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Research Article

The Release of Egg White Lysozyme Containing EDTA from Composite Edible Film Based on Whey Protein, Konjac Flour and Lipid

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Abstract: The objectives of this research were to find out the effect of EDTA addition on antibacterial spectrum broadening of lysozyme on Gram negative bacteria and the release of lysozyme from composite edible film made of whey protein, konjac glucomannan and several lipids type and content. The research were conducted with 2 steps. Step I: The addition of EDTA on lysozyme aquaeous (Lysozyme (mg/mL): EDTA (mg/mL) = 11.14:8.14; 11.14:11.14 and 11.14:14.14) using Randomyzed Block Design, the variables were, antibacterial of lysozyme on *Micrococcus lysodeikticus* and *Escherichia coli.* Step II: Lipid content (5 and 10%) and kind of lipid (butter, margarine, palm oil and beeswax) using nested Randomyzed Block Design, the variables were lysozyme release, Water Vapor Permeability (WVP), protein solublity and microstructure of composite edible film. The results were, step I: the treatment didn't gave significantly effect ($p>0.05$) on lysozyme activity. EDTA decrease cell membrane stabilization and lysozyme made lysis of cell membrane. EDTA chelate Ca^{2+} and Mg^{2+} salts as bridge between Lypopolysachcharide (LPS) in outer membrane so LPS released from cell wall of Gram negative bacteria. Step II: The treatment didn't gave significantly effect ($p>0.05$) on release of lysozyme and water vapour permeability, but gave significantly effect ($p<0.05$) on protein solubility. The release of lysozyme from composite edible film gave the best lysozyme release from beeswax 10% addition.

Keywords: Antibacterial activity, composite edible film, EDTA, lipid, lysozyme

INTRODUCTION

Antibacterial activity of lysozyme limited against Gram positive bacteria, thus requiring the addition of physical treatment to trigger a conformational change of enzyme molecules resulting in broader activities of bacteriostatic against Gram positive bacteria and Gram negative. Another treatment was the use of chemical compounds that are synergistic with lysozyme (Radziejewska *et al*., 2003). Treatment of chemical compounds that was synergistically on lysozyme with the addition of detergents and chelator that served as a destroyer of the membrane so that it work effectively on Gram negative bacteria. One of the ingredients of Ethylenediamine Tetraacetic Acid (EDTA) chelator which had properties via inhibition of the cation. Compounds EDTA destabilized the cell membranes of bacteria through the formation of the complex cation divalen that works as a bridge between salt membrane macromolecules, such as lipopolysaccharide, which was found in Gram negative bacteria.

Effect of lysozyme could be done by adding them on media restricting the growth of bacteria that was edible films. Based on studies of Waty (2007), edible film of whey protein powder with the addition of lipids could be lower WVP because the hydrophobic interaction between the substance and the emulsification between lipid emulsion with whey protein in edible film. Lipids had a characteristic of hydrophobic proteins that gave the nature of barrier against moisture. Based on this, it needs to do research on the diffusion control chicken egg white lysozyme by addition of EDTA which applicated in composite edible film. The role of lysozyme that have been added to EDTA as a detergent to the entry of bacteria in foodstuffs is expected to run well and can be known through test of lysozyme activity of *M. lysodeikticus* (Gram positive bacteria) and *E. coli* (Gram negative bacteria) with spectrophotometric method (Lee and Yang, 2002). According to Waty (2007), edible film with the addition of lipid to increase the value of hidrofobic on edible film that the lower WVP and protein solubility. Scanning Electron Microscopy was used to view the microstructures of composite edible film with the addition lipids and to know ability release of lysozyme on edible film. This study was aimed to investigate that the release of egg white lysozyme containing EDTA from composite edible film based on whey protein, konjac flour and lipid with synergistic effect on WVP, protein solubility and microstructures.

MATERIALS AND METHODS

Materials: The materials used in the study were hen egg white lysozyme extracts, SiO_2 (PT. Panadia

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Corporation Indonesia), EDTA (Merck), aquadest, glacial acetic acid (PT. Panadia Corporation Indonesia), whey protein powder (Prostar), konjac glucomannan flour (PT. Perhutani), several lipids are butter (Anchor), margarine (Blue Band), palm oil (Bimoli) and beeswax, culture of *E. coli* (culture by Medical Faculty University of Brawijaya), *M. lysodeikticus* cells (Sigma Chemical), 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 (Memmert Germany), oven (Memmert Germany), sentrifuge (Bench top hettich centrifuge model microliter refrigerated micro centrifuge 22R), spirometer, a ruler, 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 SiO_2) 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 NH_4Cl. The solution containing hen egg white dissolved in SiO_2 solution, stirred for 5 min, left over night at 4°C then stirred for 5 min, centrifuge at 6000 rpm, 4°C for 20 sec (Sharegi *et al*., 2012). Supernatant was heated at 40°C for 20 min at water bath. The supernatant containing lysozyme was analyzed (Sharegi *et al*., 2012).

Lysozyme-EDTA solution: Lysozyme and EDTA prepared according to the treatment and homogenized for 5 min, heated at 40°C for 20 min to be cool down a bit and ready to be analyzed.

Composite edible film whey protein konjac glucomannan making (Modified by Manab, 2008): Three g/mL protein whey and 3 g/mL konjac glucomannan flour was heated at 90°C in hot plate and stirred at 250 rpm for 30 min, cooled until 30°C. The solution containing lysozyme and EDTA was added to composite edible film solution, then casted and dried at 40°C. Lysozyme activity and release and the characteristic of composite edible film were analyzed.

Lysozyme activity (Lee and Yang, 2002): The activity of lysozyme was calculated from the slope of the time course by linear regression of data points. The A450 nm of a blank tube, i.e., cell suspension without lysozyme, usually does not change during the 7 min measurement course; when it decreased perceptibly, its slope value was subtracted from those of the experimental values. A unit of enzyme was defined as that quantity of enzyme that caused the decrease in absorbance of 0.001/min, under our set of specified conditions of pH 6.0 (0.67 M buffer phosphate) and 25°C. Results can be calculated:

$$\text{Lysozyme Activity (U/minute)} = \frac{(\Delta\ 450\ \text{nm/minute})}{(0{,}001/30{,}02\ \mu\text{L})}$$

Lysozyme release assay (Park *et al*., 2004): Lysozyme release assay was measured using the spectrophotometric turbidity assay. Samples of edible film weighed 0.03 g. Phosfat buffer solution prepared as many as 100 mL with a pH of 6.2. *M. lysodeikticus* suspensions prepared with absorbance 0.65 on A 450 nm. The sample was dissolved in a solution of edible film phosphate buffer and rocking in a waterbath at time 0, 1 and 4 h, respectively. Phosfat buffer inserted as much as 2.5 mL suspension and added the cuvet *M. lysodeikticus* as 2,98 μL. The sample solution was mixed with edible film suspension *M. lysodeikticus* as much as a 1 μL in kuvet and dihomogenkan. Measured by UV-Vis spectrometer with 450 nm for 40 sec. Note the number that appears on the A450 nm of 0.001/min recorded as 1 unit of enzyme activity (U):

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

Protein solubility (Silva, 2001): Protein Solubility was measured using the spectrophotometric turbidity assay. About 0.5-1 g samples of edible film put into test tubes, added 5 mL of TCA in test tubes and then mixed with a vortex. Solution of incubated the room temperature for 24 h, then solution centrifuged at 4000 rpm for 30 min. Supernatant was measured by Nano Drop spectrophotometer for knowing the value of the solubility of protein.

Water Vapour Permeability (WVP) Gennadios *et al*. (1994): WVP was determined using a cup method at 25°C and 100%/50% RH gradient, following ASTM E 96 (ASTM 2000). Distilled water was placed in each test cup with a 57 mm inside 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 cm^2. 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 (modified by Tanaka *et al*. (2001)):

WVP = The weight of *edible film* (g) × thickness of *edible film* (mm) /the area expose of *edible film* (m^2) × the time of gain (h) × ΔP (KPa)

Microstructure of composite edible film (Kusumo, 2011): The surface and internal structure of the films were evaluated using a Scanning Electrone Miscroscope (SEM). The sample was put very thin evenly on the plate aluminum has two sides, then the sample was coated with a layer of metal powders in gold with a time of coating 30 sec. The sample were observed using SEM with voltage 15 kV and 5000 x magnification.

Statistical analysis: All data were analyzed using Analysis of Variance (ANOVA) and the LSD test to determine significant difference between the treatment (Yitnosumarto, 1991).

RESULTS AND DISCUSSION

Effect of EDTA addition on lysozyme activity: The EDTA addition on the lysozyme didn't gave significantly effect ($p>0.05$) on the lysozyme activity against *M. lysodeikticus* and *E. coli.* The average results of the lysozyme activity in each treatment (Table 1) showed that influence of EDTA addition on lysozyme made no distinction on lysozyme activity towards *M. lysodeikticus* as the antimicrobial activity of lysozyme against Gram positive bacteria. Padgett *et al*. (1998) and Branen and Davidson (2004) lysozyme to break the bond between N-acetylmuramic and N-acetylglucosamine acid in the cell walls of Gram positive bacteria. As well as on the activity of lysozyme against *M. lysodeikticus* also provide didn't gave significantly effect ($p>0.05$) due to the EDTA addition will be sensitive to impair Gram negative bacteria. EDTA had antimicrobial properties to reduce the availability of cations and make bridges between lipopolysaccharides in the microbial outer membrane.

The results (Table 1) showed that increasing ratio of lysozyme: EDTA from 11.14:8.14 mg/mL to 11.14:14.14 mg/mL gave different lysozyme activity against *M. lysodeikticus* and *E. coli*. The highest lysozyme activity of *M. lysodeikticus* at 11.14: 11.14 mg/mL, because the lysozyme activity against *M. lysodeikticus* at equal ratio of lysozyme with EDTA was working effectively. Branen and Davidson (2004) claimed that when EDTA and lysozyme are combined, bactericidal activity against *E. coli* increases. Gill and Holley (2003) reported that even though lysozyme and EDTA enhanced antibacterial activity against Gram positive bacteria, their combination did not show antimicrobial activity against some Gram-negative bacteria.

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 teicoat acid which is acidic polysaccharide containing deuteronomy chains of glycerol or ribitol. According to Radziejewska *et al*. (2003) the lysozyme enzyme as antimicrobial degrades cell walls of all bacteria, H_2O_2 decomposition catalyze the way produce oxygen. This is the core of the process of hydrolysis to break down links. In contrast to the value of the highest of the lysozyme activity *E. coli* at 11.14:11.14 mg/mL, because the ratio of lysozyme activity against *E. coli* on the ratio of lysozyme with EDTA was working effectively. The EDTA addition on lysozyme antimicrobial agents due to inhibition of nutrients. The inhibition of these nutrients as a buffer system based on antimicrobial (Stevens *et al*., 1991, 1992; Cutter and Siragusa, 1995a, b; Kalchayanan *et al*., 1992). Buffer system is a resulting condition by inhibition of nutritional needs that prevent the improvement of cells. In addition, the cell are log or stationary phase so as to antibacterials in food products.

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 (Vaara, 1992). Lysozyme catalyzes the hydrolysis of 1,4-glycosidic linkages between *N*-acetylmuramic acid 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 (Gill and Holley, 2003). The addition of EDTA to weaken the outer membrane before lysozyme treatment. Given that the presence of EDTA and M_g^{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 (Madden *et al*., 2004).

The release of lysozyme from composite edible film made of whey protein, konjac glucomannan and several lipids type and content:

Release of lysozyme: Table 2 showed that used the addition of lipid type and content didn't gave significantly effect ($p>0.05$) on the release of lysozyme

Table 1: Effect of lysozyme activity (U/minute) on *M. lysodeikticus* and *E. coli*

Lysozyme: EDTA (mg/mL) Ratio	Lysozyme activity accumulation (U/min)	
	M. lysodeikticus	*E. coli*
Control	45504688.16	389827113.63
11.14: 8.14	27562416.29	290258800.94
11.14: 11.14	32618083.95	235894640.88
11.14: 14.14	31365462.35	234894041.50

Table 2: The release of egg white lysozyme containing EDTA from composite edible film based on whey protein, konjac flour and lipid

Lipid type	Lipid content (%)	Release of lysozyme (U/min)
Control	0	45.6388
Margarine	5	36.5960
	10	34.9460
Butter	5	44.8434
	10	33.0611
Palm oil	5	46.0687
	10	39.7802
Beeswax	5	35.4145
	10	27.0755

Table 3: Characteristics of composite edible film whey protein and konjac glucomannan flour using lipids

Lipid type	Lipid content (%)	WVP ($g.mm/m^2.h.kPa$)	Protein solubility (mg/mL)
Control	0	3.5829	0.0667
Margarine	5	4.9199	0.5667
	10	4.6755	0.0833
Butter	5	3.2483	0.0733
	10	4.6983	0.0667
Palm oil	5	5.3166	0.1000
	10	3.8389	0.0900
Beeswax	5	5.2652	0.0700
	10	2.7401	0.0900

from composite edible film of whey protein and konjac glucomannan flour. The high value of the release of lysozyme was the addition of palm oil was suspected because of hydrophobic interactions and emulsification solution of edible film. That wasn't optimum, so that edible film formed become brittle. Treatment of this lipid type and content is an important element for release of lysozyme, thus achieving a measure of lysozyme control as antibacterial effect in the face of the fluctuations that do not necessarily.

At the time of the release of lysozyme on composite edible film of whey protein and konjac glucomannan flour expands due to the diffusion of water molecules that fit into the structure of the polymer film. Slowly release occurred on the lysozyme then enters in a more dilute so out of the matrix film (Park *et al*., 2004). The release of lysozyme is expected to be able to work effectively when used for a long time in the composite edible film of whey protein and konjac glucomannan flour.

Composite edible film which had composition 20% konjac glucomannan flour, so the glucomannan content was also influential in the release of lysozyme. Based on the research of Fan *et al*. (2008) that a mixture of konjac glucomannan and dispersal in the matrix gum shows high potential for resistance on stability gel phases by the presence of intermolekuler hydrogen bond network between two polymer to slow diffusion of film.

WVP of composite edible film: Table 3 showed that the addition of lipid type and content didn't gave significantly effect ($p>0.05$) on composite edible film of whey protein and konjac glucomannan flour on WVP because of lipid controlling the transport of moisture in edible film. According to Tanaka *et al*. (2001) that the double bond in unsaturated fatty acid molecules can reduce the thickness of the structure of the composition edible films. Decrease in WVP with the addition of lipid controls the moisture transport, caused the increased mileage water molecules absorbed on edible film surface to prevent the occurrence of evaporation of food products which are coated. According to McHugh *et al*. (1994a) and Tanaka *et al*. (2001) that the growing range of water molecules that diffuse through the edible films lead the WVP decline with increase of lipid emulsion on edible films.

The presence of unsaturated long-chain fatty acids in edible films solution can lose WVP because hidrofobicity of the composition of fatty acids regulate WVP in edible film so as to increase the mobility of the edible film structure. According to McHugh *et al*. (1994b) and Rhim *et al*. (1999), WVP of edible films are expected to decline with the rise of long chain hydrocarbons from oil palm, due to hidrofobicity of fatty acids regulate directly Water Vapor Transmition on fatty acid composition of edible films and long-chain fatty acids have a great mobility in forming the structure of the composition of the edible films. According to Perez-Gago and Krochta (2000) and Kester and Fennema (1989) that the addition of hydrophobic group into hydrophilic protein group in edible film that is by forming a lipid emulsion stability edible film of lipids was able to improve the capabilities in blocking the onset of evaporation of water.

The content used 5 and 10% margarine, butter, palm oil and beeswax were likely to decline the WVP (Table 3). The results indicate that the addition of lipid on composite edible film controls the moisture transport, so the value of the WVP declined by the interaction between hydrophobic group proteins and lipid. The high value of the WVP showed that edible film easily penetrated by water vapour, if the value of edible film shows low WVP hard penetrated moisture. Low values indicate that the hydrophobic group WVP in beeswax work well so as to impede the moisture movement in edible film. According to Tanaka *et al*. (2001) that the ability of edible film prevents the evaporation of water can be enhanced by adding the component lipids such as neutral lipid, fatty acids or night (wax).

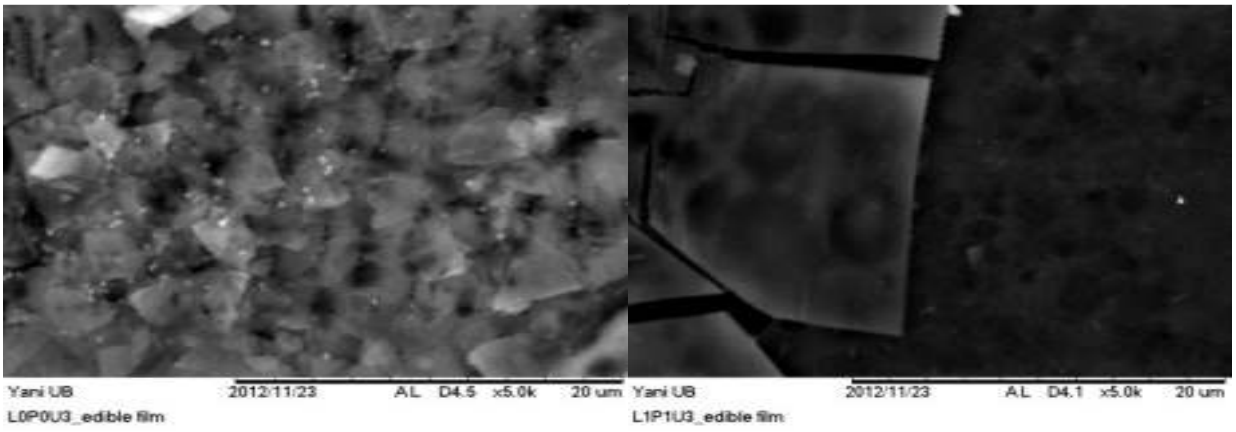
The addition of 5% palm oil has the highest value because the magnitude volume of the CH_2 group owned by palm oil causing edible films generated more easily traversed by water vapor, so that the value of the WVP increases. This is in accordance with the opinion of the Prodpran *et al*. (2005) that the group of aliphatic liquid bonding CH_2 has a large volume when compared to the crystal form (dense) so easily traversed water vapor.

Protein solubility: The results showed that lipid type and content gave significantly effect ($p<0.05$) on the

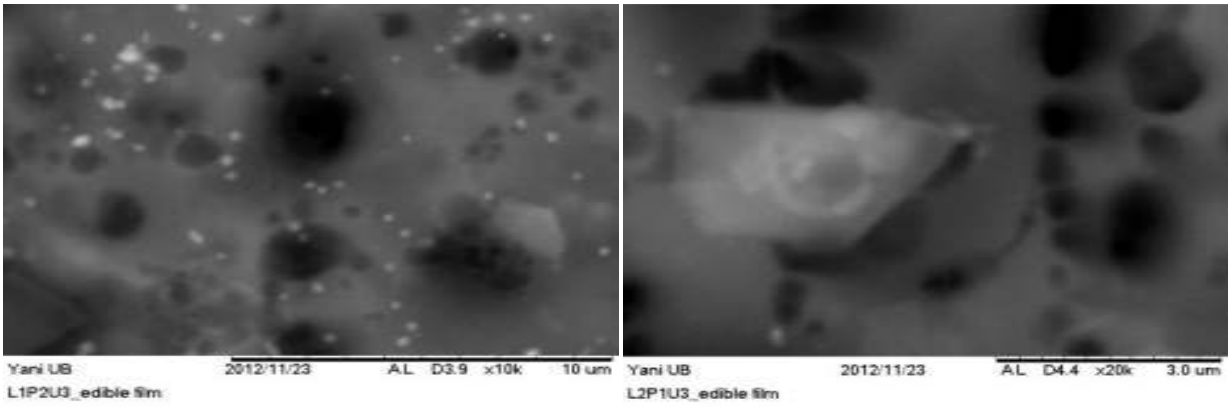
protein solubility of edible film, this means that lipids type and content gave a real influence, because all lipid type and increasing lipid content will increase hydrophobic interaction between hydrophobic group of whey protein and lipid. Table 2 showed that the addition of 5 and 10% margarine, butter, palm oil and beeswax to indicate a decrease in protein solubility value. The highest protein solubility value in the addition of 5% margarine and the lowest value in the addition of 10% butter. The addition of butter because of the increasing content of saturated fatty acids it will be increasingly difficult soluble in water. According to Prodpran *et al*. (2005) that the decrease on protein solubility due to increased lipid used. Hydrophobic lipids to disperse proteins in a very polar, so as to lower the protein solubility.

In line with the decrease in protein solubility occurs by increasing the addition of lipid and improving hidrofobicity of edible film, so that it can reduce the loss of protein due to dissolved in the water. Composite edible film of whey protein and konjac glucomannan without the addition of lipid has a value that is too low. This is due to the finite groups with long-term treatment hidrofil and hydrophobic molecules of protein that is hydrophobic cluster after denaturation on the outside. The addition of lipid which has properties of hydrophobic molecules will increase hidrofobitas in edible film of whey protein and konjac glucomannan, it is in accordance with the opinion of Kim and Ustunol (2001) that the emulsion of whey protein with lipids in edible film will increase hidrofobicity.

According to Tanaka *et al*. (2001) that the low point of the liquid from the lipid produced edible film fragile, less well as a barrier against humidity this is caused due to the increased content of unsaturated fatty acids in solution edible films. According to Prodpran *et al*. (2005) that the hydrophobic lipid is dispersed protein in a highly polar, so as to lower the solubility of the protein. Edible films added palm oil showed decreasing protein solubility lower than using butter or shortening is white. The existence of cross-ties from ties that variations of hydrogen bonds, hydrophobic interactions, can prevent the loss of disulfide bonds in proteins from edible film. According to Kim and Ustunol (2001) that in emulsion film of edible protein whey with the addition of lipids can increase nature hidrofobicity.



(a) (b)



(c) (d)

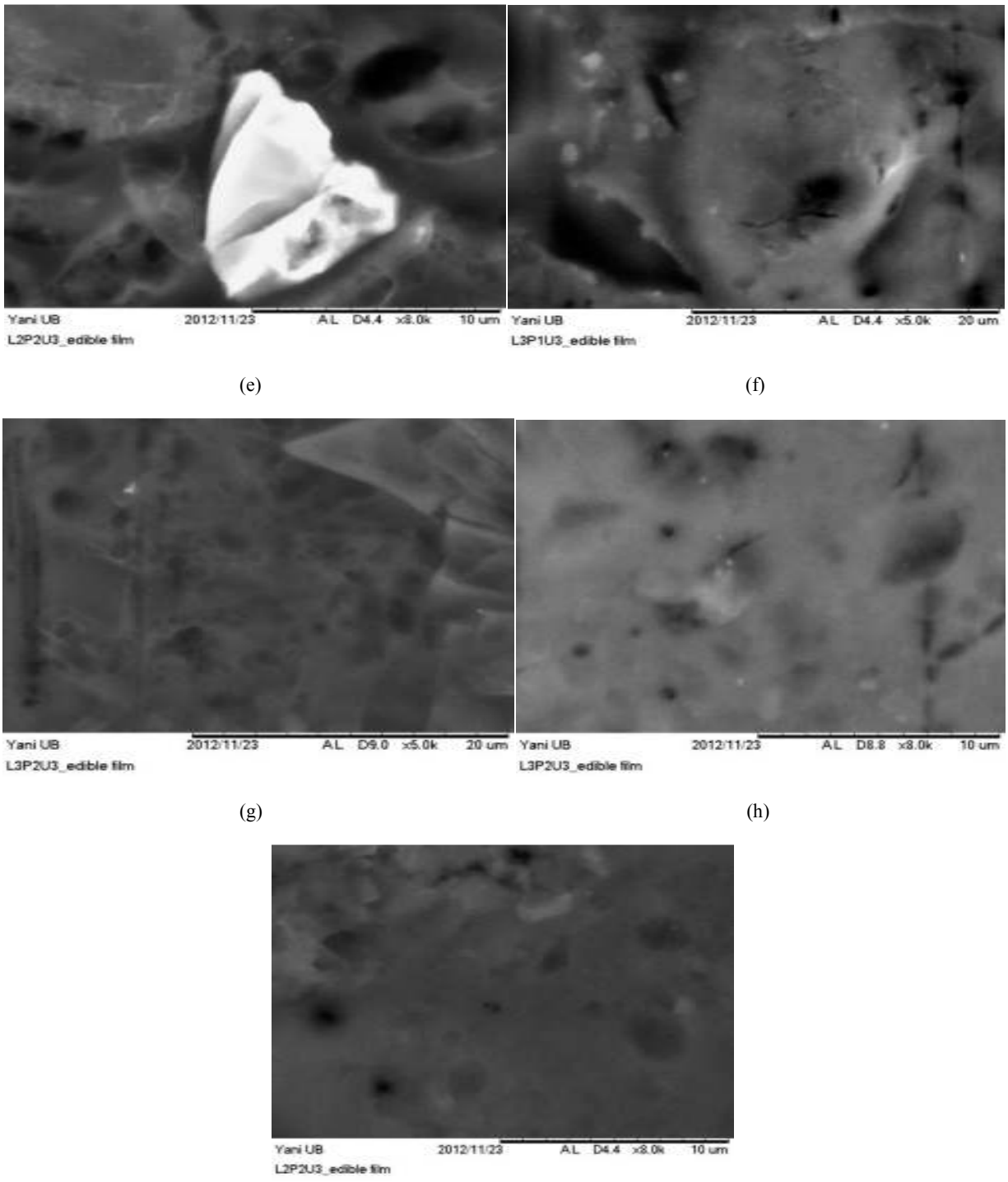


Fig. 1: Microstructure of composite edible film of whey protein and konjac glucomannan powder
a: Control (no added lipid); b: 5% margarine; c: 10% margarine; d: 5% palm oil; e: 10% palm oil; f: 5% butter; g: butter 10%; h: 5% *Beeswax*; i: 10% *Beeswax*

Microstrutures of composite edible film: Figure 1 shown the outer surface exposed to air during formation and cross section at a magnifation of 5000-10000x. Microstructures in Fig.1a to i were varies by the heteregeneous structures and rough surfaces observed in all the emulsion films studie. The presence ruggedness and pores on film was caused by the formation of protein gel complex from whey protein

and polysaccharide of konjac glucomannan starch. Figure 1a to g shown increased surfaced irregularity because the increasing of lipids content caused vapour form the gel, but in Fig. 1i the addition of 10% beeswax look a lot different microstructures, it was compact and not much hollow.

Figure 1d shown surface microstructures look flat, solid and not hollow with the addition of 10% palm oil. According to Bravin *et al*. (2004) that microstructures of edible film with the addition of palm oil showed the structure of a thin layer. Ruggedness and pores on the edible film caused by the formation of proteins gel complex depends on the pH. Isoelectric pH closer to the complex dissolved so that a gel was formed producing woven structure. pH 5.2 on edible film was isoelectric pH of whey protein that causes the gel formation. The addition of 10% palm oil shown surface were droplets spread because it had the best WVP and protein solubility as a barrier moisture and was easily dissolve in water. Oil droplets clearly visible on the surface with the ever increasing the addition of palm oil. According to Prodpran *et al*. (2005) that the oil droplets on the surface of ordinary edible film linked with decreased WVP on film with ever increasing the addition of palm oil.

The Microstructures of edible films (Fig. 1i) shown the structure was smal holes. The more the hole or the size tend to increase the value of the WVP because the addition of 10% beeswax caused the release of lysozyme was slow and the WVP and protein solubility was low.

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