INTRODUCTION

For many years, natural sources have been an important subject in drug discovery (Subramani and Sipkema, 2019). Indonesia, a biodiversity country endowed with thousand species of flora (Sholikhah, 2016), holds an enormous potential for natural sources-based bioactive compound discovery. Canarium indicum L., also known by the locals as kacang kenari or Canarium nut, is an endemic species of Canarium genus under Bruseraceae family which is grown in eastern part of Indonesia, especially in Maluku (Tamalene et al., 2016). Apart from its utilization as food, nuts were found to also contain bioactive peptide (Wang et al., 2018; Tang, et al., 2012). Bioactive peptides, sometimes termed hydrolysates, are fragments of a protein which are released after hydrolysis (Sanchez and Vazquez, 2017). Reports found that Canarium nut also antioxidant and anti-inflammatory activities (Leakey et al., 2008).

Several parameters such as the specificity of hydrolyzing agent, temperature, pH and time of hydrolysis should be carefully optimized (Chakrabarti et al., 2018; Oshimura and Sakamoto, 2017) to avoid complete degradation of the protein. Sometimes, microwave irradiation is used to accelerate hydrolysis (Kabaha et al., 2011; Zhong et al., 2005). Although the harsh condition may lead to destructive reaction such as oxidation, yielding undesired product (Halldorsdottir et al., 2014).

Currently, most bioactive peptide analysis focused mainly on the measurement of activity. It is well known that biological activity could be directly correlated to the amount of chemical compound. For example, antioxidant activity may be correlated to the amount of chemical compound. Instrumental methods such as flurometry, UV spectrophotometry and high-performance liquid chromatography (HPLC) have been employed.
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(Barth, 2007). However, said methods are costly, not environmentally friendly, takes longer time and require great effort (Mallah et al., 2015; Siregar et al., 2018). Fourier transform infrared (FTIR), in comparison, is easier to operate. The instrument enables solid analysis and requires less pretreatment. It has been used widely, especially in qualitative protein analysis for structure elucidation, binding interaction and conformation determination including in post-degradation state (Barth, 2007; Alhazmi, 2019; Vichi et al., 2019). Quantitative analysis using FTIR has also been employed, especially in the field of pharmaceutical science (Bunaciu et al., 2010; Fanelli et al., 2018).

In this study, we aim to develop an innovative FTIR method for peptide content estimation in Canarium nut protein and its hydrolysates, based on absorbance in characteristic region. Furthermore, since the instrument can distinguish between a compound and its resulting product after chemical reaction (Oleszko et al., 2015), we also explore the role of FTIR in microwave-assisted protein degradation kinetic study.

**MATERIAL AND METHODS**

**Chemicals**
Canarium nut (Canarium indicum L) protein, Bovine Serum Albumin (BSA), Tryptophan (Sigma Aldrich), Tyrosine (Sigma Aldrich), Phenylalanine (Sigma Aldrich), Methionine (Sigma Aldrich), Proline (Sigma Aldrich), Cysteine (Sigma Aldrich), distilled water, Papain (Merck), Pepsin (Merck), NaOH (Merck), HCl (Merck), KBr (Merck).

**Instruments**
FTIR spectrophotometer (JASCO 4200 Type A, USA), Microwave oven (Samsung R-230R(S), Korea) set at 199.5 Ws, analytic scale (Mettler Toledo, USA), pH meter (Mettler Toledo, USA)

**Determination of characteristic wavenumber**
Amino acids, BSA and Canarium nut protein in KBr (1% w/w) pellet was scanned using FTIR spectrophotometer at the range of 4000-400 cm⁻¹. The spectra were examined to determine distinctive band of protein.

**Method validation**
The method was validated by evaluating linearity, precision, accuracy, limits of detection and limit of quantification. Linearity test was conducted by measuring peak area of BSA in a series of concentration (1.25-2.5%) and calculating correlation coefficient of the resulting graph. Accuracy test was conducted by measuring the response of 1.8%, 2.0% and 2.2% of BSA and calculating percent recovery. Precision was considered by measuring the response of 2.1% BSA at 3 different times for 3 days and calculating relative standard deviation (%RSD). Limit of detection and limit of quantification were calculated from calibration curve as 10S/Slope and 3S/Slope, respectively, where S is residual standard deviation.

**Estimation of peptide content**
For enzymatic hydrolysis, defatted Canarium nut was homogenized in 0.9% NaCl to obtain a concentration of 30% w/v. Homogenate was filtered and centrifuged at 12.000 rpm for 20min. The supernatant was adjusted to reach optimum condition for each enzyme (papain: 55°C, pH 7; pepsin: 37°C, pH 2 for pepsin) and was left for 30min. The enzymatic mixture was mixed for 1h, agitated with magnetic stirrer at 300rpm. Then, enzyme was deactivated by heating at±100°C for 15min. Hydrolysates were cooled to room temperature and centrifuged 4°C at 12.000 rpm for 20min. The supernatant was collected and freeze-dried.

For alkaline hydrolysis, defatted Canarium nut was mixed with 4N NaOH to obtain a concentration of 30% (w/v). Homogenate was filtered and centrifuged at 12.000rpm for 20min. Supernatant was placed inside a hydrolysis tube and incubated at 100°C for 4h. Hydrolysate was cooled down in ice bath and neutralized with 6N HCl. The hydrolysate was centrifuged at 10.000 rpm for 5min. The supernatant was collected and dried in the oven at 50°C for 1h. Peptide content was estimated by calculating the peak area of Canarium nut protein, papain, pepsin and alkaline hydrolysates at amide I band.

**Reaction kinetic study**
Defatted Canarium nut (100mg) was added with NaOH 4 N dropwise in an evaporating dish and microwaved for 120min. Samples were taken at 0, 1, 15, 30, 60 and 120min. The spectrum of each sample (1%) were scanned and the peak area were calculated. A plot between period and peak area was constructed to determine kinetic profile.
RESULT AND DISCUSSION

Determination of characteristic wavenumber

Food components such as carbohydrate and fat have characteristic intramolecular bonds. Carbohydrates generate a peak around 1200–900 cm\(^{-1}\) indicating C-O-C and C-OH stretch while fat generates a peak around 1700 cm\(^{-1}\) indicating C=O stretch in ester linkage (Bagcioglu et al., 2017; Birkel and Rodriguez-Saona, 2011). We observed distinctive peaks at 2100 cm\(^{-1}\) generated by amino acids, representing free -NH\(^3+\) bond. In protein and proline, the peak disappeared due to bond formation between -NH\(^3+\) and -COO\(^-\) (Leifer and Lippincott, 1957). BSA and Canarium nut protein showed two distinctive peaks, termed amide I (1724.05-1619.91 cm\(^{-1}\)), corresponding to C=O stretch and amide II (1480–1575 cm\(^{-1}\)), corresponding to the combination between C-N stretch and N-H bend (Bagcioglu et al., 2017).

The intensity of amide II band was much lower compared to amide I and overlapped with aromatic C=C band (Figure 1). Meanwhile, amide I peak appeared strongly in both BSA and Canarium nut protein. Eventhough proline C=O band (1600 cm\(^{-1}\)) seemed to occupy the same region, Canarium was found to not contain this amino acid (Djarkasi et al., 2017). Marcone et al. (2002) also stated that proline was not detected in Canarium ovatum. Therefore, amide I peak was chosen for peptide content analysis in this research.

Method validation

Figure 2a showed an overlay of secondary derivative BSA spectra in a series of concentration ranging from 1.25% to 2.5%. Within this range, linear profile was observed (Figure 2b), generating a correlation coefficient of 0.9977, which is acceptable for linearity test (Peris et al., 2017).
A method is considered accurate if the percent recovery is between 80-110% (Peris et al., 2017). Percent recovery of BSA using this method is between 99.59-100.99%. The RSD obtained from intra-assay precision test are 1.6882%, 1.7686% and 1.1868% while intermediate precision test resulted in RSD value of 1.5512%. A method is considered precise if the value of RSD is ≤2% (Peris-Vicente et al., 2017). Therefore, the precision test of this method met the requirement. The LOD and LOQ obtained for this method are 0.12% w/w dan 0.35% w/w.

### Estimation of peptide content

FTIR analysis of Canarium nut protein and its hydrolysates shown no notable changes apart from diminishing intensity of amide bands (Figure 3a-b). The absence of νNH band in all hydrolysates indicated that hydrolysis yield peptide instead of amino acid. Amide II band intensity decreased more greatly due to the cleavage of C-N bond (Figure 3b). Meanwhile, C=O bond did not break, but the intensity of amide I band decreased due to the influence of C-N bond loss (Barth, 2007).

Peak area of 1% w/w of Canarium nut protein and its hydrolysates exhibited greater value compared to BSA in the same concentration (Table I). The presence of asparagine and glutamine, which are abundant in food, may increase the intensity of amide I and amide II bands due to the overlap between intra- and intermolecular amide bond (Barth, 2007). Peptide content in papain and pepsin hydrolysates decreased and left only 38.24% and 33.67% of the initial concentration while alkaline hydrolysate left only 28.53%. This result confirmed that nonselective reagent was slightly more efficient compared to enzymes in cleaving peptide bonds.

### Reaction kinetic study

As previously mentioned, FTIR enables qualitative and quantitative analysis in stability study. In this research, the method was employed to study microwave assisted protein degradation. Microwave could accelerate hydrolysis reaction to yield peptide fragment (Quitain et al., 2006). However, in the presence of alkali and oxidants, along with high temperature generated from the irradiation, degradation occurs (Fan et al., 2016). Canarium nut protein turned brownish due to oxidation after microwaving, thus confirmed this theory.

Peptide content (represented by amide I peak area) declined steeply in the first 30min and continue to decrease in slower rate.

**Table I. Peptide content in Canarium nut protein and its hydrolysates**

| Sample                | % in KBr (w/w) | Peak area | Peptide content (%) |
|-----------------------|----------------|-----------|---------------------|
| Canarium nut protein  | 0.4            | 1.2848    | 2.0324              |
| Papain hydrolysate    | 0.8            | 0.9102    | 1.5544              |
| Pepsin hydrolysate    | 0.8            | 0.7644    | 1.3684              |
| Alkaline hydrolysate  | 1              | 0.8281    | 1.4497              |

Figure 3. Overlay of a) the spectra of BSA, Canarium nut protein and its hydrolysates (1% w/w in KBr) indicating amide I (▲) and amide II (♦), b) second order derivative spectra of Canarium nut protein and its hydrolysates.
The reaction exhibited negative logarithmic profile (Figure 4) which indicates decreasing rate over time. An equation of $y = -0.148 \ln(x) + 0.9591$ were constructed with the value of $R^2 = 0.963$. From this result, we expect complete degradation after 600-700 minutes of microwaving.

Figure 4.Kinetic profile of microwave-assisted degradation of Canarium nut protein

Protein hydrolysis kinetic studies have been reported (Motyan, 2013 and Warner, 1942). However, the utilization of FTIR in the construction of reaction profile is scarce. Currently, vibrational spectroscopy has been used for qualitative analysis to observe changes in protein structure after degradation (Barth, 2007; Vichi et al., 2019). Meanwhile, in this research, we obtained more information regarding protein degradation, such as reaction profile and optimum period for complete reaction.

CONCLUSION

FTIR as rapid method for estimation of peptide content was successfully developed and validated. In addition, compared to bioactive assay and other instrumental analysis such as liquid chromatography, FTIR is simpler, easier to operate, requires little to no pretreatment and more environmentally friendly due to the absence of solvent usage. The method was applied in quantitative analysis of Canarium nut protein as well as its hydrolysates and found useful to determine the efficiency of each hydrolyzing agent. From degradation kinetic study, we generated a profile which could predict the end point for microwave-assisted alkaline hydrolysis of protein.

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