



Antibacterial and Physical Properties of Composite Edible Film Containing Modified Lysozyme and Sodium Cyanoborohydrate

Abdul Manab^{1*}, Manik Eirry Sawitri¹,
Khotibul Umam Al Awwaly¹, Aris Sri Widati¹ and Yuny Susanti Haniyah²

¹Animal Product Technology Study Program of Animal Husbandry Faculty Brawijaya University, Jalan Veteran Malang 65145, East Java, Indonesia

²Agriculture and Animal Husbandry Polytechnic, MAPENA Tuban East Java, Indonesia

Abstract: The aim of this research were to find out the effect of sodium cyanoborohydrate addition on the release of modified lysozyme from composite edible film based on whey protein and porang flour. The research was the addition of sodium cyanoborohydrate on modified lysozyme aqueous (control ; 0.005%; 0.007% and 0.009%) using randomized block design, the variables were, the release of modified lysozyme, antibacterial of modified lysozyme on *Latobacillus bulgaricus* and *Escherichia coli*, water vapor permeability, protein solubility and microstructure of composite edible film. The results were the treatment didn't gave significantly effect ($P>0.05$) on release of modified lysozyme, antibacterial of modified lysozyme, water vapor permeability, protein solubility and microstructure of composite edible film. Increasing sodium cyanoborohydrate produced decreasing the release of modified lysozyme from composite edible film, however, the treatment produce stable antibacterial activity, water vapour permeability and protein solubility of composite edible film containing modified lysozyme and sodium cyanoborohydrate. Formation of more dense structure with increased sodium cyanoborohydrate content. It concluded that Sodium cyanoborohydrate contributed to controlled release rates of modified lysozyme from composite edible film by reducing film porosity. The film showed antimicrobial activity against *Latobacillus bulgaricus* and *Escherichia coli*.

Keywords: release, antibacterial activity, composite edible film, modified lysozyme, sodium cyanoborohydrate.

Introduction

Antimicrobial packaging is an alternative method to overcome the limitations of antimicrobial agent as food preservative, because antimicrobial agent is interacted in the complex food system and it cannot selectively target the food surface where spoilage reactions occur more intensively. ^[1] Antimicrobial packaging incorporating antimicrobials agent can retard the release of antimicrobials into the food, target mainly the food surface on which microbiological growth occurs, consequently improve safety of food while using minimum amounts of antimicrobial agents. ^[1, 10, 25]

The natural antimicrobial agents frequently employed in active packaging include antimicrobial enzymes, bacteriocins, essential oils and phenolic compounds. ^[1, 21, 28] Lysozyme from hen egg white is potential

for antimicrobial packaging since it has a GRAS status and it shows good stability and activity in edible films and food systems.^[29, 42] Lysozyme (1,4-beta-N-acetylmuramidase, 14.4 KDa), is a hydrophilic enzyme and widely used as a natural biopreservative for packaging applications.^[34] This enzyme shows antimicrobial activity mainly on Gram-positive bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in the cell walls, it does not show antibacterial activity against Gram-negative bacteria. The application of lysozyme in active packaging mainly inhibition of the critical Gram-positive pathogenic bacteria.^[11, 30] However, when lysozyme is combined with ethylenediaminetetraacetic acid (EDTA), the outer membranes of Gram-negative bacteria are destabilized by EDTA and the obtained lysozyme which show antibacterial activity against on pathogenic bacteria including *Escherichia coli* O157:H7 and *Salmonella typhimurium*.^[17, 29, 31, 42]

Sufficient antimicrobial effect could be achieved if the release rate of antimicrobial compounds from the packaging materials to food surface could be adjusted.^[18] Matrices with entrapped lysozyme showing good antimicrobial properties are sodium alginate,^[9] fish-skin gelatine^[6] and whey protein isolate.^[30]

Edible film derived from proteins and polysaccharides potentially replace some synthetic packaging materials used to preserve and protect foods.^[4, 36] On the other hand, polysaccharide-protein mixed systems are increasingly used in various food, pharmaceutical, and biotechnology applications. The protein-polysaccharide complexes could exhibit better functional properties than that of the proteins and polysaccharides alone,^[33] but most of them were not able to release the antimicrobial agent in a controlled way.

The release of modified lysozyme from edible film need controlling to maintain their antibacterial activity on edible film. Some chemical used in attachment of lysozyme, one of the ingredient is sodium cyanoborohydride which have ability to create a covalent attachmen between enzyme and polyethylene. Sodium cyanoborohydride stabilized attachment of modified lysozyme on edible film through the formation of the covalent interaction between modified lysozyme and edible film.

Experimental

Materials

The materials were hen egg white lysozyme extracts, SiO₂ (PT. Panadia Corporation Indonesia), sodium cyanoborohydride (Sigma), *Micrococcus lysodeikticus* (Sigma) aquadest, glacial acetic acid (PT. Panadia Corporation Indonesia), whey protein powder (Prostar), porang flour (PT. Perhutani), beeswax, culture of *E. coli* and *L. bulgaricus* (Medical Faculty University of Brawijaya), sodium phosphate buffer (Merck) and trichloroacetic acid (TCA) (Brataco). Some of the tools used in this research include glassware, analytical balance (Ohaus BC series and Mettler Instrumente type AJ150L Switzerland), hot plate stirrer (Labinco), vortex (Vibrofix VF, Electronic), pH meter (Schoot Gerate), sentrifuge (Jovan, Japan), waterbath (Mettmert Germany), oven (Mettmert Germany), sentrifuge (Bench top hettich centrifuge model microliter refrigerated micro centrifuge 22R), Scanning Electron Microscope (Hitachi 300 CE), UV-Vis spectrophotometer (Unico), Nano Drop spectrophotometer (ND-1000) and Hamilton syringe 10-1000 µl.

Lysozyme extraction

0.851 g SiO₂ dissolved in 1 M sodium phosphate buffer, 20 ml hen egg white was adjusted at pH 3 using 1 N acetic acid, stirred for 5 min, dissolved in 60 ml 0.5 M NH₄Cl. The solution dissolved in SiO₂ solution, stirred for 5 min, left over night at 4°C then stirred for 5 min, centrifuge at 6000 rpm, 4°C for 20 s.^[37]

Lysozyme modification

Lysozyme and EDTA at ratio 11.14:11.14 mg/mL prepared and homogenized for 5 min, heated at 50°C 20 min.^[2, 39]

Lysozyme- sodium cyanoborohydride solution

Modified lysozyme in 1/15M pH 6.8 potassium phosphate buffer containing 0.005, 0.007 and 0.009 % sodium cyanoborohydride. The solution homogenized for 5 min, cooled down until room temperature to be prepare with composite edible film.^[16, 2]

Preparation of Composite edible film whey protein porang flour containing lysozyme and sodium cyanoborohydride

Edible film solution containing 3 g/ml whey protein isolate and 3 g/ml porang flour was heated at 90°C and stirred at 250 rpm for 30 min, cooled down until 30°C. The solution of modified lysozyme and sodium cyanoborohydride was added to composite edible film solution, casted and dried at 40°C for 24 hours. Antibacterial of composite edible film containing modified lysozyme and sodium cyanoborohydride, the release of lysozyme, and the characteristic of composite edible film was analyzed. [27]

Antibacterial activity of composite edible films containing modified lysozyme and sodium cyanoborohydride

Antibacterial activity test on films was carried out using the agar diffusion method and the antibacterial effects of films containing modified lysozyme and sodium cyanoborohydride. *L. bulgaricus* and *E. coli* were carried out using zone of inhibition assay on solid media. The composite edible films were cut into a circle form with 7.1 mm diameter and placed on the surface of the solid media which had been inoculated with 0.1 ml culture, approximately containing 10^6 to 10^7 cfu/ml. The plates were then incubated at 37°C for 24 h, and after incubation time, the inhibiting zone was measured on the film discs. [31]

Lysozyme release assay

Substrate solution of lyophilized *M. lysodeikticus* suspensions was prepared in 0.15 M phosphate buffer solution (absorbance of 0.65 at 450 nm). 0.03 g of edible film was submerged in 20ml of 0.15M phosphate buffer pH 6.2 and shaken in a waterbath at time 0, 1, and 4 h. 1 μ L of each cell suspension shaken with film specimens was taken at time intervals of 0, 1, and 4, mixed with 2.5 mL of *M. lysodeikticus* substrate into a cuvette, and then immediately read for 40 s at 25 °C using a UV-Vis 2100 spectrophotometer. Activity rates of lysozyme were determined by measuring the decrease in solution absorbance at 450 nm, which reflects the hydrolysis of the cell wall substrate. [32]

$$\text{Release of lysozyme (U/minute)} = \frac{(\Delta 450 \text{ nm/ minute})}{(0,001/30,02 \mu\text{l})}$$

Protein Solubility

1 g of composite edible film put into a test tube, 5 ml of TCA was added then mixed with a vortex. The solution was incubated for 24 h at room temperature, centrifuged at 4000 rpm 30 min. Supernatant was measured by Nano Drop spectrophotometer to measure the value of protein solubility. [38]

Water Vapour Permeability (WVP)

WVP was determined at 25°C and 100%/50% RH gradient, following ASTM E 96 (ASTM 2000). 11 ml of distilled water was placed in each test cup with a 57-mm inside dia and a 15-mm inner depth. The distance between water and the film was 10.7 mm, and the effective film area was 25.5 cm². Test cup assemblies were placed in the environmental chamber (25°C and 50% RH). Each cup assembly was weighed every hour for 6 h using the electronic balance (0.0001 g accuracy) to record moisture loss over time. Water vapor permeability was then corrected for resistance of the stagnant air gap between the film and the surface of water using the WVP correction method WVP was calculated as follows: [14, 40]

$$\text{WVP} = \frac{\text{the weight of edible film (g)} \times \text{thickness of edible film (mm)}}{\text{the area expose of edible film (m}^2\text{)} \times \text{the time of gain (h)} \times \Delta P \text{ (KPa)}}$$

Microstructure of composite edible film

Composite edible film coated with a layer of metal powders in gold for 30 s. The surface and internal structure were observed using Scanning Electron Microscope (SEM) with voltage 15 kV and 5000 x magnification. [22]

Statistical analysis:

All data were analyzed using analysis of variance (ANOVA) and the LSD test to determine significant difference between the treatment. ^[44]

Results and Discussion

The Release of modified lysozyme from composite edible film

Table 1 showed that addition of sodium cyanoborohydride didn't give significantly effect ($P > 0.05$) on the release of modified lysozyme from composite edible film of whey protein and porang flour, increasing sodium cyanoborohydride content showed decreasing modified lysozyme release from composite edible film, addition of sodium cyanoborohydride at 0.005 (mg/ml) gave highest release of modified lysozyme. There is an indication that increasing sodium cyanoborohydride content produced more lysozyme entrapment and covalent interaction between modified lysozyme with matrix of composite edible film. Lower release of modified lysozyme from film at higher sodium cyanoborohydride is caused by the increase of dense layer and the decrease of pore size of film. ^[13]

N-terminal α -amines and lysine ϵ -amines from lactase were covalently attached to surface aldehyde groups via reductive amination. ^[19] The interaction of amine group of modified lysozyme and whey protein within matrix edible film induced morphological changes and reduced porosity of edible film caused slower release of modified lysozyme from composite edible film than those of control composite edible film.

At the time of diffusion of water molecules into composite edible film, whey protein and porang flour swell and expand, ^[32] so modified lysozyme released from film matrix. The release of modified lysozyme is expected to be able to work effectively when used for a long time in the composite edible film of whey protein and porang flour.

Table 1. The Release of modified lysozyme containing sodium cyanoborohydride from composite edible film

Lysozyme: Sodium Cyanoborohydride (mg/ml) Ratio	Release of Lysozyme (U/minute)
Control	40.04
0.005	45.44
0.007	32.20
0.009	37.09

Antibacterial of composite edible film

The addition of sodium cyanoborohydride on the edible film containing modified lysozyme didn't give significantly effect ($P > 0.05$) on the antibacterial activity of edible film- against *L. bulgaricus* and *E. coli*. The average results of the antibacterial edible film containing modified lysozyme in each treatment (Table 2) showed that addition sodium cyanoborohydride at 0.005 (mg/ml) gave higher antibacterial of edible film containing modified lysozyme than control and other treatments towards *L. bulgaricus*, but increasing sodium cyanoborohydride content on edible film containing modified lysozyme increase antibacterial activity towards *E. coli* but lower than control.

The are indication that lysozyme activity depends on the structure of the films. The change from porous to dense structure caused an increase in the amount of trapped and immobilized lysozyme in the films. This is an expected result since the porous surfaces of f the films gave higher soluble lysozyme activity during release tests. The porous surfaces of films may be attributed to protein aggregates formed in these films by lysozyme which may block the pores. ^[13]

Lysozyme to break the bond between N-acetylmuramic and N-acetylglucosamine acid in the cell walls of Gram positive bacteria. ^[31, 7] The cell wall of Gram positive bacteria have only one thick layer of

peptidoglycan. The structure of the cell walls of Gram positive bacteria including teichoic acid which is acidic polysaccharide containing deuteronomy chains of glycerol or ribitol. Lysozyme as antimicrobial agent degrades cell walls of all bacteria. [35]

Modified lysozyme have bactericidal activity against *E. coli* increases, [7] modified lysozyme enhanced antibacterial activity against Gram positive bacteria. [15]

Modification of hen egg white lysozyme using thermal method increased the antibacterial spectrum mainly on gram-negative bacteria especially *E. coli*, [39] is suspected due to polymerization of lysozyme. [23] Lysozyme oligomers interaction with the cell membrane of gram-negative bacteria resulting in increased surface hydrophobicity of lysozyme. [24] Increased surface hydrophobicity of egg white protein solution occurs above the temperature of 50°C. [41] Bacteriolytic lysozyme activity against gram-negative bacteria through the destruction of the function of the phosphate groups of phospholipids with lipopolysaccharide in the outer membrane of gram negative bacteria. [20]

The EDTA addition on lysozyme aims to antibacterial spectrum broadening of lysozyme on Gram negative bacteria. EDTA destabilizes the outer membrane of Gram negative bacteria by chelating Ca^{2+} and Mg^{2+} salts, as bridges between lipopolysaccharides (LPS) in the microbial outer membrane, resulting in the release of LPS from Gram negative bacteria. [43] Lysozyme catalyzes the hydrolysis of 1,4-glycosidic linkages between *N*-acetylmuramic and *N*-acetylglucosamine acid in cell wall peptidoglycan. Because the cell walls of Gram negative bacteria are protected by an outer membrane, Gram negative microorganisms are relatively resistant to the antimicrobial activity of lysozyme; thus, the application of lysozyme in foods has been limited. [15] The addition of EDTA to weaken the outer membrane before lysozyme treatment. Given that the presence of EDTA and Mg^{2+} inhibited NADH dehydrogenase activity, but that their absence may have resulted in poor cell breakage, we developed alternative methods of weakening the outer membrane to allow lysozyme access to the peptidoglycan layer. [26]

Table 2. Antibacterial of composite edible film containing modified lysozyme and sodium cyanoborohydride

Lysozyme: Sodium Cyanoborohydride (mg/ml) Ratio	Inhibition (mm)	
	<i>Lactobacillus bulgaricus</i>	<i>E. coli</i>
Control	32.70	34.73
0.005	34.35	32.39
0.007	32.61	33.72
0.009	32.00	34.25

Modification lysozyme using EDTA had antimicrobial properties to reduce the availability of cations and make bridges between lipopolysaccharides in the microbial outer membrane, increase spectrum broadening of lysozyme on Gram negative bacteria. [2] When EDTA and lysozyme are combined, bactericidal activity against *E. coli* increases. [7] EDTA destabilizes the outer membrane of Gram negative bacteria by chelating Ca^{2+} and Mg^{2+} salts, as bridges between Lipopolysaccharides (LPS) in the microbial outer membrane, resulting in the release of LPS from Gram negative bacteria. [43]

WVP of composite edible film

Table 3 showed that the addition of sodium cyanoborohydride on the edible film containing modified lysozyme didn't give significantly effect ($P > 0.05$) on WVP of composite edible film. Sodium cyanoborohydride addition on edible film containing modified lysozyme decrease WVP, this is due to with decreasing water vapor diffusion within film, This result was attributed with increasing interaction between modified lysozyme with film components, especially with whey protein.

Table 3. Physical properties of composite edible containing modified lysozyme and sodium cyanoborohydride

Lysozyme: Sodium Cyanoborohydride (mg/ml) Ratio	Water vapour permeability (g.mm/m ² .h.kPa)	Protein Solubility (mg/mL)
Control	0.00000118	0.4223
0.005	0.000000706	0.4074
0.007	0.000000839	0.3938
0.009	0.000000329	0.3915

These results suggest that a denser and tighter polymer network was achieved when modified lysozyme was incorporated into films covalently with sodium cyanoborohydride. when lysozyme was incorporated into whey protein in films, exhibiting lower wvp than the control films without sodium cyanoborohydride. It seem that sodium cyanoborohydride exerts a protective effect toward water vapour on the film. This protective effect due to the capacity of sodium cyanoborohydride to interact between modified lysozyme and film components together forming complexes. This result similar with previous research. ^[12]

Increasing degree of interaction between modified lysozyme and film by sodium cyanoborohydride, water vapour penetration into the film decreases. Increasing degree of crosslink of the polymeric matrix, the water penetration dept into the film decreases.^[8] Lower wvp of composite edible film containing modified lysozyme and sodium cyanoborohydride decreasing the release of modified lysozyme.

Protein Solubility of composite edible film

The results at Table 3 showed that the addition of sodium cyanoborohydride on the edible film containing modified lysozyme didn't gave significantly effect ($P>0.05$) on protein solubility of composite edible film. Increasing sodium cyanoborohydride content on edible film containing modified lysozyme decrease protein solubility of composite edible film. This can attributed to complete dissolution of modifies lysozyme and film component in composite edible film that result in homogenous distribution of suspended modified lysozyme aggregate and film component within the final film structure.

Lysozyme and film component complexes can be formed covalently using sodium cyanoborohydride. The association of these complexes which each other through a bridging mechanism which caused a change in particle and aggregate formed. After the individual complexes formation, some lysozyme cationic sites remained free at the surface of complexes and allowed to establish bridges between complexes. ^[5] Consequently, protein solubility of edible film containing modified lysozyme and sodium cyanoborohydride decreased. The addition of sodium cyanoborohydride on the edible film containing modified lysozyme decrease protein solubility, this supporting that modified lysozyme was covalently attached to the film. ^[16] Lower protein solubility of composite edible film containing modified lysozyme and sodium cyanoborohydride decreasing the release of modified lysozyme.

Microstrutures of composite edible film:

The SEM image of composite edible film were obtained to understand the morphological changes in film occurred by covalent interaction between modified lysozyme with film matrix using sodium cyanoborohydride. As seen in Fig 1 shows the outer surface (left) and cross section (right), Fig 1A showed composite edible film without sodium cyanoborohydride have higher porosity and gave loose film, the particles and aggregates formed small. Fig 1B, 1C and 1D showed big particles and agregates. Incorporation of sodium cyanoborohydride into composite edible film containing modified lysozyme reduced the porosity of films and gave denser films.

Thus, it appeared that the reduce release rates of modified lysozyme from composite edible film containing sodium cyanoborohydride are in part due to the entrapment or covalent interaction of modified lysozyme within matrix of composite film wichh increase the barriers against lysozyme diffusion, this result similar with previous research. ^[3] Formation of more dense structure with increased sodium cyanoborohydride

content in the film causes decrease in the release rate due to higher mass transfer resistance in the matrix film. It is expected that the influences of pore size and porosity of the matrix are more critical for the release of a large molecule such as lysozyme. ^[13]

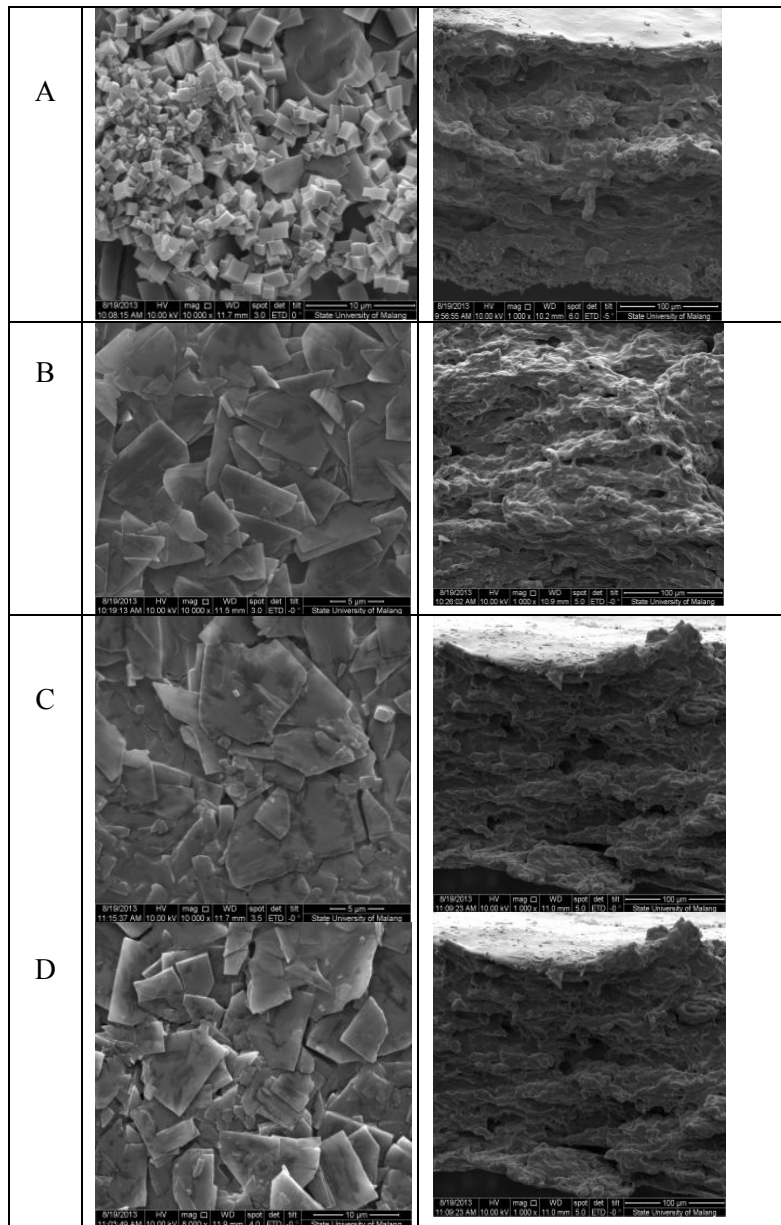


Figure 1. Microstructure of composite edible film containing modified lysozyme and sodium cyanoborohydride

Note : A. Control (No Sodium Cyanoborohydride), B: 0.005% Sodium Cyanoborohydride, C: 0.007% Sodium Cyanoborohydride, D: 0.009% Sodium Cyanoborohydride

Conclusion

Sodium cyanoborohydride contributed to controlled release rates of modified lysozyme from composite edible film by reducing film porosity. The film showed antimicrobial activity against *Latobacillus bulgaricus* and *Escherichia coli*.

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