

Research Article

Antimicrobial Activity and Preliminary Characterization of κ -Carrageenan Films Containing Cinnamon Essential Oil

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Abstract: κ -Carrageenan (CA) based films containing Cinnamon Essential Oil (CEO) at 0.025, 0.05, 0.1, 1 and 2%, respectively (v/v) levels were evaluated their physical and antimicrobial properties. The addition of 1 and 2% CEO significantly ($p < 0.05$) increased film thickness and opacity. Only CA-2% CEO film showed inhibition effect against *Escherichia coli* (inhibition zone diameter was 1.59 mm) and *Staphylococcus aureus* (inhibition zone diameter was 12.64 mm), possibly by damaging cell membrane. Wrapping of pork slices with CA-based films reduced Total Viable Count (TVC) by 0.71-2.82 logarithm units at the end of 4°C storage, when compared with the control. The lowest TVC value (6.90 log CFU/g) was detected in samples wrapped with CA-2% CEO film. This study pointed to the effectiveness of CA-based films for antimicrobial purposes in pork preservation.

Keywords: Antimicrobial activity, carrageenan film, cinnamon essential oil, physical property

INTRODUCTION

Microbial growth on the surface is principally responsible for food spoilage, inducing enormous economic losses each year all over the world (Ojagh *et al.*, 2012a). In this context, various physical and chemical processes have been used to prolong food shelf life over the years (Dutta *et al.*, 2012). However, in recent years, the demand for natural antimicrobials as effective alternatives to the aforementioned methods has been soaring in response to increased social concern on 'green' consumerism and food safety.

Essential Oils (EOs), oily liquids extracted from aromatic plants, have attracted increasing attention in food additive industry. They possess well documented insecticidal, antimicrobial and antioxidant activities (Bakkali *et al.*, 2008). Cinnamon Essential Oil (CEO), a GRAS material, is amongst the most active EOs. Its antimicrobial-antioxidant potential has been indicated in earlier studies (Du and Li, 2008; Ojagh *et al.*, 2012a, b). Nevertheless, when applied to foods, effective antimicrobial concentrations of EOs may induce organoleptic alteration (Burt, 2004). Incorporation of EOs into edible films is one appealing approach because the decreased diffusion rate of EOs will reduce the effective dose required for *in vivo* test (Zinoviadou *et al.*, 2009).

Films made of polysaccharides or proteins are usually utilized to carry bioactive ingredients.

Specifically, several polysaccharides (carrageenan, chitosan, starch, cellulose, alginate, etc.) exhibit excellent film-forming abilities (Seol *et al.*, 2009; He *et al.*, 2014). Additionally, there are increasing applications of utilizing polysaccharide-based films for pork preservation. The shelf life of stewed-pork could be extended using chitosan coating loaded with 5% aqueous extract of onion, ginger and garlic, respectively (Cao *et al.*, 2013). Similarly, our previous study showed that chitosan coating enriched with clove oil could promote the microbial and sensory quality of pork slices (He *et al.*, 2014). In this case, edible films based on other polysaccharides need to be developed.

Carrageenans (CAs), derived from red seaweed, are galactose polymers consisting of a linear chain of sulphated galactans. Interestingly, Shojaee-Aliabadi *et al.* (2012) found that incorporating *Satureja hortensis* EO into CA films could contribute to their application due to the reduced affinity toward water and enhanced antimicrobial-antioxidant properties. However, the information on the preparation of EO-incorporated CA films is quite limited. Additionally, no reports exist on the application of CA-EO composite films for antimicrobial purposes in pork. Consequently, the current work aimed to:

- Develop a biodegradable film made from CA and CEO
- Determine the film's physical and antibacterial properties

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- Evaluate effectiveness of the films on total viable count of raw pork slices during cold storage
- Assess the effect of these films on membrane permeability of *Escherichia coli* and *Staphylococcus aureus*

MATERIALS AND METHODS

Bacterial strains: *E. coli* (CICC20729) and *S. aureus* (CICC23478) were purchased from China Center of Industrial Culture Collection and maintained refrigerated (4°C) on Luria-Bertani (LB) agar slants. The target bacteria were resuscitated by two successive transfers to LB agar for 24 h at 37°C. The resultant culture was grown in LB broth and incubated at 200 rpm/37°C for 24 h, yielding a population of ca. 10⁹ CFU/mL. In further study, the required viable population was obtained by dilution with sterile normal saline (SNS, 0.85%).

Materials: Food-grade CEO was procured from Yunfeng Fragrance and Flavor Co., Ltd. (Shanghai, China). Its predominant components were found to be E-Cinnamaldehyde (92.16%), eugenol (3.12%) and benzaldehyde (1.06%) in the preliminary test. κ-carrageenan was kindly provided by Shanghai Beilian Biological technology Co., Ltd., China. All other chemicals were obtained from Sinoreagent Co., Ltd. (Shanghai, China).

Preparation of CA-based films: The solution casting method was employed to prepare CA-based films (Seol *et al.*, 2009; Ojagh *et al.*, 2012a). κ-CA (2%, w/v) was dispersed in hot distilled water (90°C) with 1.5% (v/v) glycerol under constant stirring for 1 h. The solution was cooled to 60°C and tween 80 (0.2%, v/v) was added to distribute and incorporate CEO. Then CEO was added to meet desired concentrations of 0, 0.025, 0.05, 0.1, 1 and 2% (v/v), respectively. After 30 min of stirring, the mixtures were homogenized at 10,000 rpm for 2 min (FLUKO FA25, Shanghai, China) and 80 mL was casted on glass plates (20×20 cm) with a 3 cm spacer, uniformly spread and then dried at 40°C for 24-28 h. Dried films were then carefully removed and conditioned inside a desiccator at 25°C for 3 days over a saturated magnesium nitrate solution (53% relative humidity) prior to testing. In all, six kinds of films were prepared:

- CA+0% CEO (CA)
- CA+0.025% CEO (CA-C_{0.025})
- CA+0.05% CEO (CA-C_{0.05})
- CA+0.1% CEO (CA-C_{0.1})
- CA+1% CEO (CA-C₁)
- CA+2% CEO (CA-C₂)

Film thickness: The thickness was determined on three films per treatment with a digital micrometer (Su Zhou, China). At least five random measurements were

performed for each sample. The mean thickness (mm) was utilized for subsequent determination of opacity values.

Film opacity: A WFZ UV-2100 UV-VIS spectrophotometer (Shanghai, China) was used to evaluate the light barrier properties of films (Tunç and Duman, 2011). One rectangle cut strip (1.0×4.0 cm) was placed in a colorimetric cup to measure the absorbance at 600 nm (A₆₀₀). An empty spectrophotometer cell was used as the reference. Five film pieces were analyzed per treatment. The opacity values of the films were calculated as A₆₀₀/Film thickness.

Assessment of *in vitro* antibacterial activity of films: Antimicrobial properties of CA-based films were assessed via agar diffusion method (Seol *et al.*, 2009). Films were aseptically punched into circular discs (7 mm in diameter). An overnight culture (approximately 10⁹ CFU/mL) of target strains was spread on nutrient agar plates (100 μL/plate). Then the sample discs were placed on the center of the inoculated plates. After 24 h incubation at 37°C, inhibitory zones around the film pieces were measured. The diameter of the whole zone subtracted that of the film disc was reported as “inhibition zone diameter”. The measurements were conducted in triplicate.

Application of films to raw pork:

Treatment of pork samples: Lean pork pieces from the same cut of meat were procured from Haiwan retail market nearby and transported to our laboratory in ice. According to Ahmad *et al.* (2012), both sides of the slice were covered with films of the same type. The perimeters of the 2 films were attached closely. Subsequently, the treated slices were placed on polystyrene trays, sealing with polyethylene bags prior to 7 days’ storage at 4°C. The non-wrapped pork samples served as controls. It was observed that the films kept their integrity and were perfectly adhesive to the surface of the slice at the end of 4°C storage.

Determination of Total Viable Count (TVC): To determine TVC, pork sample (10 g) was homogenized (FLUKO Equipment Co. Ltd., Shanghai, China) with SNS (90 mL). Appropriately diluted homogenates (100 μL) were spread on solidified plate count agar. For TVC enumeration, plates were kept for 48 h at 28°C. TVC values (CFU/g) were log-transformed. Each treatment had three plates and the experiment was replicated twice.

Effect of films on the release of cell constituents: Permeability of cell membranes was checked based on Zhang *et al.* (2009) with modifications. The cultures grown overnight were washed and re-suspended in

SNS. Cell suspension (1×10^5 cells/mL) was incubated at 200 rpm/37°C for 1 h in the presence of films (CA, CA-C₂) or SNS (control), respectively. The absorbance of the mixture at 260 nm (A1) was measured after filtering through 0.22 µm. Bacteria suspension was also treated with Triton X-100 and monitored at 260 nm after 1-h incubation (A2). Leakage rate of cell constituents was calculated as $A1/A2 \times 100\%$. The measurements were conducted in triplicate.

Statistical analysis: The resulting data were evaluated by SAS version 8.0, via one-way ANOVA. Duncan's test ($p < 0.05$) was then applied to detect the differences between physical and antibacterial properties of CA-based films.

RESULTS AND DISCUSSION

Film thickness: The thickness is a critical value for edible film. For this reason, the influences of CEO on the thickness of CA-based films were initially evaluated (Table 1). Film thickness of all treatments ranged from 0.045 to 0.066 mm. Comparable thickness (0.031-0.068 mm) were observed by Shojae-Aliabadi *et al.* (2012) in films made from κ-CA and *Satureja hortensis* EO. In the scientific literature, however, a wide range of thickness has been reported in polysaccharide-based films. The processing parameters and film composition may explain the differences.

Film opacity: Low opacity (transparency) of films is of prime importance when utilized as packaging materials. As shown in Table 1, film transparency increased with the incorporation of CEO at low levels (0.025, 0.05 and 0.1%, respectively) but drastically decreased at high levels (1 and 2%). This phenomenon may be attributed to film thickness, light scattering provoked by oil droplets distributed in the CA matrix and selective absorption of some EO components (Sánchez-González *et al.*, 2011). Moreover, the opacity of CA-CEO composite films (2.24-3.35) in the current work was evidently lower than that of CA-*Satureja hortensis* EO films (4.12-7.35) reported by Shojae-Aliabadi *et al.* (2012). These data indicated that CEO-enriched CA films are clear enough to be utilized as packaging materials.

In vitro antibacterial activity of films: Effects of CEO incorporation on antibacterial activity of CA-based films are displayed in Table 2. When films are directly exposed to agar gel with a high moisture content, water molecules may interpenetrate into the hydrophilic CA polymer's matrix, thus resulting in swelling and ultimately leading to diffusion of CEO into the surroundings (Shojae-Aliabadi *et al.*, 2012). CA control film exhibited no inhibition against *E. coli* and *S. aureus*. The result agrees with the findings of Seol *et al.* (2009) and Shojae-Aliabadi *et al.* (2012).

Table 1: Thickness and opacity of CA-based films

Treatment	Thickness (mm)	Opacity
CA	0.051±0.0038 ^c	2.65±0.10 ^c
CA-C _{0.025}	0.047±0.0033 ^c	2.24±0.02 ^d
CA-C _{0.05}	0.045±0.0018 ^c	2.36±0.04 ^d
CA-C _{0.1}	0.045±0.0033 ^c	2.47±0.09 ^{ed}
CA-C ₁	0.059±0.0083 ^b	2.93±0.31 ^b
CA-C ₂	0.066±0.0043 ^a	3.35±0.12 ^a

Different lowercase letters within a column indicate significant differences ($p < 0.05$)

Table 2: Antibacterial effect (inhibition zone diameter, mm) of CA-based films against *E. coli* and *S. aureus*

Treatment	<i>E. coli</i>	<i>S. aureus</i>
CA	0.00±0.00 ^b	0.00±0.00 ^b
CA-C _{0.025}	0.00±0.00 ^b	0.00±0.00 ^b
CA-C _{0.05}	0.00±0.00 ^b	0.00±0.00 ^b
CA-C _{0.1}	0.00±0.00 ^b	0.00±0.00 ^b
CA-C ₁	0.00±0.00 ^b	0.00±0.00 ^b
CA-C ₂	1.59±0.38 ^a	12.64±0.90 ^a

Different lowercase letters within a column indicate significant differences ($p < 0.05$)

Films containing 0.025, 0.05, 0.1 and 1%, respectively of CEO were not effective against both target bacteria. Clear inhibition zones were only observed around the CA-C₂ film cuts. One possible reason is that CEO may be lost during film preparation and drying due to oil evaporation. Important aroma losses have been reported during film formation (Monedero *et al.*, 2010). On the other hand, chemical interactions between CA and CEO may hinder the release of CEO to inhibit the bacteria surrounding the film cuts. During agar diffusion test, loss of antibacterial effect has been found in chitosan-CEO films due to the compactness of film network (Hosseini *et al.*, 2009). Contrary to our observation, Shojae-Aliabadi *et al.* (2012) reported that κ-CA film containing only 1% of *Satureja hortensis* EO exhibited strong antibacterial effect against both *E. coli* and *S. aureus* in the direct contact test. This discrepancy may be due to the film composition, film processing parameters and variations in the concentration of tested bacteria.

In addition, CA-C₂ film was more active against *S. aureus* (Gram-positive) than *E. coli* (Gram-negative). This may be because the outer membrane outside *E. coli* cell wall restricted diffusion of hydrophobic CEO through its lipopolysaccharide covering (Burt, 2004).

Changes in TVC of pork slices: Variations in TVC of pork samples wrapped with and without CA-based films during 4°C storage are depicted in Fig. 1. Some microorganisms were present in pork slices at day 0 as indicated by the initial TVC value (4.50 log CFU/g). This may be possibly due to contamination during animal slaughtering, slice preparation, etc. At day 3, there was no profound difference between wrapped and control pork slices in TVC value. Hence, we assume that the concentration of CEO released from CA film

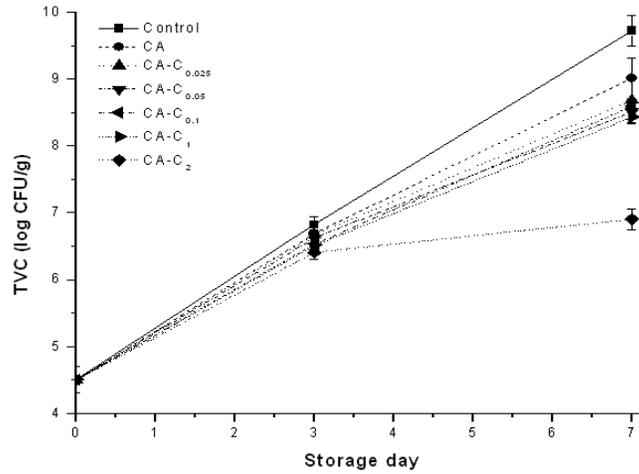
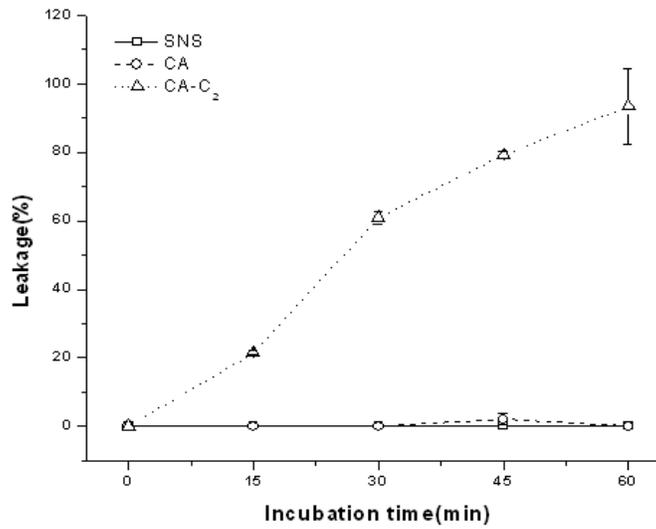
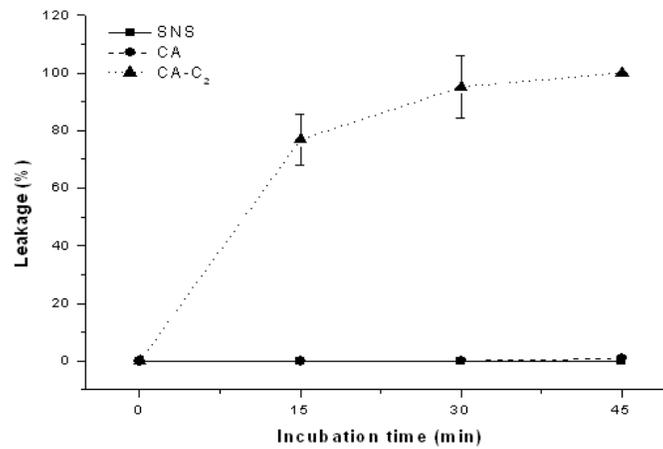


Fig. 1: TVC of raw pork wrapped with CA-based films during refrigerated storage (control: unwrapped samples)



(a)



(b)

Fig. 2: Leakage of 260 nm absorbing material from; (a): *E. coli*; (b): *S. aureus* treated with SNS and CA-based films (CA and CA-C₂)

matrix at day 3 may not be enough to effectively inhibit the growth of total microbes. At the end of refrigerated storage, however, the packaging treatments reduced TVC in pork by 0.71-2.82 log CFU/g, compared with the control slices. Antimicrobial activity of CEO-enriched films on pork slices was much more effective than that of pure CA film. Similar results were obtained in refrigerated rainbow trout (Ojagh *et al.*, 2010b) and sea bass slices (Ahmad *et al.*, 2012).

It is worth noting that the use of CA-C₂ film resulted in the highest log-reduction (2.82) in TVC. This phenomenon may be partly associated with elevated thickness of CA-C₂ film. In addition, the *in vivo* antimicrobial activity of CA-C₂ film was to be expected since it exhibited inhibitory effect against both target bacteria *in vitro*. These results implied that the release of active compounds was partly dependent on the initial level of CEO loaded in the film matrix. Many other factors, including water content of pork slices, interaction force between CEO and polymer matrix and hydrophobicity of the external environment, may also affect the migration of CEO to pork surface (Seol *et al.*, 2009).

Based on the antimicrobial activity (both *in vitro* and *in vivo*) of the films, CA and CA-C₂ films were selected for subsequent evaluation of film activity on the release of cell constituents.

Effects of CA and CA-C₂ films on membrane integrity of bacteria: The cytoplasmic cell membrane of bacteria is a target for many antimicrobial agents. If bacterial membrane is damaged, release of intracellular components (RNA, DNA, etc.) can be estimated by detection of 260-nm absorbance.

For *E. coli* (Fig. 2a), CA-C₂ film resulted in a constant leakage increase, reaching 93.4% in 60 min. When CA-C₂ film was added to *S. aureus* suspensions, leakage dramatically increased to 100% within 45 min (Fig. 2b). SNS and CA film did not cause release of cytoplasmic constituents in both target bacteria. Interestingly, these findings were in good agreement with those for *in vitro* antibacterial activity.

Because of the lack of information on the antibacterial mode of films containing EOs, our results were compared with those reported for free EO. The mechanism of antibacterial action of CEO in terms of cell membrane permeability has been reported by Oussalah *et al.* (2006). They found that Chinese cinnamon could destroy the membrane integrity of Gram-positive *Listeria monocytogenes* and Gram-negative *E. coli* O157:H7. Similarly, our results evidenced that CA-C₂ film induced the release of 260 nm absorbing materials in *E. coli* and *S. aureus*, which was quite similar to its raw material CEO. Pronounced antibacterial activity of CA-C₂ film may be potentially due to the hydrophobicity of CEO released from film that enables it to partition in the membrane lipids of

bacteria, destroy the structures and render the membrane more permeable (Burt, 2004).

CONCLUSION

CA-based antimicrobial active films could be achieved by CEO incorporation. CEO addition at levels of 1 and 2% led to an increase in film thickness and opacity. The CA-C₂ film exhibited the strongest inhibition effect against *E. coli* and *S. aureus* in agar diffusion test and against total microbes in pork during 4°C storage, possibly by damaging bacterial cell membranes. Hence, CA-C₂ film is the most recommended one to be utilized as a type of antimicrobial pork-packaging material. Nevertheless, further investigations are required to evaluate the mechanical, oxygen permeability and antioxidant properties of CA-CEO films.

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