystatin/Lysozyme Preparation as Protective Edible Films Components

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This work characterizes biological, physical, and chemical properties of films formed from an aqueous solution of hydroxypropyl methylcellulose (HPMC), with different concentrations of chitosan (CH) and bioactive cystatin/lysozyme preparation (C/L). The properties of biocomposites were examined by Dynamic Mechanical Analysis (DMA), Fourier's transfer infrared spectroscopy (FTIR), water vapour permeability (WVP), and tensile testing. Antimicrobial activity against *Micrococcus flavus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Candida famata* was conducted. Films glass transition and storage modulus were dependent on the C/L and CH concentration. Modulus values decreased during the temperature scan and with higher reagents levels. An increase of CH and C/L concentrations in the films resulted in a decrease in tensile strength from 2.62 to 1.08 MPa. It suggests the hydrolyzing influence of C/L, also observed in smaller peak size of α relaxation. C/L addition caused shifting T_g to higher temperature. DMA and FTIR analysis proved that HPMC and CH are compatible polymers. Water resistance was improved with rising CH concentration from $1.08E-09$ to $7.71E-10$ g/m $*$ s $*$ Pa. The highest inhibition zone in *M. flavus* and *C. famata* was recorded at the highest concentration of CH and C/L.

1. Introduction

The growing interest and demand of producers as well as consumers for environmental friendly, biodegradable, biocompatible, and bioactive materials to produce edible films and coatings are observed. Most polymers and bioactive substances have very good film forming properties. These include polysaccharides: chitosan [1], cellulose [2], starch [3], and carrageenan [4], proteins: gelatin [5], collagen [6], and lipids [7]. Enzymes are also used to improve or change features of polymers: transglutaminase [8], lysozyme [9], or cellulase [10].

Derivatives of cellulose are composed of the same β-(1 $\rightarrow$ 4)-glycosidic units with different substituents methyl, hydroxypropyl, or carboxyl. Hydroxypropyl methylcellulose is cellulose ether with hydrophilic groups, hydroxyl groups, which provide good interaction of HPMC with water [11]. The mechanical and thermal properties of HPMC are influenced by presence of these groups and water uptake [12]. HPMC exhibits thermogelation and has excellent film-making properties, high solubility, efficient oxygen, and lipid barrier properties [13, 14].

Chitosan is a biopolymer, which has a β-(1 $\rightarrow$ 4)-D-glucopyranose backbone similar to cellulose. The difference is that chitosan possesses acetamide group instead of the hydroxyl group in C2 position of glucose residue. The similarity of primary structure of both polymers suggests possibility of formation of homogenous edible films based on chitosan and cellulose derivatives [15]. The modification of chitosan with different polysaccharides or proteins may be an effective way to improve mechanical properties of chitosan for which antimicrobial [16] and oxygen barrier properties were reported [17].

Interactions of the polymers with bioactive substances have been recently studied extensively [18–20]. Egg white is

rich natural source of bioactive proteins such as lysozyme, ovotransferrin, avidin, ovoflavoprotein, and proteinase inhibitors (ovoinhibitor, ovomucoid, ovostatin, and cystatin). Classical separation methods of proteins are based on the salting out of solution or precipitation with alcohol. These techniques have been extended by the ion exchange chromatography and membrane separation in recent years. Nowadays, researchers are looking for separation methods, which will be cheap, easy, nontoxic, and maintaining the highest biological activity of isolated proteins. They are important factors in commercial production and in food and pharmaceutical applications in view of their health activities [21]. The lysozyme damages cell walls, especially Gram-positive bacteria by catalyzing hydrolysis of β-(1 $\rightarrow$ 4) linkages between particles of N-acetylmuramic acid and L-glucosamine [22]. The cystatin is a protein inhibitor of cysteine proteinases [23] and has very high antimicrobial [24, 25] and antiviral properties [26]. A special challenge is to develop a cheap method of cystatin isolation, because cystatin is present in small amount (around 0.05%) in egg white. Skiba et al. have proposed a thermochemical method of initial isolation of bioactive proteins of egg white, especially cystatin [27]. They used diversity of chemo- and thermoresistance property of egg proteins for their separation. Lysozyme, cystatin, and ovomucoid are acid soluble and thermally stable, even when heated up to 100°C. The rest of the egg white proteins (ovotransferrin, ovalbumin) in such conditions will coagulate. The production process of bioactive preparation can be carried out in processing plants, but the obtained mixture could be used just for foodstuff. Extension of application in pharmaceutical usage is possible after purification by chromatography methods. Possibility to obtain cystatin/lysozyme preparation with ovomucoid was proved [27] and bioactivity of these single proteins was confirmed by researchers [28].

Applicable opportunities of bioactive preparation in HPMC biocomposites were assessed, in view of the aim of this study, which was to develop HPMC films with addition of cystatin/lysozyme preparation and chitosan as well as evaluate their antimicrobial activity and thermomechanical, barrier, and chemical changes.

2. Materials and Methods

2.1. Materials.
The materials included HPMC (Walocel HM 100 PA 2208 FG, Wolff Cellulosics), chitosan (low molecular weight, DD 75–85%, Mw = 150×10^{-3}, low viscosity ranged between 20 and 200 cps, Aldrich), DL-lactic acid (85%, Sigma), and glycerol 99% (Aldrich).

2.2. Cystatin/Lysozyme Isolation.
A substrate of C/L preparation was homogenous hen egg white, which was diluted with an equal 0.25% NaCl following the procedure described by Skiba et al. [27]. The solution was brought to pH 3.0 with 1 N HCl and heated to 60°C for 30 minutes. Protein suspension was obtained and centrifuged at 9300 ×g for 20 minutes to precipitate ovomucin and other eggs' protein. Supernatant containing bioactive substances was frozen at −40°C, then lyophilized, and stored at 4°C.

2.3. Activities of Cystatin and Lysozyme.
Activity of cystatin against papain was analyzed according to colorimetric method reported by Siewiński [29], where BANA (hydrochloride Na-benzoyl-DL-arginyl-B-naftylamid) was a substrate of hydrolysis and reaction was stopped by the addition of DMBA (p-dimethylaminobenzaldehyde). Absorbance was measured at $\lambda = 450$ nm. One unit of inhibitory activity of cystatin equals one unit of papain activity, which is quantity of enzyme able to hydrolyze 1.0 mM of substrate in one minute (standard conditions, 37°C).

Lysozyme activity was analyzed by spectrophotometrical method [30]. Bacterial cells of *Micrococcus lysodeikticus* were used as a substrate for lysozyme. The dynamic turbidity changes were reported by measuring of absorbance at $\lambda = 450$ nm in 25°C, every 60 seconds during 6 minutes.

2.4. Film Preparation.
HPMC was dissolved in bidistilled heated (70°C) water for preparation of solution. Chitosan was solubilized in 2% (v/v) aqueous lactic acid solution in room temperature. Both solutions were stirred with 400 rpm for 12 hours. C/L stock solution was prepared by dissolving 20% C/L preparation in bidistilled water followed by centrifugation (9300 ×g) and filtration to remove insoluble residues. The same procedure was applied to prepare protein solution. Glycerol was used as plasticizer in amount of 25% of polymers dry mass. Thus prepared solutions of hydrocolloids, preparation of C/L, equivalent of proteins, and glycerol were mixed in suitable proportions to obtain final concentrations of the components shown in Table 1. The HPMC and CH solutions at three different levels, 0%, 1%, and 2%, were blended with 25% wt/wt of glycerol (of dry weight of the used polymers) and C/L mixture (at three different levels: 0%, 0.5%, and 1%, which correspond to 0/0, 0.35/96, and 0.7/192 U/g activity of C/L, resp.) in different ratio (wt/wt). All experimental samples were centrifuged at 8400 ×g for 20 minutes to remove air bubbles. Twelve mL of film forming solutions was then cast on leveled, coated by Teflon glass plate on an area of 66 × 77 mm, and dried at 25°C and 60% RH for 48 hours. The dried biocomposites were peeled from plates and cut into pieces for the measurements of thermal and mechanical properties.

2.5. Dynamic Mechanical Analysis.
Strips of HPMC films (5 mm length and 7 mm width) were subjected to Dynamic Mechanical Analysis using TRITEC 2000 DMA from Triton Technology. Samples were heated from −80°C to 70°C at a heating rate of 2°C/min and frequency of 1 Hz. E', storage modulus, and tan δ, loss factor, were recorded.

2.6. Tensile Test.
Tensile test was performed in a universal testing machine HSKT (Tinius Olsen). The samples were cut into 12 mm × 65 mm dumbbell-shaped test specimen with contraction of the following dimensions: 7 mm × 15 mm. The film strips were uniaxially stretched immediately after removing from the chamber (25°C, 60% RH) to minimize moisture content variability. Tensile strength (TS, Pa) values were obtained from equation of measured maximum force (N) divided by film cross-section (thickness × width) and elongation at break (EL, percent at break point) values were

TABLE 1: Experimental design of film forming hydrosols.

Variants	Variability factors			Constant factors	
	Chitosan [%]	Cystatin/lysozyme [%]	HPMC [%]	Glycerol [%]	Lactic acid [%]
CH0C/L0		0			
CH0C/L0.5	0	0.5			
CH0C/L1		1			
CH1C/L0		0			
CH1C/L0.5	1	0.5	0.5	25 (of dry weight of used polymers)	1
CH1C/L1		1			
CH2C/L0		0			
CH2C/L0.5	2	0.5			
CH2C/L1		1			

reported as deformation at break divided by the initial length and multiplying by 100.

2.7. Fourier Transform Infrared Spectrometry.

Infrared spectra were registered in FTIR-460 Plus, Specac spectrometer. The transmission spectra were collected at $4\,cm^{-1}$ resolution and by 32 scans, directly on films with a golden bridge reflection apparatus. The reference background was air.

2.8. Water Vapour Permeability.

Water vapour permeability of the film was determined following ASTM E-96 method [31]. The cups were filled with $100\,cm^3$ of distilled water each. A sample was placed in between the cup and the ring cover of each cup. Then, they were stored at 4°C and 60% RH. Cups were weighed every hour for 6 h. Water vapour transmission rate (WVTP) was estimated using the following equation:

$$WVTR = \frac{G}{(t \times A)}, \quad (1)$$

where G is the change in weight (g), t is the time (h), and A is the test area (m^2).

Water vapour permeability (WVP) was calculated as

$$WVP = \frac{(WVTR \times T)}{\Delta P}, \quad (2)$$

where T is the thickness of the test specimen (mm) and ΔP is the partial pressure difference of the water vapour across the film.

2.9. Antimicrobial Activity.

Disc diffusion test was used to determine the antibacterial activity of biocomposites. *B. cereus* B3p, *M. flavus* ATCC10240, *P. fluorescens* PCM 1994, *E. coli* PCM 2560, and *C. famata* MI1a were selected for determination. Gram-positive and Gram-negative bacteriainoculums were prepared by growing cells in enriched broth for 24 h at 37°C. Yeast was inoculated in YM broth for 24 h at 30°C. Optical density of the bacterial culture was measured in a Ray Leigh UV 1800 spectrophotometer at $\lambda = 550$ nm. The inoculum containing 10^6 CFU/mL was evenly spread on agar plates. The uniform discs with 20 mm diameter of appropriate films were sterilized by UV for 2 minutes and placed on the surface of the agar plates. The plates were incubated at 37°C for bacteria and at 30°C for yeast for 24 h. The diameter of the inhibition zone (mm) was measured using the GIMP 2 program.

2.10. Statistical Analysis.

Data were analyzed by analysis of variance (ANOVA). The differences between means were established with Duncan Test. Statistical analyses were performed using the Software STATISTICA (Version 7.1, Statsoft, Inc.). Significance of differences was defined at $p \leq 0.05$. All experiments were performed in triplicate.

3. Results and Discussion

3.1. Tensile Test.

The main and interaction effects of CH and C/L blend on tensile properties of HPMC films are showed in Table 2. There was significant impact of chitosan, C/L preparation, and interaction of blended factors on tensile strength (TS) and elongation at break (EB). TS of the films significantly increased ($p < 0.05$) from 1.80, 3.61 to 4.72 MPa with the addition of 0, 1, and 2% of chitosan, respectively. On the contrary, it decreased with the 0, 0.5, and 1% addition of cystatin/lysozyme preparation, from 6.51, 2.89 to 0.72 MPa. Chitosan films with protein addition provide much more tensile resistance than without it [32]. Lysozyme and cystatin are both proteins, but the brittleness was caused by the presence of lysozyme. Lysozyme is an enzyme with degraded properties towards β-(1 $\rightarrow$ 4) linkages of polysaccharides and could hydrolyze both chitosan and hydroxypropyl methylcellulose [33]. Simultaneously, implicated in our study, chitosan with high deacetylation degree is less susceptible to enzymatic degradation [34] but cannot be stopped completely and the products, oligomers, can still have intermediate viscosities [18]. On the other hand, the reason of weaker structure and integrity of films could be caused by disruption of crystalline structure formation during drying and weakening intermolecular hydrogen bonds of chitosan and HPMC [34]. Existence of seven and six homogenous groups in interactional effects was showed and proved high complicity of CH and C/L impact on TS and EB, respectively. The elongation at break was enlarged with chitosan 2% (60.42%), compared to its 0 and 1% addition (50.89 and

TABLE 2: Mechanical properties of films.

Main effects				Interaction effects	
CH [%]	TS [MPa]	C/L [%]	TS [MPa]	CH × C/L	TS [MPa]
				0×0	2.62 ± 0.23^{cd}
0	1.80 ± 0.29^a	0	6.51 ± 1.07^a	0×0.5	2.06 ± 0.05^c
				0×0.1	0.72 ± 0.01^{ab}
				1×0	7.09 ± 0.36^f
1	3.61 ± 0.98^b	0.5	2.89 ± 0.22^b	1×0.5	3.36 ± 0.13^e
				1×1	0.37 ± 0.03^a
				2×0	9.83 ± 0.45^g
2	4.72 ± 1.32^c	1	0.72 ± 0.10^c	2×0.5	3.25 ± 0.16^{de}
				2×1	1.08 ± 0.05^b
CH [%]	EB [%]	C/L [%]	EB [%]	CH × C/L	EB [%]
				0×0	29.23 ± 0.59^e
0	50.89 ± 5.46^b	0	51.02 ± 8.19^b	0×0.5	59.50 ± 0.55^{ab}
				0×0.1	63.93 ± 0.88^b
				1×0	40.90 ± 1.27^c
1	36.27 ± 7.80^a	0.5	58.92 ± 1.06^a	1×0.5	60.55 ± 2.43^{ab}
				1×1	7.36 ± 0.55^d
				2×0	82.93 ± 2.34^f
2	60.43 ± 6.15^c	1	37.64 ± 8.27^c	2×0.5	56.71 ± 1.82^a
				2×1	41.63 ± 2.82^c
CH [%]	WVP [g/m∗s∗Pa]	C/L [%]	WVP [g/m∗s∗Pa]	CH × C/L	WVP [g/m∗s∗Pa]
				0×0	$1.08 \pm 0.03E - 09^a$
0	$1.08 \pm 0.03E - 09^a$	0	$9.25 \pm 0.85E - 10^a$	0×0.5	$1.12 \pm 0.11E - 09^a$
				0×1	$1.05 \pm 0.02E - 09^a$
				1×0	$1.06 \pm 0.02E - 09^a$
1	$9.71 \pm 0.61E - 10^a$	0.5	$1.02 \pm 0.08E - 09^a$	1×0.5	$1.02 \pm 0.17E - 09^a$
				1×1	$8.40 \pm 0.50E - 10^{ab}$
				2×0	$6.40 \pm 1.60E - 10^b$
2	$7.71 \pm 0.87E - 10^b$	1	$8.78 \pm 0.53E - 10^a$	2×0.5	$9.30 \pm 2.00E - 10^{ab}$
				2×1	$7.40 \pm 0.70E - 10^{ab}$

Values with different letters (a–g) within the same column differ significantly ($p < 0.05$).

36.27%, resp.). EB decreased with 1% addition of C/L preparation. The highest TS and EB were registered for samples with the highest amount of CH. Hosseini et al. have reported 12.2 MPa tensile strength of film with 2% chitosan and 0.5% of glycerol [35]. However, 6% of HPMC film with no plasticizer was registered at 63 MPa [36], which is very high value because of the HPMC concentration and lack of glycerol. The same authors noted that with the increased concentration of plasticizer the TS decrease significantly. Worse resistance of our films is caused by addition of plasticizer and hydrophilic compounds, which create lower density of films and help in polymers chains movements. On the other hand, chitosan films made with lactic acid exhibit lower tensile strength and higher elongation than those made with acetic acid, which was registered by Kumar et al. [37].

3.2. Dynamic Mechanical Analysis.

Glass transition T_g identifiers of DMA test are large drop in storage modulus E' and a peak in tan δ (Figures 1(a), 1(b), 1(c), 2(a), 2(b), and 2(c)).

Storage modulus is an index of rigidity of polysaccharides [38]. The location of the glass transition was shifted to the higher temperature with addition of CH and C/L. The E' curves increased from 120 MPa for CH0C/L0 to 600 MPa for CH0C/L1 and to 450 MPa for CH2C/L1 films. One drop in E' was observed in DMA curves of polysaccharides films at −40–5°C. Wu et al. have presented study on cellulose-chitosan blend and chitosan; the curves of storage modulus of chitosan showed a drop at 0–30°C [15]. The authors suggested that drop is related with hydration of side groups (–CH$_2$OH) on chitosan. On the other hand, tan δ curves showed two peaks; the first of them is characterized as the β relaxation attributed to local mode in amorphous phase, and another one is designed as the α relaxation assigned to glass transition of amorphous phase [39]. Tan δ is a measure of polysaccharide viscoelasticity. Wetton has also suggested that tan δ peak size is correlated with the polymer in the composition of sample [40]. The α relaxation peaks were reduced after C/L addition, which confirms degradative effect of lysozyme on main chain of polymers. The viscoelastic
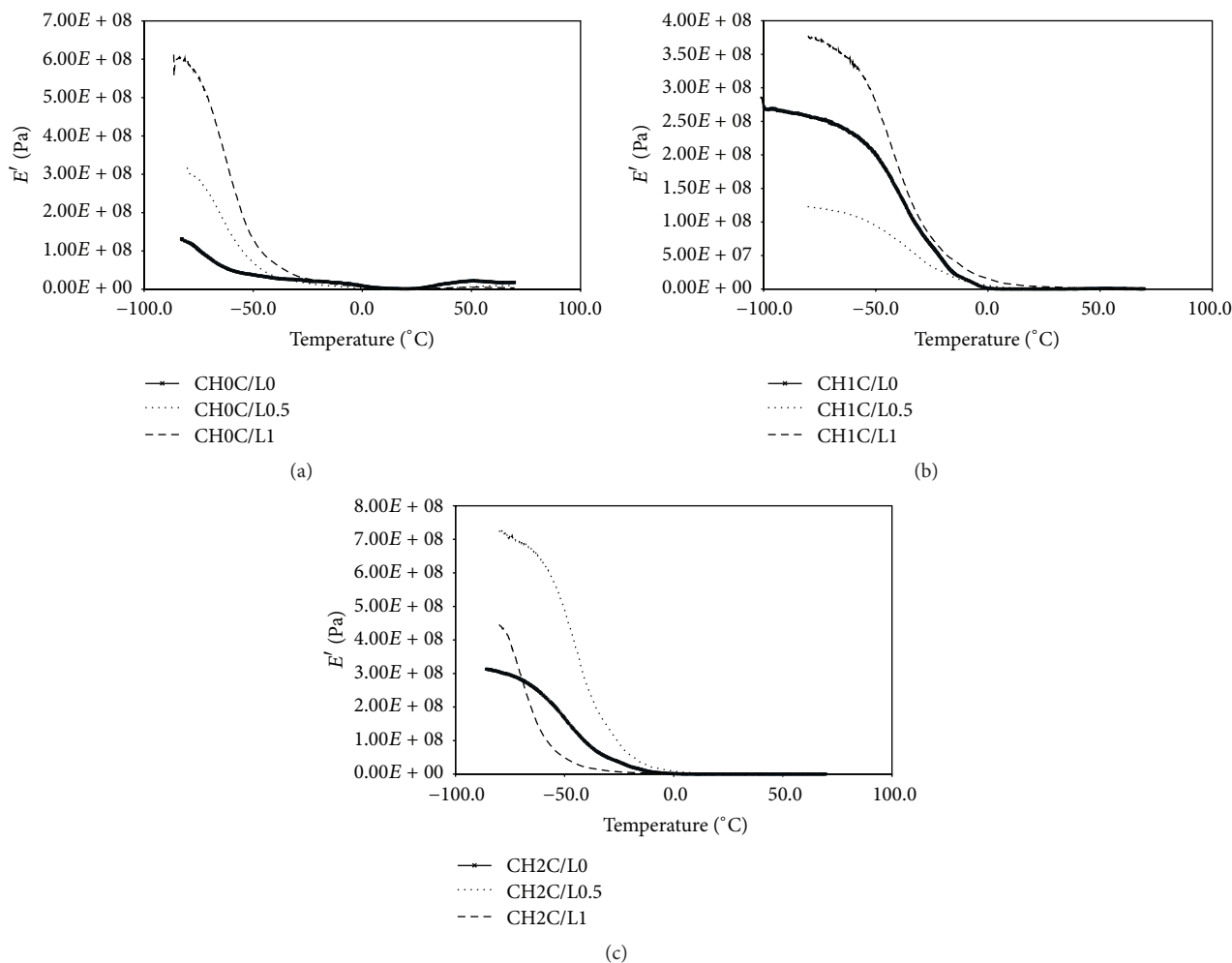
FIGURE 1: Storage modulus (E') of HMPC, CH, and C/L preparation films of (a) CH0C/L0, CH0C/L0.5, and CH0C/L1; (b) CH1C/L0, CH1C/L0.5, and CH1C/L1; and (c) CH2C/L0, CH2C/L0.5, and CH2C/L1 variants.

behavior of films was confirmed by loss factor values, which were between 0.1 and 0.9. The CH0C/L0 film represented β relaxation around -80–(-50)°C and T_g at 5–50°C samples with CH and C/L were characterized by $\tan \delta$ peaks at 0°C and between 40 and 60°C. The shift of β and α transition temperature is the result of CH addition. Addition of chitosan has changed T_g of HPMC to higher temperature but has not changed the width and height of the peak, which suggest that both polymers were mixing well. If the peak got broadness and dumping decrease, it will mean that polysaccharides are incompatible and semicompatible [41].

3.3. Fourier Transform Infrared Spectrometry. FTIR spectra of HPMC films had absorption bands at 3373.85 cm^{-1}, 2885.95 cm^{-1}, 1649.80 cm^{-1}, and 1041.37 cm^{-1} assigned to O-H stretch, combination of methyl groups and C-H stretch, C=O, and ether C-O-C stretch, respectively (Figure 3(a)). The position of the peaks of HPMC film spectrum is similar to those described by Gustafsson et al. [42]. Hydrogen bonding or other interactions between chemical groups on dissimilar polymers should theoretically cause a shift in peak position

of the participating groups [43]. This kind of behavior was observed for the OH stretching since this peak shifted from 3373.85 cm^{-1} for HPMC film toward 3293.82 cm^{-1} for HPMC-CH mixtures (Figures 3(b) and 3(c)). Also increased C/L preparation concentration changed position of the OH group from 3373.85 cm^{-1} for pure HPMC to 3350.71 cm^{-1} for HPMC-0.5% C/L and to 3331.43 cm^{-1} for HPMC-1% C/L blends. According to Wu et al. [16], FTIR spectra of CH films had absorption bands at 3400–3480 cm^{-1} that responded to OH-3 and CH$_2$OH intra- and intermolecular hydrogen bonds, 1650 cm^{-1} for amide I, and 1557 cm^{-1} for amide II vibrational mode (Figures 3(b) and 3(c)).

3.4. Water Vapour Permeability. The results of statistical analysis of water vapour permeability are presented in Table 2. No significant effect of C/L preparation on water permeability was noted. Lopes, Martins, Fonseca, and Vicente (2011) also noticed that WVP of chitosan films were not affected by the enzyme, glucose oxidase incorporation [44]. Cellulase did not change water resistance of chitosan-hydroxypropyl
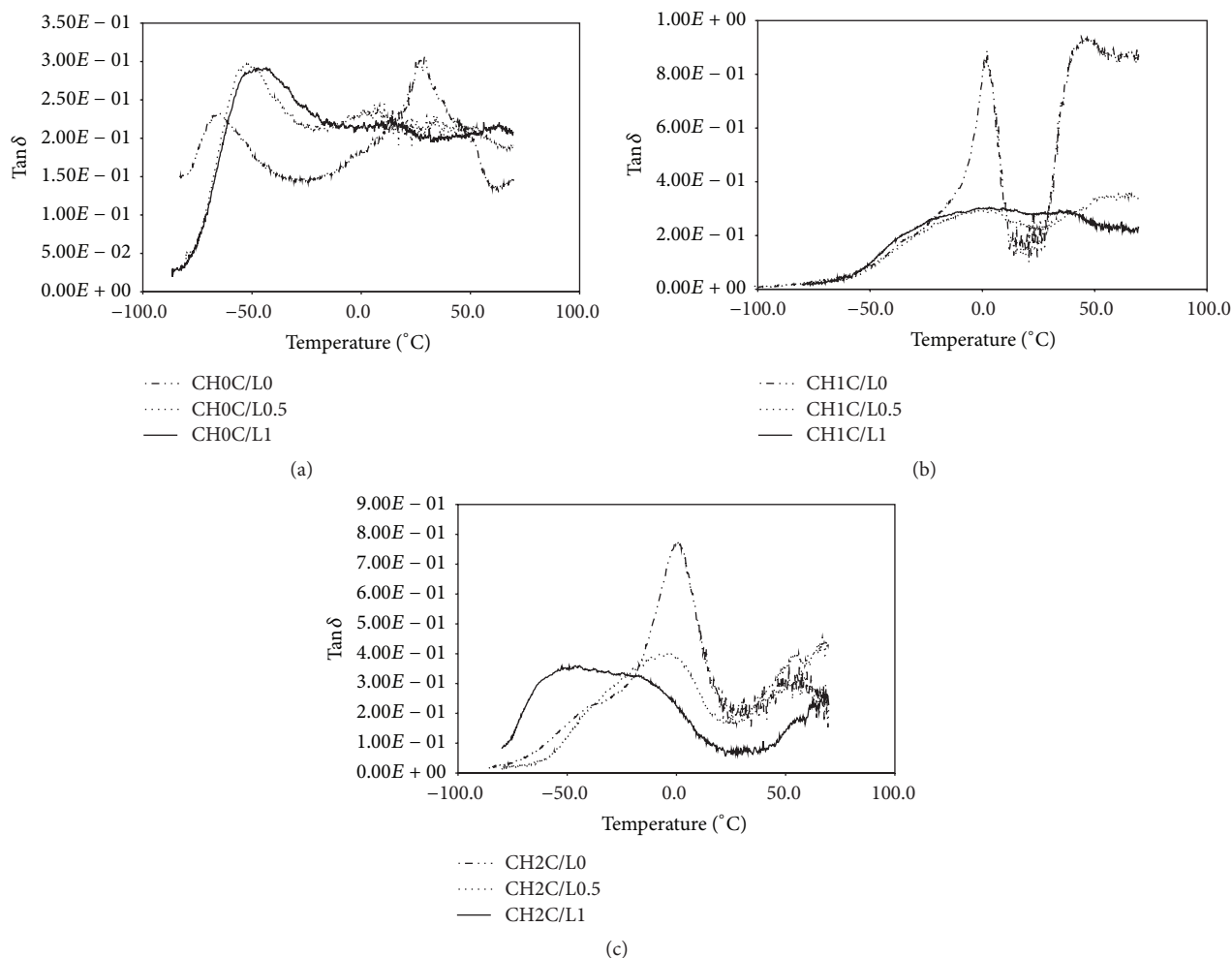
FIGURE 2: Tan δ of HPMC, CH, and C/L preparation films of (a) CH0C/L0, CH0C/L0.5, and CH0C/L1; (b) CH1C/L0, CH1C/L0.5, and CH1C/L1; and (c) CH2C/L0, CH2C/L0.5, and CH2C/L1 variants.

methylcellulose films [10]. However, interactional effect of C/L and CH blend as well as chitosan influence was significant. Water resistance of tested films was higher with increasing concentration of chitosan from $1.08E - 09$ to $7.71E - 10 \, \text{g/m} * \text{s} * \text{Pa}$ for 0 and 2% dose, respectively. Those results were confirmed by significant impact of simultaneous action of CH and C/L. The lowest WVP of $6.40E - 10 \, \text{g/m} * \text{s} * \text{Pa}$ was noticed for film with the highest addition of chitosan and lack of lysozyme/cystatin preparation.

3.5. Antibacterial Activity. Antimicrobial effect of chitosan and cystatin/lysozyme preparation incorporated into the filmogenic hydrosol composition against *M. flavus*, *B. cereus*, *E. coli*, *P. fluorescens,* and *C. famata* was presented in Tables 3 and 4. Incorporation of C/L and CH exhibited a clear inhibitory zone by the absence of bacterial and yeast growth. CH showed maximum inhibition of 33.0 mm against Gram-positive bacteria, *M. flavus,* in the highest concentration of 2%. These bacteria were selected for our test because of their high susceptibility to the various antimicrobial agents. Similar inhibiting effect of 32.8 against the same

bacteria was observed with 1% addition of C/L. The highest concentration of C/L statistically influenced inhibition of yeast growth (29.6 mm). Kolaczkowska et al. have proved antifungal activity of cystatin, affinity-purified from chicken egg white, against the most frequent human fungal pathogens of the genus *Candida* [25]. The mechanism of fungal growth inhibition by cystatin is not related to its protease inhibitory properties as Wesierska et al. suggested in their study regarding antibacterial activity of cystatin [24]. The purified recombinant proteins of tarocystatin from *Colocasia esculenta* such as N-terminus peptide have a greater antifungal activity than full-length peptide. In addition, C-terminus peptide has not showed antifungal effect, which is proof of lack of connection to the inhibitory activity [45]. Significant reduction of two Gram-negative bacteria by every tested concentration of CH and C/L was also noted. Characteristic intensification of antibacterial activity is also possible by the addition of hydrolytic enzymes, such as lysozyme to the chitosans films, which increases the inhibiting effect on *Escherichia coli* growth [33], which was also confirmed in our study. Microbiological test showed that chitooligomers obtained after
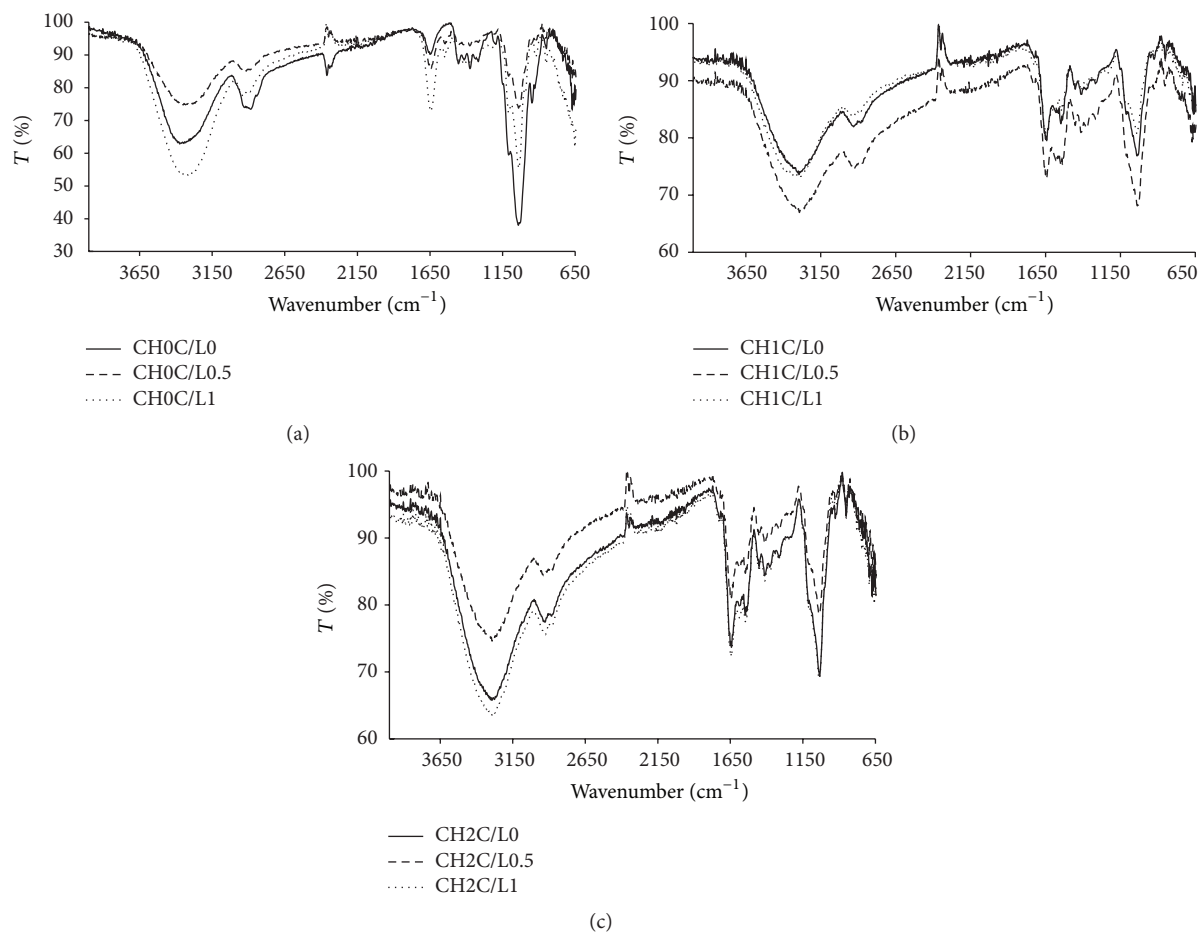
FIGURE 3: FTIR spectra of HMPC and C/L preparation films of (a) CH0C/L0, CH0C/L0.5, and CH0C/L1; (b) CH1C/L0, CH1C/L0.5, and CH1C/L1; and (c) CH2C/L0, CH2C/L0.5, and CH2C/L1 variants.

TABLE 3: Antibacterial activity of edible films modified by CH and C/L preparation (main effects).

Main effect	Inhibition zone [mm]				
	P. fluorescens	E. coli	M. flavus	B. cereus	C. famata
		Chitosan [%]			
0	21.9 ± 1.48[a]	22.1 ± 0.44[a]	26.8 ± 9.34[a]	20.0 ± 0.00[a]	22.3 ± 1.21[a]
1	22.8 ± 0.60[b]	23.1 ± 0.46[b]	31.2 ± 2.89[b]	20.0 ± 0.00[a]	29.5 ± 1.56[b]
2	24.8 ± 1.48[c]	24.4 ± 0.89[c]	33.0 ± 2.22[c]	23.7 ± 1.27[b]	33.0 ± 1.41[c]
		Cystatin/lysozyme [%]			
0	21.9 ± 1.46[a]	22.7 ± 0.88[a]	26.1 ± 8.92[a]	20.8 ± 5.65[a]	26.9 ± 4.45[a]
0.5	23.3 ± 1.70[b]	23.0 ± 1.23[a]	32.1 ± 2.60[b]	21.3 ± 6.94[b]	28.4 ± 4.64[b]
1	24.3 ± 1.19[c]	23.8 ± 1.03[b]	32.8 ± 1.53[c]	21.6 ± 6.50[b]	29.6 ± 5.16[c]

Values with different letters (a–c) within the same column differ significantly ($p < 0.05$).

lysozyme hydrolysis exhibit higher antilisterial activity than chitosan [46]. Interaction effects of statistical analysis showed that inhibition growth of *E. coli* and *P. fluorescens* is the result of simultaneous addition of tested bioactive substance. Both species are the food poisoning bacteria in chilled foods. The lack of reduction zone of *B. cereus* growth (20.0 mm, the same as film diameter) was observed for variants with 0 and 1% chitosan with C/L addition in various concentrations. It confirms what Mellegard et al. have noted that antimicrobial activity of chitosan is concentration-dependent towards *B. cereus* [47]. They have also found out that chitosan with low acetylation degree and higher molecular weight might reduce

TABLE 4: Antibacterial activity of edible films modified by CH and C/L preparation (interaction effects).

Interaction effects	Inhibition zone [mm]				
	P. fluorescens	E. coli	M. flavus	B. cereus	C. famata
CH0C/L0	20.1 ± 0.48[c]	21.7 ± 0.11[c]	20.4 ± 0.34[c]	20.0 ± 0.00[a]	21.2 ± 0.17[a]
CH0C/L0.5	22.2 ± 0.12[b]	22.0 ± 0.33[c]	30.4 ± 0.57[a]	20.0 ± 0.00[a]	22.1 ± 0.72[a]
CH0C/L1	23.4 ± 0.46[a]	22.6 ± 0.09[b]	29.7 ± 0.28[a]	20.0 ± 0.00[a]	23.7 ± 0.58[e]
CH1C/L0	22.5 ± 0.70[ab]	22.9 ± 0.51[ab]	27.5 ± 0.19[d]	20.0 ± 0.00[a]	28.2 ± 0.47[b]
CH1C/L0.5	22.9 ± 0.25[ab]	22.9 ± 0.45[ab]	32.8 ± 1.04[b]	20.0 ± 0.00[a]	29.4 ± 1.62[b]
CH1C/L1	23.1 ± 0.78[ab]	23.5 ± 0.25[a]	33.4 ± 0.99[b]	20.0 ± 0.00[a]	30.9 ± 1.09[c]
CH2C/L0	23.2 ± 0.28[c]	23.5 ± 0.64[a]	30.5 ± 0.39[a]	22.3 ± 0.70[b]	31.2 ± 0.63[c]
CH2C/L0.5	24.8 ± 0.21[d]	24.2 ± 0.28[d]	33.2 ± 0.96[b]	24.0 ± 0.70[c]	33.7 ± 0.22[d]
CH2C/L1	26.5 ± 0.77[e]	25.4 ± 0.25[e]	35.4 ± 0.66[e]	24.9 ± 0.54[d]	34.1 ± 0.11[d]

Values with different letters (a–e) within the same column differ significantly ($p < 0.05$).

B. cereus growth more effectively than with high acetylation degree and low molecular weight, which also explain very low reduction of these bacteria in our study.

4. Conclusions

HPMC films were successfully prepared with chitosan and cystatin/lysozyme preparation. Thermal properties of the HPMC-CH-C/L biocomposites are almost dominated by hydroxypropyl methylcellulose and protein preparation. Hydrolyzed impact of cystatin/lysozyme preparation was observed only in α main chain of HPMC. C/L preparation induces significant changes of tensile strength and elongation at break but has no influence on water vapour permeability. Elongation of films was improved by CH addition, which is important in coating process of elastic materials, such as raw meat. C/L preparation addition results in higher elasticity of obtained biocomposites, because very high T_g temperature provides a brittle material. Simultaneous effect of CH and C/L in microbial reduction of every tested microorganism was obtained. Implication of HPMC biocomposites with CH and bioactive preparation in packaging production system is possible. We also assumed that designed composition is suitable for meat and meat products, due to simplicity of the process to obtain film components and to achieve desired thermomechanical, barrier, and antimicrobial properties.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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