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ENCAPSULATION OF INDONESIAN PROPOLIS BY CASEIN MICELLE

MUHAMAD SAHLAN* AND TONY SUPARDI

Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Jl. Prof. Somantri Brojonegoro, Kampus UI, Beji, Depok 16424, Indonesia

ABSTRACT

Propolis is strong adhesive material with one of most valuable bees product and has wide range of biological activities such as antibacterials, antiviruses, antifungals, anti-inflammation, photoprotector and so on. Propolis has been aplied as antimicrobials agent in food and healtcare products. To improve handling properties, Indonesian propolis were encapsulated by casein micelle with a homogenizer followed a sonication, and separated by micro- and ultra-filtration system, created micro- and nano- particles. These micro- and nano-particles exhibited high flavonoids and moderate polyphenols capacities (encapsulations efficiency, 94% and 67% for flovonoids and polyphenols, respectively) Size of particles was analyzed by particle size analyzer (PSA) showed that the average size of particles are 1,3 micrometer and 300 nanometer. The morphologies of particles analyzed by transmission electron microscopy (TEM). The results also shown that encapsulated Indonesian propolis have antibacterial activity. Therefore, these micro- and nano-propolis might be used as antimicrobials agent or others in food or healtcare products.

KEYWORDS: Propolis, casein micelle, encapsulation, micro- and nano-propolis

Corresponding author

MUHAMAD SAHLAN
Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Jl. Prof. Somantri Brojonegoro, Kampus UI, Beji, Depok 16424, Indonesia

INTRODUCTION

Propolis (bee glue) is dark sticky, strongly adhesive compounds with one of the most valuable and enigmatic by product [1]. The bee combined propolis with beeswax and used by bee worker to seal and sterilize the colony nest. It has been shown that propolis has wide range of biological activities due to different propolis constituents, such as antimicrobials, antiviral, anti-inflammatory, antitumor, antioxidant and photoprotector etc [2; 3; 4; 5; 6; 7]. Propolis from different region is chemically diverse, but in general consists of polyphenol (flovonoids, phenolic acids, esters and aldehydes, alcohols and ketones), terpenoids, steroids, amino acids, and various inorganic compounds [8; 9; 10]. Propolis is found in market with many different forms such as capsule, tablets, liquid drop, and mouthwash, toothpaste, mixing with honey, face creams and ointments [11; 12]. The medical applications of propolis led to an increased interest in its chemical composition as well as its origin. Furthermore, ethanol extract of propolis (EP), alone or incorporated in another dosage is commonly utilized as therapeutics. However, the high ethanol concentrations, unpleasant odor and difficult to form in solid are disadvantage of EP, resulting in difficulties on the administration and also the patient compliance to the therapeutics is committed too [13]. Cow milk contains 0.30-0.35 % w/v of protein. 80% of milk protein is casein which organized in micelles [14]. Casein micelles are fractionated in nature to concentrate, stabilize and transport essential nutrients, mainly calcium and protein, which mean casein micelle is natural nano-delivery system [15]. Several studies indicated that casein micelle able to encapsulate several compounds such as vitamin D2 and curcumin [16]. Thus, the aim of study was to prepare micro/nano- particle containing Indonesian propolis with encapsulation the propolis with casein micelle.

MATERIALS AND METHODS

Cow milk was obtained commercially from market store around University of Indonesia, Indonesia. Beehives were obtained from local honey based company, Cibubur, Jakarta, Indonesia.

(i) Preparation and characterization of Propolis

Propolis was extracted from beehive isolated from Indonesia by Hamada et al., method (Hamada et al., 1996) with slight modification. Weight 130 g beehive extracted with 1 litre ethanol for 16 hours, filtered and removes insoluble material. To separate propolis with wax, the extract added by water until 50, 60, 65, 70 and 80% ethanol water v/v, incubated on water bath 50^{O}C for 30 mins. Than, the solution freezed on refrigerator overnight. Incubate in room temperature until propolis separated with wax clearly; separate the wax and propolis by filtration. Separation degree between propolis and wax was analyzed by Kuno Kazuya Method (Kuno, Kazuya, 1987). The concentration of polyphenol and flavonoid of the propolis analysed by Folin-phenol Ciocalteu and aluminum chloride methods, respectively.

(ii) Preparation and characterization of the casein

The cow milk purchased from market store adjusts pH until 6.4 using hydrochloride acid 1 N, incubated at 30^{O}C for 1 hour. Added rennet with agitation for 15 mins at 30^{O}C, the casein will aggregate. To increase size of particle the aliquot incubated in same temperature for 15 mines. The casein and others separated by filtration. The remained rennet inactivated by hot water (70 ^{O}C) for 5 mins and separated casein and water by filtration. The molecular weight of casein analyzed by 12 % SDS PAGE.

(iii) Preparation of micro- and nana-particle Propolis

5 g casein diluted in 50 mL 10 mM phosphate buffer pH 10. While stirred add 5 mL propolis (in ethanol). The mixture added 1 mL 10% $CaCl_2$ six times every 5 mins. The pH of mixture adjusted in pH 7 with 0.1 N HCl or 0.1 N NaOH. The mixture also sonicated for 5

mins. The mixture separated by microfiltration (Whatmann paper No. 42). Ultrafiltrate the permate by 10 kDa cut off. The retentante of micro and ultra filtration were diluted by phosphate buffer. The permeate of ultrafiltration contain unencapsulated propolis, and then measured the total of polyphenols and total flavonoids.

(iv) Total polyphenols or flavonoids encapsulated

The efficiency of encapsulation process was calculated as follow:

$$Encapsultion\ efficiency = \frac{A - B}{A} \times 100\%$$

$A = Total\ polyphenols\ or\ flavonoids\ added\ initially$

$B = Total\ unencapsulated\ polyphenols\ or\ flavonoids$

(v) Particle Size analysis and microstructure study

The nano/micro particles sizes were determined using laser light scattering granularity analyzer (Delsa^{tm} Nano, Beckman Coulter). The sample was analyzed in triplicate. The microstructure of particles was observed using transmission electron microscopy (TEM) operated at 80 kV. The sample prepared by placing one preparation drop on a collodion support on grids.

(vi) High Performance Liquid Chromatography

The sample propolis before and after encapsulation processes was analyzed qualitatively by high performance liquid chromatography HPLC.

(vii) ***Antibacterial assay***

Antibacterial activity analyzed by Kirby-Bauer methods with chloramphenicol was the standard. The bacterial used in this study were *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*.

(viii) Others.

Total polyphenol analyzed by Folin-phenol Ciocalteu method with galic acid as standard. Total flavonoid measured by alumunium chloride method with quercetin as standard. Concentration of protein was analyzed by Lowry method with Bovine serum albumin as standard. Molecular weight of protein was analyzed by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS AND DISCUSSIONS

(i) Properties and composition of Indonesian propolis

Propolis from Indonesia was isolated by Hamada et al., method (Hamada et. al., 1996). The propolis extracted by ethanol, the wax separated from the extract by added water until the concentration of ethanol 40, 50, 70 and 80% ethanol: water v/v followed by heating and freezed for overnight than decanted or filtration. The separation degree between propolis and beeswax were analyzed by Kuno kazuya method (Kuno, Kazuya, 1987). The separation degree between propolis and wax is calculated by dividing the maximum peak in UV absorbance that represent to amount of bioactives and absorbance at 660 nm that represent to the wax (Fig. 1). The result shown that the separation degree of 70% ethanol: water is the highest compare with others (see Table 1). Thus, the propolis used in this study was extracted by 70% ethanol: water. the extract contain 200 µg/ml and 370 µg/ml of polyphenols and flavonoids, respectively. These results showed that the Kuno kazuyo method is the simplest method to identify quality of raw material of propolis.

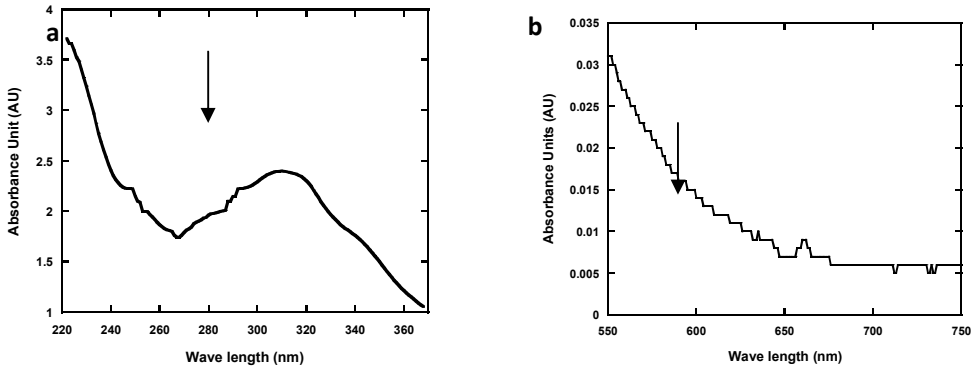

Figure 1

UV-Vis Spectrum of Indonesian propolis. (a) UV spectrum scanning of Indonesia propolis. (b)

Visible spectrum of Indonesia propolis, absorbance at 660 nm corresponding to the wax.

Table 1

The separation degree between propolis and wax obtained by Kuno Kazuyo Method.

Propolis in % Ethanol	Dilution Factor (DF)	Absorbance (λ = 310 nm)	Absorbance (λ = 310 nm) × DF	Absorbance (λ = 660 nm)	Separation Degree ($\frac{(A\,\lambda 310)}{(A\,\lambda 660)}$)
40	10	0.588	5.880	0.022	267
60	10	0.917	9.170	0.005	1834
65	10	1.387	13.870	0.008	1734
70	10	1.441	14.410	0.007	2059
80	10	1.515	15.150	0.009	1638
96	10	1.614	16.140	0.011	1467

(ii) Isolation of Cow Milk Casein

About 80% of cow milk proteins is casein. Casein found in milk as a suspension of particles called casein micelle that shows hidrophilic parts reside at the surface. Casein in the micelle are held together by calcium ion and hydrophobic interactions. Caseins in milk containing several kinds of casein such as alpha (s-1), alpha (s-2), beta and kappa casein [17]. Although the casein micelle is fairly stable, the rennet containing cyhmosin could induce casein aggregation [18]. In this study, Rennet used to separated casein from the others. The extracted casein was analysed by 15 % SDS PAGE, the result shown that all kinds of casein extracted by this method, all bands appear ranging from 19 to 29 kDa (Fig. 2) represent to all kinds of casein.

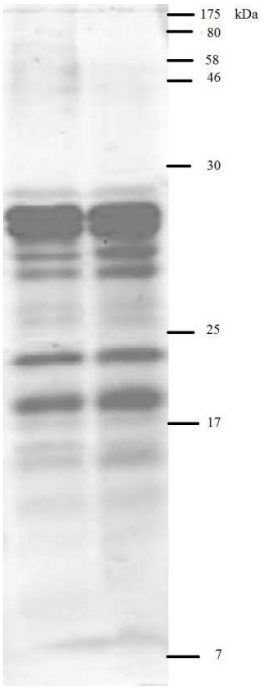

Figure 2
Image of 12% SDS PAGE of the extracted casein.

(iii) Encapsulation Process

Isolated casein micelles were redispersed as a suspension in phosphate buffer (pH 6.4) that containing 10 mM $CaCl_2$. Ion calcium are necessary for a stable micelle suspension [19]. After homogenization process, the sample were sonicated (25 KHz), keep cool the sample with ice. We suggested that the sample was treated by sonication could increase the efficiency of encapsulation and also produced nanoparticle. The efficiency of the encapsulation process determined by made comparison between total polyphenols and flavonoids before and after encapsulation process. To separate micro-/nano- propolis particle with unencapsulated propolis in the end step of process, the sample was separated by ultrafiltrasion 10 kDa cut off, the permeate of the ultrafiltration step was the solution containing unencapsulated propolis and measured the total polyphenols and flavonoids, then compare with before encapsulation process. The result shows that casein micelle encapsulate 94% flavonoids compounds more effective compare with polyphenol compounds that only 67% of it was encapsulated by casein micelle (see table 2). Based on basic structure of flavonoid more hidrophobic compare polyphenols, which have many hidroxies regions. These data further support the possibility that the casein micelle was bound to the hydrophobic regions of casein micelle, which are located in the submicelles.

Table 2
Efficiency of encapsulation process

	Total Polyphenols	Total Flavonoids
Before Encapsulation	954.3 µg	1846.1 µg
Unencapsulated sample	314.5 µg	112.6 µg
Encapsulation Efficiency	67 %	94%

We also analysis the samples qualitatively by using high pressure liquid chromatography (see fig. 3), many peaks found in propolis sampel before encapsulation process, after encapsulation many peaks were disappear. The peak in retention time 2.94 and 4.92 mins totally dissapear that shows that those componds encapsulated by casein completely, otherwise the peak in retention time 3.35 min is still remain and the peak in retention time 5.57 min, the absorbance of it sligthly decrease. It is suggested that the compounds in retention time of 2.94 and 4.92 mins are flavonoids and compounds in 3.35 and 5.57 min are other polyphenols. Flavonoids such as galangin, chrysin, and pinocembrin commonly detected in propolis have the capacity to induce oxidative DNA damage, hepatoprotective effects, anti-inflammation, anti-cytotoxic and also protect keratinocytes from ultraviolet A and B spectrum and so on. These results also shown the posibility of new biological activities of propolis, the activities caused by flavonoids will be strongest compare with unencapsulated propolis.

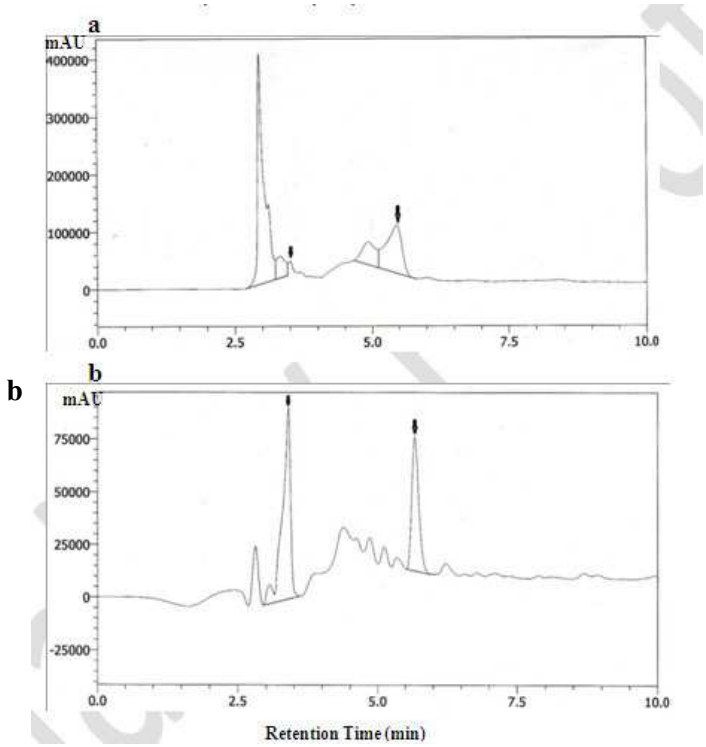

Figure 3

Indonesia propolis measured by HPLC, a) and b) refer to before and after encapsulation, respectively. (a) Before the encapsulation process, Indonesia propolis was analysed by HPLC. (b) The unencapsulated Indonesia propolis was analysed by HPLC. The arrow showed the retention time of 3.35 and 4.92 min.

(iv) Micro- and nano-particles size distribution and microstructure study

In the process, the encapsulated propolis separated by two step filtrations, microfiltration and ultrafiltration (10 kDa cut off), the retentate of microfiltration and ultrafiltration determined and produced micro- and nanoparticle, respectively. The means of micro- and nanoparticles determined as 1,3 µm and 320 nm as shown in fig. 4. The microparticle size varied from 1 µm to 1.6 µm. Whereas, the nanoparticle size varied from 252 nm to 530 nm. TEM micrographs showed rod for microparticel and

Retention Time (min)

trapezium for nanoparticle with a diameter of around 1 µm and 300 nm, respectively fig. 5. Production of nanoparticle bound casein micelle usually used high pressure homogenizer, the results shown that sonicated of casein micelle bound propolis sample also to be one method alternative to produce micro- and nano-particles. Therefore, propolis, which is poorly soluble in water, can be formulated in a colloidal system by the use of casein micelle. Furthermore, to compare antibacterials of free and casein micelle-bound propolis, we applied three kinds of bacterials to a series of equivalent concentration of free propolis or the casein micelle propolis complex with Kirby-Bauer method. the result shown that free and casein micelle bound propolis have similar antibacterial activity for *B. subtilis, M. luteus* and *S. aureus* (data not shown).

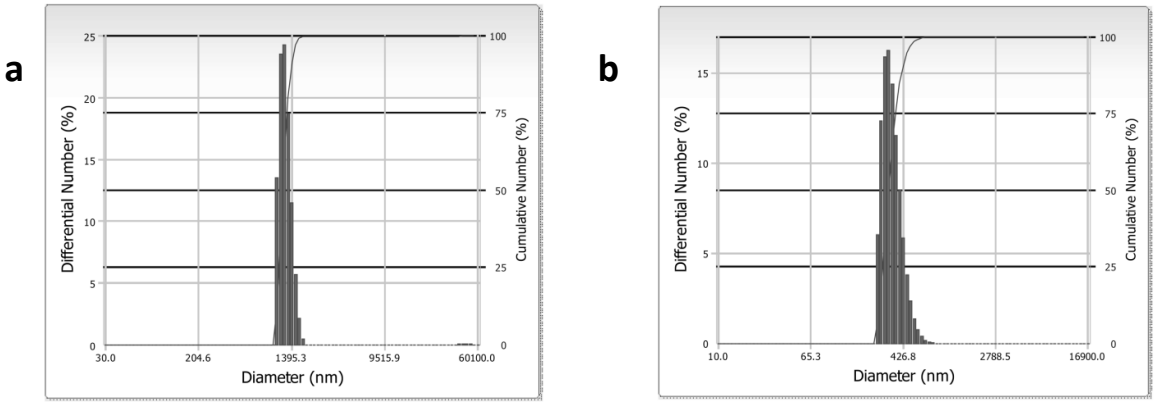

Figure 4
Particle size distributions of micro- and nano-propolis measured by Delsa^{tm} Nano, Beckman Coulter. (a) the retentate of microfiltration prosess produced micropropolis. (b) the retentate of microfiltration prosess produced nanopropolis.

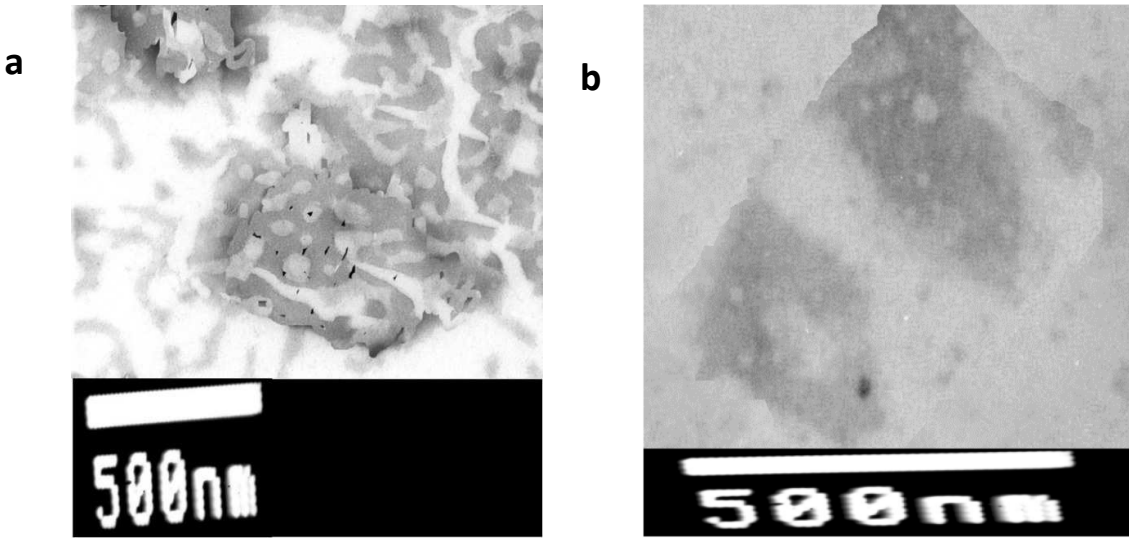

Figure 5
Micro- and Nano- Propolis measured by Transmission Electron Microscopy. (a) Micropropolis. (b) Nanopropolis.

CONCLUSION

We successfully prepared the casein micelle complex, which can be alternative formulation of propolis for antimicrobial compounds with have to size micro and nano particles. It was observed that flavonoids which have lower polarity compare with polyphenols more effective encapsulated by casein micelle. The complex also show antibacterials activity similar with free propolis. Because casein is an common protein, the complex has the potential for many applications such as applying in kids formula milk as bioactive and antimicrobial, antimicrobial oral drug and bioactive for sunscreen lotion. However, further studies are required to confirm those hypothesis.

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