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# Optimization of active antioxidative defatted *Canarium indicum* L. (Canary) protein hydrolysate production

Cintya Nurul Apsari[1,2]*, Ilma Nugrahani[1], Sukrasno[1], Tutus Gusdinar[1]

[1]Department of Pharmacochemistry, School of Pharmacy, Bandung Institute of Technology, Ganesha St. no. 10 40132, Bandung, Indonesia.
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara St. 55281, Yogyakarta, Indonesia.

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**ABSTRACT**

Defatted Canary (*Canarium indicum* L.) as an oilcake was used as a nutraceutical due to its high protein content (36.039%). One of its applications is the preparation of protein hydrolysate using enzymes, namely, papain, flavourzyme, and pepsin. The production was performed using the Box–Behnken response surface methodology, which involves three factors and three levels, namely, concentration [E/S], hydrolysis time, and hydrolysis temperature which are all factors to be considered. The antioxidant test on the sample was used as a parameter for determining the optimal conditions. In addition, other properties, namely, protein content, degree of hydrolysis, and SDS-PAGE electropherogram profile, were also analyzed to determine the character of the product. The results showed that the optimum process for producing antioxidative protein hydrolysate with papain (A13), flavourzyme (B1), and pepsin (C14) was performed at 60°C, 50°C, and 42°C, with an enzyme concentration of 0.55%, 0.55%, and 0.1% (w/v) for 3, 1, and 3 h, respectively. Furthermore, the antioxidant activity ($IC_{50}$) values of each hydrolysate were 2.622 ± 0.072 (A13), 0.426 ± 0.008 (B1), and 0.195 ± 0.001 mg/mL (C14). This implies that the protein hydrolysate produced by pepsin has the highest antioxidant activity.

## 1. INTRODUCTION

Protein hydrolysate is a product of digestion containing high-quality amino acids. It is used as nutraceuticals or functional food [1]. Furthermore, it is known that hydrolyzed protein products have higher biological activity and functional qualities than non-hydrolyzed [2]. This bioactivity is influenced by the content of peptides, which have a specific amino acid structure and sequence [3].

Canary (*Canarium indicum* L.) is an Indonesian indigenous plant that grows primarily in the country's Eastern region, specifically in Northern Maluku [4]. The deffated Canarium is a by-product of Canary oil extraction (Nangai oil), conducted using mechanical pressure. The protein content of the sample is increased by the defatted procedure [5]. Defatted walnuts are still limited to animal feed [6]. Therefore, *C. indicum* L. defatted is used as a functional food supplement or an active cosmetic component containing antioxidative peptide with additional procedures, including hydrolysis.

The protein hydrolysate production was optimized with an antioxidant activity using three different enzymes, namely, papain, flavourzyme, and pepsin. The specificity of the enzyme to the substrate results in several advantages in the production of protein hydrolysates with proteases, which were conducted in mild settings to avoid side reactions. Furthermore, the hydrolysate produced does not reduce the nutritional value of the protein [7]. Several factors can affect the yield and quality of the protein hydrolysate, including the properties of the substrate, the nature of the enzyme, and the process conditions (temperature, pH, hydrolysis time, and addition of water) [2]. The aim is to determine which method produces the protein hydrolysate with the highest antioxidant activity, measured by a low $IC_{50}$ value, and also analyze the factors that influence the production of antioxidative protein hydrolysates.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Canary (*C. indicum*) plant samples were collected from Makian Island, North Maluku, Indonesia. Papain (Merck), Flavourzyme (Merck), Pepsin (Merck), Sodium dodecyl sulfate (Merck), $CuSO_4.5H_2O$, Bovine Serum Albumin (Sigma-Aldrich), Ninhydrin (Merck), L-Leucine (Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazy (Himedia), and Ascorbic acid (Merck) were used in the analyses.

### 2.2. Sample Preparation

The Canary seeds (Nut in Testa) were roasted at 60°C for 60 min. After which, the testa was peeled to obtain the canary kernels/nuts. The kernel was pressed at a pressure of 100 kN/m$^2$ for 5 min, later increasing to 250 kN/m$^2$, resulting in a defatted canary seed/kernel. The defatted canary seed was grounded into powdery form and sieved to achieve a uniform particle size.

*Corresponding Author:
Cintya Nurul Apsari,
Department of Pharmacochemistry, School of Pharmacy, Bandung Institute of Technology, Ganesha St. no. 10 40132, Bandung, Indonesia.
E-mail: cintya_apsari @ students.itb.ac.id

### 2.3. Extraction of Canary Seed Protein

200 g of defatted canary seed flour was extracted with 1000 mL of 0.15 M Phosphate Buffer pH = 7 or 0.15 M HCl Buffer pH = 2. Furthermore, the extraction was performed in a blender for 2 min. The extract was filtered using a filter cloth and refrigerated for at most 12 h before being used as material to produce Canarium protein hydrolysate.

### 2.4. Production of Canary Seed Protein Hydrolysate

Proteases including papain, flavourzyme, and pepsin were used to hydrolyze canary seed protein. The response surface methodology with a Box–Behnken design optimized the protein hydrolysate production. In which, 17 trials were conducted randomly involving several factors, such as hydrolysis time (hours), enzyme/substrate concentration ([E/S]) (%), and temperature (°C) [Table 1]. The optimization was performed using the trial version of Design-Expert software (Stat-Ease Inc., Minneapolis, USA). To produce protein hydrolysate, the pH of papain and flavourzyme was adjusted to 7, while pepsin was used at a pH of 2.0. This experiment's factors and levels were based on a literature research of protein hydrolysate and preliminary experimental data.

### 2.5. Proximate Analysis

The Association of Official Analytical Chemist [8] method was used to conduct proximate analysis of Canary kernel, as well as roasted and defatted Canary kernel.

### 2.6. Lowry Protein Assay

The protein content of the sample was determined using Lowry's method in accordance with [9-11]. First, a stock solution of Reagent A was prepared using 2% $Na_2CO_3$, 0.4% NaOH, 0.16% sodium potassium tartrate, and 1% SDS, while the stock solution of Reagent B was produced using 4% $CuSO_4.5H_2O$. There were combined in a ratio of 100:1 (Reagent A: Reagent B, yielding Reagent C or alkaline copper reagent). Afterward, 1 mL of the sample (containing 50–250 µg of protein) was added to 3 mL of Reagent C and incubated for 60 min at room temperature. Then, 0.3 mL of 50% Folin–Ciocalteu solution was added, and the mixture was set for an additional 45 min. Finally, an absorbance reading at λ = 737 nm (maximum wavelength) was recorded.

### 2.7. Degree of Hydrolysis (DH)

The evaluation of the DH was modified from Moore and Stein, Pearce *et al*., and Zhang *et al*. [12-14] based on ninhydrin methods (1). Solution A preparation involved 80 mg of $SnCl_2$ dissolved in 50 mL of Citrate buffer (0.2 M; pH=5.0) and purified using $N_2$ gas (2). Solution B preparation involved 0.5 g of Ninhydrin dissolved in 10 mL of DMSO (3). Quenched Buffer preparation contains 12% (w/v) PEG 6000 and 25mM EDTA (4). Preparation of Ninhydrin Reagent involved the combination of solution A and B in a volume ratio of 1:1 (5). The preparation of a Standard Solution of L-Leucine includes dissolving 25 mg L-Leucine in 25 mL of phosphate buffer (0.2 M; pH = 8) at a concentration of 1 mg/mL (stock solution). Then, a series of concentrations of 0.500, 0.100, 0.075, 0.050, 0.025, and 0.01 mg/mL were prepared from the stock solution (6). Reaction: 50 µL of sample/standard solution (L-Leucine) and 50 µL of Quenched Buffer were added to the microtube, followed by 500 µL of ninhydrin reagent. The reaction was performed in a water bath at 80°C for 10 min (ensure the microtube cover is tightly closed). After the microtube reached room temperature, 1000 µL of distilled water was added and vortexed. Finally, the spectrophotometer UV-Visible measured the absorption at a maximum wavelength of 570 nm. The DH was calculated using the following equation.

$$DH = \frac{L_t - L_0}{L_{max} - L_0} \times 100\%$$

Where DH denotes the degree of hydrolysis, $L_t$ denotes the amount of α-amino in a sample after hydrolysis under specified conditions, $L_0$ denotes the amount of α-amino in non-hydrolyzed samples, and Lmax = the maximum amount of α-amino in the sample after 24 h of hydrolysis with 6 M HCl at 100°C.

### 2.8. Antioxidant Activity

Antioxidant tests were conducted using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method, as described in Zhang *et al*., He *et al*., Yarnpakdee *et al*., and Erdoğan *et al*. [15-18]. In the dark vial, 1 mL of the sample/standard solution (Ascorbic acid) was combined with 1 mL of 0.1 mM DPPH. The mixture was incubated for 30 min at room temperature. The absorbance value was determined at a maximum wavelength of 515 nm. Next, a standard curve was constructed using ascorbic acid at concentrations ranging from 1 to 20 ppm. Finally, the following equation was used to calculate the percentage of inhibition.

$$\%Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

Where Acontrol = control absorbance (DPPH solution+methanol), Asample = sample absorbance. The $IC_{50}$ value for each sample was determined using the regression equation between the sample concentration (x-axis) and the %inhibition (y-axis) obtained from the calibration curve.

### 2.9. Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein sample's molecular weight profile was determined using a slightly modified method [19,20]. Furthermore, electrophoresis was performed using 12% and 4% of polyacrylamide separating gel and stacking gel, respectively. A sample of 25 µL containing 40 µg of protein was mixed with 25 µL Laemmli sample buffer and heated for 10 min at 90°C. After the injection of aliquots into the wells, electrophoresis was performed at 150 V, followed by staining with Coomassie blue R-250. Finally, the molecular weight of the sample was determined by comparing it to the standard band.

## 3. RESULTS AND DISCUSSION

### 3.1. Proximate analysis

The Canary nut in testa [Figure 1a] was pre-processed before being used to produce protein hydrolysate. The processes are roasting, removing testa, and pressing, including all steps in the production of defatted canary kernels [Figure 2]. Furthermore, the proximate compositions of the samples are listed in Table 2, and a comparison of the processed canary compositions is shown in Figure 3.

The most abundant components in the samples (canary kernel [Figure 2b], roasted canary kernel [Figure 2c], and defatted canary kernel [Figure 2d]) were fat and protein. However, protein, moisture, and ash levels increased after processing (roasting, pressing, and grinding). As shown in Figure 3, proteins are the composition with a higher

**Table 1** : Experimental design for the production of protein hydrolysates using various enzymes.

| Papain | | | | Flavourzyme | | | | Pepsin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Code | Time (h) | [E/S] (b/v) (%) | Temperature (°C) | Code | Time (h) | [E/S] (b/v) (%) | Temperature (°C) | Code | Time (h) | [E/S] (b/v) (%) | Temperature (°C) |
| A1 | 3 | 1 | 55 | B1 | 1 | 0.55 | 50 | C1 | 3 | 0.1 | 37 |
| A2 | 3 | 0.1 | 55 | B2 | 3 | 0.1 | 50 | C2 | 5 | 0.55 | 37 |
| A3 | 5 | 0.55 | 55 | B3 | 5 | 0.55 | 50 | C3 | 3 | 1 | 37 |
| A4 | 1 | 0.55 | 55 | B4 | 3 | 1 | 50 | C4 | 1 | 0.55 | 37 |
| A5 | 3 | 0.55 | 60 | B5 | 3 | 0.55 | 55 | C5 | 3 | 0.55 | 39.5 |
| A6 | 5 | 0.1 | 60 | B6 | 3 | 0.55 | 55 | C6 | 5 | 0.1 | 39.5 |
| A7 | 3 | 0.55 | 60 | B7 | 5 | 0.1 | 55 | C7 | 3 | 0.55 | 39.5 |
| A8 | 3 | 0.55 | 60 | B8 | 3 | 0.55 | 55 | C8 | 3 | 0.55 | 39.5 |
| A9 | 1 | 0.1 | 60 | B9 | 3 | 0.55 | 55 | C9 | 1 | 1 | 39.5 |
| A10 | 5 | 1 | 60 | B10 | 1 | 0.1 | 55 | C10 | 1 | 0.1 | 39.5 |
| A11 | 3 | 0.55 | 60 | B11 | 1 | 1 | 55 | C11 | 3 | 0.55 | 39.5 |
| A12 | 1 | 1 | 60 | B12 | 5 | 1 | 55 | C12 | 3 | 0.55 | 39.5 |
| A13 | 3 | 0.55 | 60 | B13 | 3 | 0.55 | 55 | C13 | 5 | 1 | 39.5 |
| A14 | 3 | 0.1 | 65 | B14 | 3 | 1 | 60 | C14 | 3 | 0.1 | 42 |
| A15 | 3 | 1 | 65 | B15 | 1 | 0.55 | 60 | C15 | 3 | 1 | 42 |
| A16 | 1 | 0.55 | 65 | B16 | 5 | 0.55 | 60 | C16 | 1 | 0.55 | 42 |
| A17 | 5 | 0.55 | 65 | B17 | 3 | 0.1 | 60 | C17 | 5 | 0.55 | 42 |

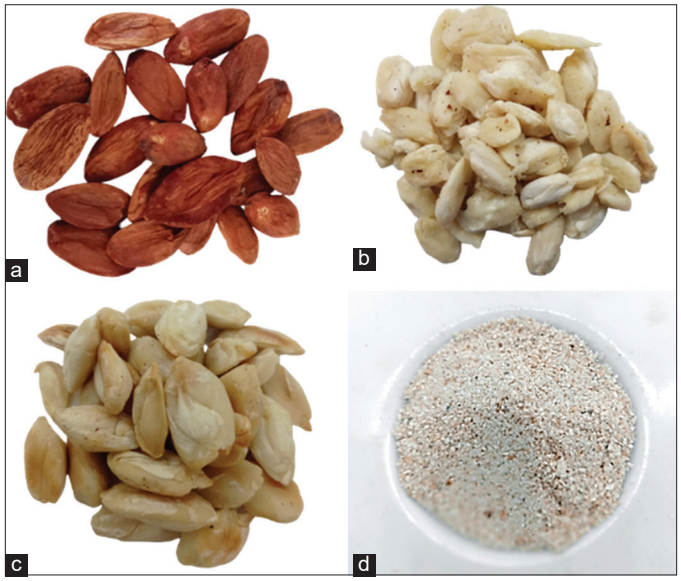


**Figure 1:** Canary (*Canarium indicum* L.) *nut.* (a). Canary Nut in Testa (NIT) (b). Canary kernel (c). Roasted canary kernel (d). Defatted canary kernel (powder).

increase in the defatted canary seed (from 12% to 36%), implying that the canary pressing procedure increases the proportion of protein in the samples. According to Xing *et al*., Melo *et al*., Filho and Egea [5,21,22], fat removal's mechanical pressing process enriches protein and eliminates other non-protein matrices. Protein yield was significantly higher in deffated canary kernel (36.039 ± 0.003%) compared to canary kernel (12.611 ± 0.061%, $P < 0.05$) and roasted canary kernel (13.650 ± 0.332%, $P < 0.05$). Furthermore, the protein content of the roasted canary kernel was significantly higher than the canary kernel ($P < 0.05$). The defatted canary kernel significantly ($P < 0.05$) has more enormous mineral resources than the canary seed/kernel [Figure 3]. The defatting process causes the sample to have a higher ash concentration [21,23,24].

As a result of the pressing process, the content of most nutrients (fat, fiber, and carbs) was significantly reduced ($P < 0.05$). Unlike moisture content, the value of the water component in the defatted canary kernel (powder) is increased since the powder form has a higher hygroscopicity than entire kernels [25-27]. This allows the defatted canary sample to absorb moisture from the environment more readily. Therefore, extra caution is needed while storing ground samples to avoid moisture reabsorption, leading to microbial contamination (bacteria, molds, and yeasts) [26,28].

### 3.2. Protein Content of Canarium Protein Hydrolysate

Lowry's technique was used to determine the protein content of the hydrolysate. The dissolved protein in a hydrolysate solution is measured using this approach. Meanwhile, $Cu^{2+}$ ($CuSO_4$) binds to peptide bonds (O=C-N-H) under alkaline conditions, reducing it to Cu. The folin phenol reagent (phosphomolybdic-phosphotungstic acid) interacts with Cu ions, resulting in a blue-green complex that can be measured using a wavelength of 650–750 nm [29-31]. According to the Lowry method's analysis results in Table 3, different protein content values were acquired from three enzymes, namely, papain, flavourzyme, and pepsin. The protein content of hydrolysate generated by flavourzyme (2.608–4.151 mg/mL) and pepsin (0.871–6.802 mg/mL) was statistically insignificant ($P > 0.05$). In contrast, the protein hydrolysate produced by papain had higher concentrations (21.805–38.334 mg/mL). This indicates that there was a statistically significant difference ($P < 0.05$) between hydrolysates hydrolyzed by papain and those produced by flavourzyme and pepsin.

According to the 3D surface response graph in Figure 4, the optimal model for maximizing protein hydrolysate production created by papain and flavourzyme is linear. On the other hand, pepsin's protein

**Table 2**: Proximate compositions of canary (*Canarium indicum L.*).

| Samples | Moisture Content (%) | Fat (%) | Protein (%) | Crude Fiber (%) | Ash (%) | Carbohydrate | Energy (Kal/100 g) |
|---|---|---|---|---|---|---|---|
| Canary kernel | 3.492±0.071[b] | 67.502±0.058[c] | 12.611±0.061[a] | 8.459±0.024[b] | 3.615±0.019[a] | 4.320±0.052[b] | 681.377±0.578[c] |
| Roasted canary kernel | 2.239±0.204[a] | 63.224±0.147[b] | 13.650±0.332[b] | 11.358±0.169[c] | 3.583±0.043[a] | 5.946±0.557[c] | 653.353±0.599[b] |
| Defatted canary kernel (powder) | 3.331±0.044[b] | 45.439±0.038[a] | 36.039±0.003[c] | 3.204±0.249[a] | 10.781±0.116[b] | 1.206±0.142[a] | 569.749±0.878[a] |

*a-c within column, least square means without a common superscript differ ($p < 0.05$)

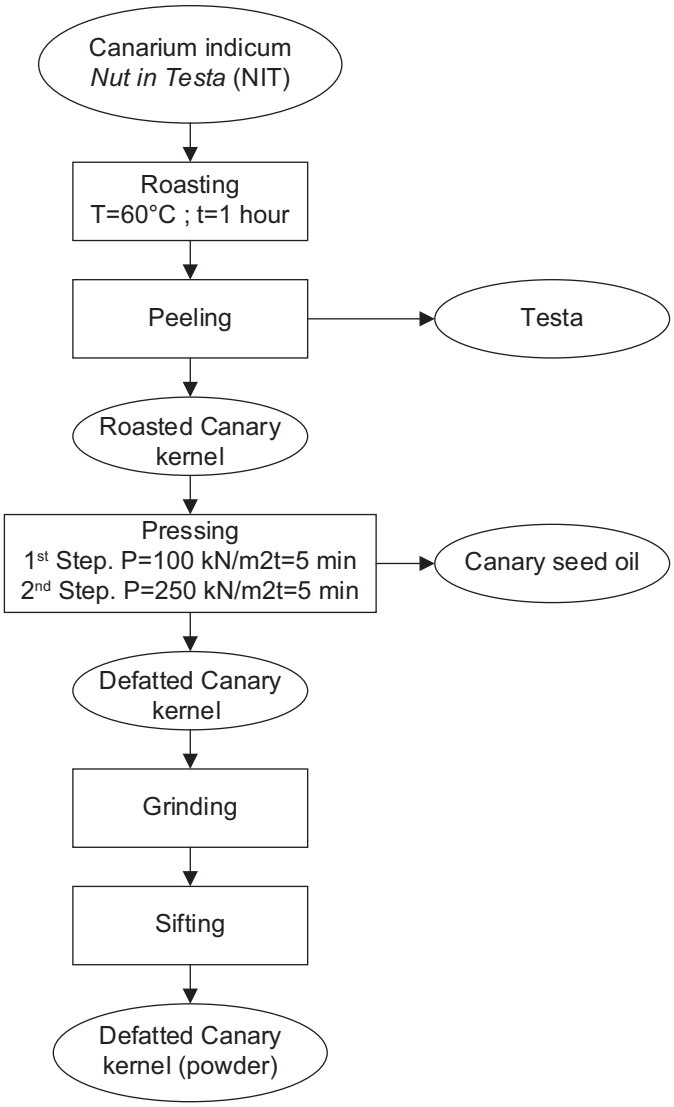


**Figure 2:** Preparation of the Canary (*Canarium indicum* L.) seed.

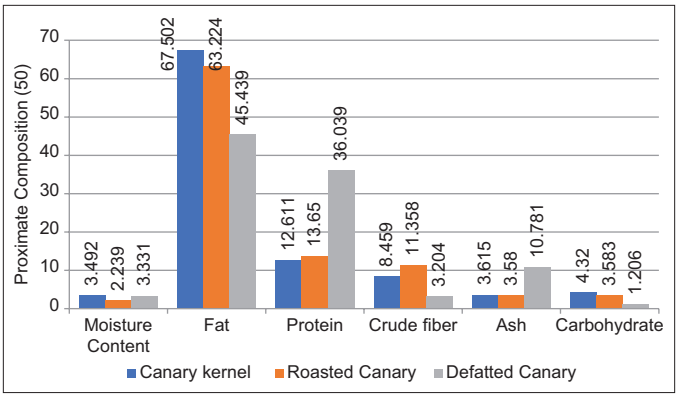


**Figure 3:** Proximate composition in processed *Canarium indicum* L.

hydrolysate follows a quadratic model. The enzyme concentration factor ([E/S]) was discovered to be a significant ($P < 0.05$) factor impacting the protein content of the overall hydrolysate produced by the three enzymes in the analysis of variance (ANOVA). However, time hydrolysis and temperature were statistically insignificant ($P > 0.05$) due to the narrowness of the two components' ranges.

In protein hydrolysate production using papain, flavourzyme, and pepsin, the ideal condition that achieves maximum protein content is coded A10, B11, and C15, respectively. The measured protein content is proportional to the number of peptide bonds in the sample, influenced by enzyme activity in hydrolyzing the existing protein into lower molecular weight fragments. Several factors that play a role in the performance of proteases as biocatalysts include hydrogen ion concentration (pH), temperature [32-34], substrate and enzyme concentration [35], enzyme activator, and inhibitor [36].

### 3.3. DH

The DH was calculated to determine the number of peptide bonds cleavaged in the protein hydrolysate due to the proteolysis process (in this case enzymatically) [37,38]. Furthermore, the percentage of peptide bonds broken during the hydrolysis process divided by the total number in the protein substrate is known as the DH [39]. This research used the Ninhydrin method to detect the amount of α-amino nitrogen in protein hydrolysate. The principle is that the reaction between the ninhydrin reagent and the amino group was used to quantify the number of α-amino nitrogen in protein hydrolysate [37,40]. The purple-blue color produced by the complex formed between Ninhydrin and the primary α-amino group is known as "Ruhemann's purple," and its intensity was measured at a wavelength of 570 nm. In addition, the intensity observed in the sample is related to the quantity of primary α-amino acids present [41]. Table 4 shows the DH measured in the samples of *C. indicum* L.

The DH content of canary protein hydrolysate prepared with papain, flavourzyme, and pepsin ranged from 1.335% to 23.140%. According to the statistical results, pepsin treatment showed the lowest DH level ($P < 0.05$, significant difference). In comparison, the DH levels in the papain and flavourzyme treatments were statistically similar ($P > 0.05$). Several factors determine the difference in DH values on the same substrate (Canarium protein). However, the enzyme type is an essential determinant of DH and the resulting protein fragments [40]. Due to variances in enzyme specificity, each has varying proteolytic activity on the same substrate [42]. This is directly connected to the affinity of the substrate for the catalytic site. Exopeptidases and endopeptidases are two types of proteases, depending on where the enzyme is located [43]. Due to its catalytic site, papain belongs to the cysteine protease group of endopeptidases. Cysteine proteases

**Table 3**: Protein concentration of canary (*Canarium indicum L.*) protein hydrolysate under varied conditions (type of enzyme, time, [E/S], and temperature).

| Code | Protein Content (mg/mL) | Code | Protein Content (mg/mL) | Code | Protein Content (mg/mL) |
|---|---|---|---|---|---|
| A1 | 38.107±0.464 | B1 | 2.608±0.026 | C1 | 4.492±0.238 |
| A2 | 32.998±0.493 | B2 | 2.364±0.061 | C2 | 5.133±0.161 |
| A3 | 32.658±0.157 | B3 | 3.232±0.034 | C3 | 5.985±0.541 |
| A4 | 27.981±0.851 | B4 | 3.958±0.225 | C4 | 5.230±0.277 |
| A5 | 30.206±0.587 | B5 | 2.988±0.049 | C5 | 3.533±0.786 |
| A6 | 35.905±0.275 | B6 | 3.016±0.035 | C6 | 2.931±0.435 |
| A7 | 28.321±0.312 | B7 | 2.148±0.130 | C7 | 2.789±0.648 |
| A8 | 32.340±0.236 | B8 | 3.221±0.055 | C8 | 3.839±0.832 |
| A9 | 28.775±0.258 | B9 | 3.255±0.020 | C9 | 4.674±0.310 |
| A10 | 38.334±0.605* | B10 | 2.114±0.130 | C10 | 2.085±0.378 |
| A11 | 34.270±0.669 | B11 | 4.151±0.129* | C11 | 3.170±0.225 |
| A12 | 36.972±0.360 | B12 | 3.794±0.475 | C12 | 4.151±0.665 |
| A13 | 27.527±0.416 | B13 | 3.215±0.123 | C13 | 3.164±0.789 |
| A14 | 21.805±0.239 | B14 | 3.731±0.309 | C14 | 0.871±0.084 |
| A15 | 34.996±0.477 | B15 | 2.982±0.130 | C15 | 6.802±0.035* |
| A16 | 35.201±0.360 | B16 | 3.067±0.064 | C16 | 5.729±0.351 |
| A17 | 33.067±0.807 | B17 | 2.273±0.121 | C17 | 2.977±0.491 |

**Table 4**: Hydrolysis degree of canary (*Canarium indicum L.*) protein hydrolysate under varied conditions (type of enzyme, time, [E/S], and temperature)

| Code | Degree of hydrolysis (%) | Code | Degree of hydrolysis (%) | Code | Degree of hydrolysis (%) |
|---|---|---|---|---|---|
| A1 | 11.345±0.534 | B1 | 16.009±0.231 | C1 | 2.237±0.060 |
| A2 | 4.137±0.029 | B2 | 4.733±0.026 | C2 | 3.122±0.085 |
| A3 | 9.447±0.193 | B3 | 16.769±0.122 | C3 | 3.301±0.065 |
| A4 | 8.860±0.177 | B4 | 21.935±0.177 | C4 | 3.164±0.092 |
| A5 | 10.955±0.060 | B5 | 15.225±0.174 | C5 | 3.055±0.003 |
| A6 | 13.278±0.241 | B6 | 15.984±0.062 | C6 | 1.335±0.010 |
| A7 | 8.632±0.060 | B7 | 4.002±0.095 | C7 | 2.817±0.071 |
| A8 | 12.541±0.347 | B8 | 16.329±0.083 | C8 | 3.117±0.085 |
| A9 | 4.137±0.029 | B9 | 16.154±0.043 | C9 | 3.499±0.096 |
| A10 | 23.140±0.320* | B10 | 4.175±0.045 | C10 | 2.796±0.082 |
| A11 | 11.814±0.099 | B11 | 17.183±0.162 | C11 | 2.896±0.046 |
| A12 | 13.525±0.294 | B12 | 22.753±0.265* | C12 | 3.257±0.034 |
| A13 | 9.875±0.223 | B13 | 15.879±0.061 | C13 | 3.842±0.102* |
| A14 | 12.704±0.464 | B14 | 16.489±0.165 | C14 | 2.830±0.057 |
| A15 | 20.699±0.256 | B15 | 5.558±0.036 | C15 | 3.513±0.096 |
| A16 | 10.765±0.163 | B16 | 9.256±0.125 | C16 | 2.889±0.111 |
| A17 | 13.259±0.919 | B17 | 3.624±0.052 | C17 | 3.322±0.018 |

have a Cys-His-Asn triad at the active site [44]. Flavourzyme is a combination of exopeptidase [45] and endopeptidase [46], a type of protease that hydrolyzes peptide bonds at the N- or C-terminal ends of protein chains [43,47]. The dual-action mechanism of flavourzyme in cleaving peptide chains enables the average value of DH flavourzyme to be higher than other enzymes with different treatments. On the other hand, Pepsin functions by cleaving internal peptide bonds (endopeptidase) [48], a member of the aspartic protease subclass. The aspartic acid residues in this subclass have two catalytic regions, including Asp-Thr-Gly-Ser in the N terminal domain and a matching Asp-Thr-Gly-Ser/Thr in the C terminal domain [49].

Figure 5 shows that the optimal DH using papain is obtained through a linear model. In contrast, flavourzyme uses a quadratic model, and pepsin utilizes a 2FI (two-factor interaction) model. The three variable factors in the production of protein hydrolysates using papain and flavourzyme, namely, time, substrate concentration ([E/S]), and temperature, were statistically significant ($P < 0.05$) in altering the DH. In pepsin (2FI model), the interaction between hydrolysis time and substrate concentration was the most significant factor ($P < 0.05$) affecting the DH produced. Based on the test results, samples with codes A10 (23.140%), B12 (22.753%), and C13 (3.842%) had the maximum DH produced using papain, flavourzyme, and pepsin, respectively.

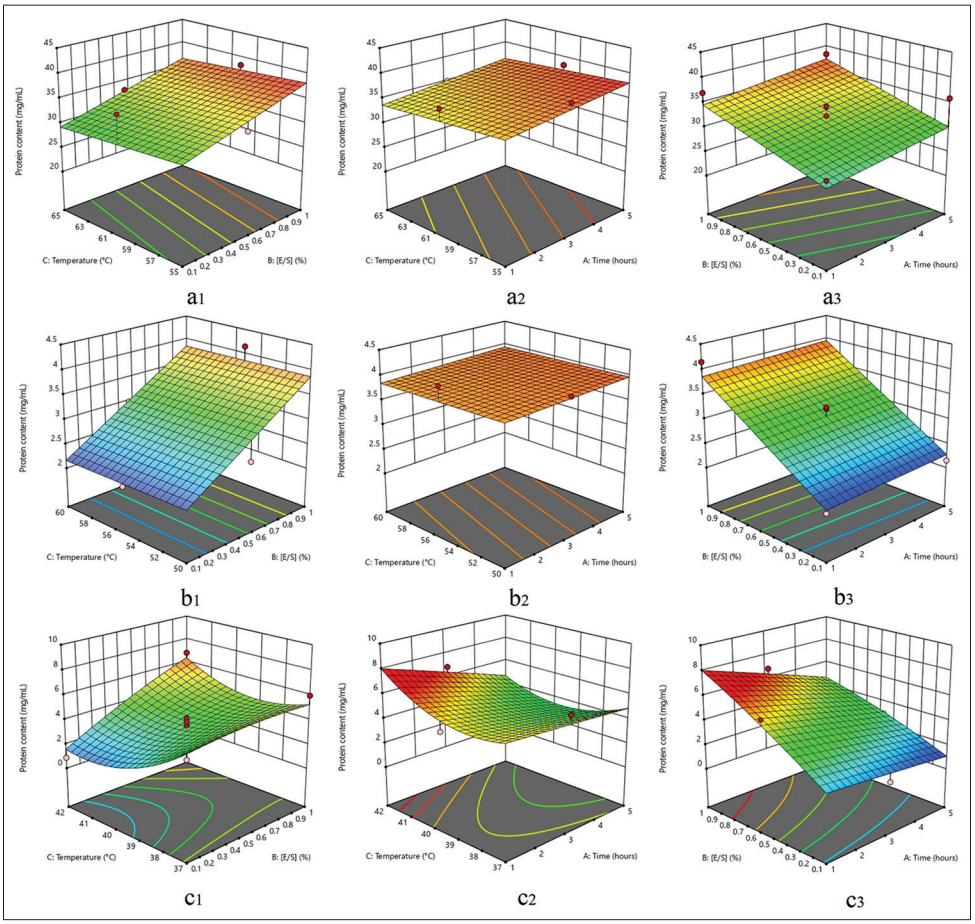


**Figure 4:** Two parameters affect the 3D surface response protein content (mg/mL). ($a_1$). Papain: temperature and [E/S]; ($a_2$). Papain: temperature and time; ($a_3$). Papain: [E/S] and time; ($b_1$). Flavourzyme: temperature and [E/S]; ($b_2$). Flavourzyme: temperature and time; ($b_3$). Flavourzyme: [E/S] and time; ($c_1$). Pepsin: temperature and [E/S]; ($c_2$). Pepsin: temperature and time; ($c_3$). Pepsin: [E/S] and time.

### 3.4. Antioxidant Activity

Antioxidant activity is used as a decision-making parameter to select the most optimum production process. This is defined by protein hydrolysate production with a more excellent antioxidant activity (lowest $IC_{50}$ value) [50]. The DPPH free radical scavenging method was used to conduct the antioxidant tests. Furthermore, the DPPH is a highly stable free radical chromogen with maximal absorbance at 515 nm (purple color) [51]. Its reaction with antioxidants was observed through decreased absorbance due to DPPH reduction and a color change from purple to yellowish [52-54].

The data in Table 5 were used to perform statistical calculations. Each group of protein hydrolysate produced by different enzymes, such as papain, flavourzyme, and pepsin, showed a statistically significant mean value ($P < 0.05$) for DPPH radical scavenging activity. According to the post hoc test, each of the three enzymes has a distinct subset. Based on ANOVA, the enzyme concentration factor (E/S) and hydrolysis temperature had a significant influence ($P < 0.05$) on the antioxidant activity of the final product. In contrast, hydrolysis duration had no significant effect ($P > 0.05$).

The response surface model was plotted according to the minimum function of the $IC_{50}$ value. The best optimization model for canary protein hydrolysate generated with papain and pepsin is quadratic, while the linear model is better for flavourzyme. Meanwhile, within a 95% confidence interval, enzyme concentration ([E/S]) and hydrolysis temperature had the most significant effect on antioxidant activity ($IC_{50}$). The antioxidant activity of protein hydrolysates generated from papain and pepsin was significantly affected by enzyme concentration (ratio [E/S]). At the same time, the hydrolysis temperature had a significant effect on the synthesis with flavourzyme and papain ($P < 0.05$).

Figure 6a1-a3 shows that the antioxidant activity ($IC_{50}$) of papain protein hydrolysate (PH) is the lowest (2.622 mg/mL) at the highest ratio [E/S], indicating that the hydrolysate product was the best to scavenge DPPH radicals (sample code A13). The best protein hydrolysate produced by pepsin is sample code B1 (0.426 mg/mL). Figure 6c1-c3 shows that the higher the ratio [E/S] and the lower the temperature, the lower the $IC_{50}$ value. In flavourzyme, C14 shows the lowest $IC_{50}$ value of the entire sample (0.195 mg/mL), the hydrolysis temperature is the factor that has a significant effect on the antioxidant activity, and this is shown in Figure 6b1-b3 the lowest hydrolysis temperature of 50ºC produce the lowest $IC_{50}$ result (best antioxidant activity).

The antioxidant activity of hydrolyzate is determined by peptide characteristics such as production process conditions, protease type, amino acid composition, amino acid sequence, peptide molecular structure, and peptide concentration [55,56]. Antioxidants' method of action in scavenging DPPH radicals is known as single electron transfer (SET) or hydrogen atom transfer (HAT) [52,53]. Furthermore, aromatic amino acids perform the SET mechanism on radical

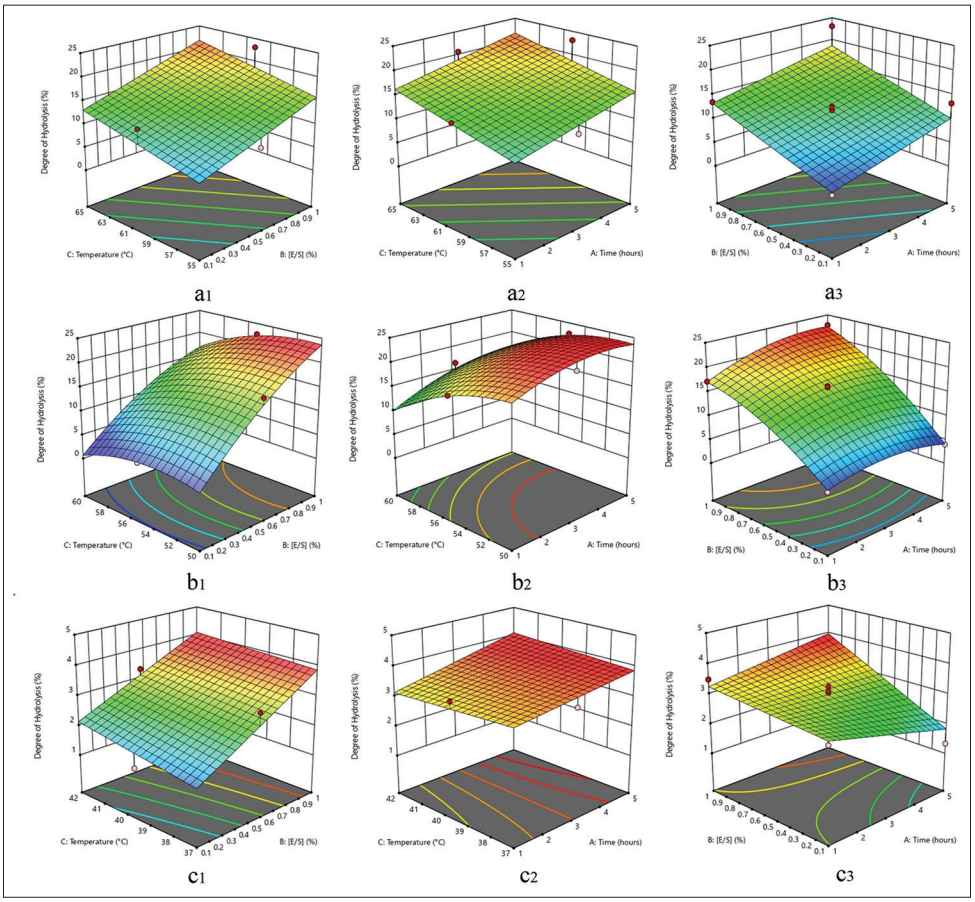


**Figure 5:** 3D surface response degree of hydrolysis (%) affected by two parameters. ($a_1$). Papain: temperature and [E/S]; ($a_2$). Papain: temperature and time; ($a_3$). Papain: [E/S] and time; ($b_1$). Flavourzyme: temperature and [E/S]; ($b_2$). Flavourzyme: temperature and time; ($b_3$). Flavourzyme: [E/S] and time; ($c_1$). Pepsin: temperature and [E/S]; ($c_2$). Pepsin: temperature and time; ($c_3$). Pepsin: [E/S] and time.

**Table 5**: Antioxidant activity of canary (Canarium indicum L.) protein hydrolysate under various conditions, such as type of enzyme, time, [E/S], and temperature).

| Code | $IC_{50}$ (mg/mL) | Code | $IC_{50}$ (mg/mL) | Code | $IC_{50}$ (mg/mL) |
|---|---|---|---|---|---|
| A1 | 3.803±0.213 | B1 | 0.426±0.008* | C1 | 1.136±0.088 |
| A2 | 4.794±0.034 | B2 | 0.555±0.021 | C2 | 1.102±0.043 |
| A3 | 3.918±0.029 | B3 | 0.457±0.022 | C3 | 1.374±0.045 |
| A4 | 3.640±0.015 | B4 | 0.438±0.050 | C4 | 1.279±0.106 |
| A5 | 3.184±0.112 | B5 | 0.658±0.099 | C5 | 0.794±0.016 |
| A6 | 3.230±0.051 | B6 | 0.605±0.057 | C6 | 0.642±0.032 |
| A7 | 3.503±0.016 | B7 | 0.529±0.009 | C7 | 0.627±0.033 |
| A8 | 3.189±0.017 | B8 | 0.624±0.017 | C8 | 0.745±0.017 |
| A9 | 3.968±0.060 | B9 | 0.578±0.018 | C9 | 0.863±0.020 |
| A10 | 3.338±0.117 | B10 | 0.611±0.010 | C10 | 0.402±0.012 |
| A11 | 3.271±0.115 | B11 | 0.752±0.007 | C11 | 0.605±0.014 |
| A12 | 3.087±0.116 | B12 | 0.644±0.065 | C12 | 0.632±0.009 |
| A13 | 2.622±0.072* | B13 | 0.673±0.012 | C13 | 0.631±0.025 |
| A14 | 4.151±0.066 | B14 | 0.777±0.008 | C14 | 0.195±0.001* |
| A15 | 3.242±0.182 | B15 | 0.668±0.023 | C15 | 1.523±0.010 |
| A16 | 3.885±0.244 | B16 | 0.671±0.016 | C16 | 1.081±0.014 |
| A17 | 3.197±0.068 | B17 | 0.610±0.007 | C17 | 0.637±0.035 |

scavenging by donating protons to electron-deficient radical entities and stabilizing them through electron resonance processes. On the other hand, the amino acids Asp and His operates as HAT since they have carboxylic and amino groups on their side [3].

### 3.5. SDS-PAGE

SDS-PAGE analysis was performed to determine the fragment ladder pattern on protein hydrolysate samples prepared with different treatments. Figures 7-9 showed the characteristics of protein hydrolysates prepared with papain, flavourzyme, and pepsin, respectively. The primary band appeared in the sample codes A2 and A9 at 20 kDa in various protein hydrolysates produced by papain [Figure 7], while the protein flavourzyme band appeared in the range of 10–35 kDa [Figure 8]. The main protein band in the hydrolysate formed by pepsin ranged from 10 kDa to 20 kDa [Figure 9]. Practically, no protein bands were observed on the electropherogram of the papain hydrolysate sample. Furthermore, the absence of protein bands shows that almost all large molecular weight proteins had been digested into peptide fragments of molecular weight <10 kDa. This result is consistent with [57], stating that hydrolysis of ovotransferrin yielded papain peptides of MW<10 kDa.

The effect of catalytic sites of each enzyme has a significant impact on the termination pattern of the protease peptide bond to the protein substrate. As previously stated, papain is an endolytic cysteine

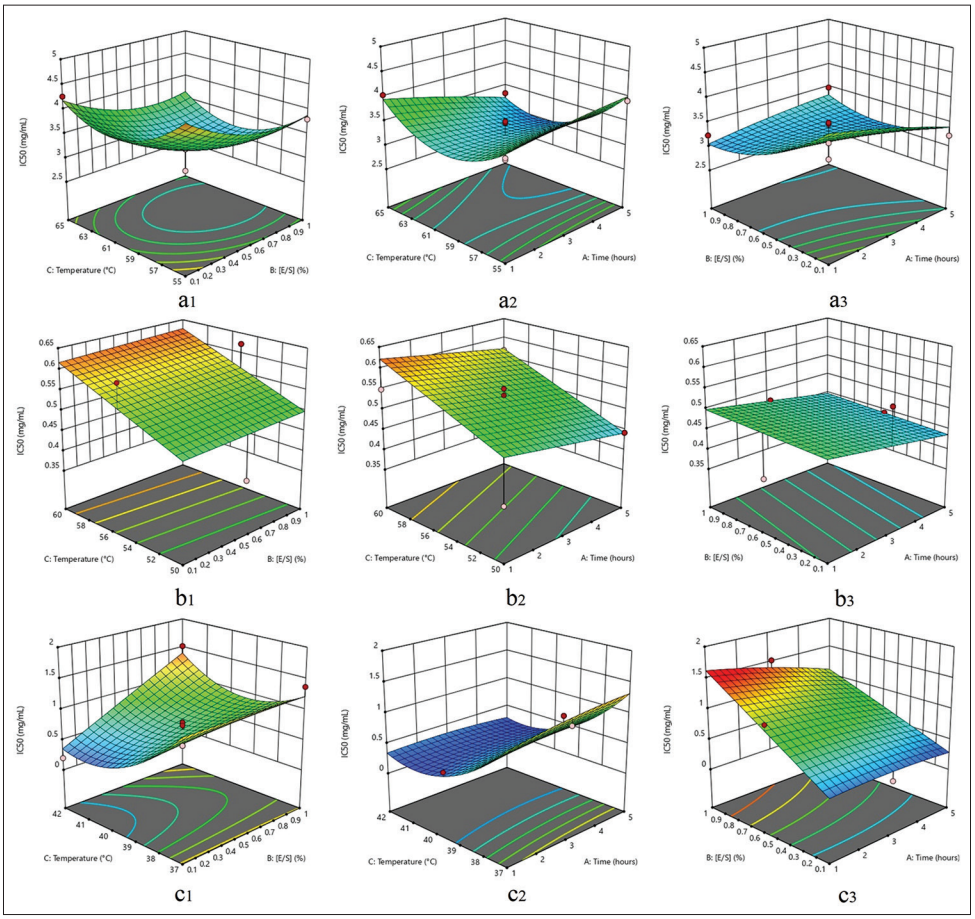


**Figure 6:** 3D surface response antioxidant activity (IC50) (mg/mL) affected by two parameters. ($a_1$). Papain: temperature and [E/S]; ($a_2$). Papain: temperature and time; ($a_3$). Papain: [E/S] and time; ($b_1$). Flavourzyme: temperature and [E/S]; ($b_2$). Flavourzyme: temperature and time; (b3). Flavourzyme: [E/S] and time; ($c_1$). Pepsin: temperature and [E/S]; ($c_2$). Pepsin: temperature and time; ($c_3$). Pepsin: [E/S] and time.

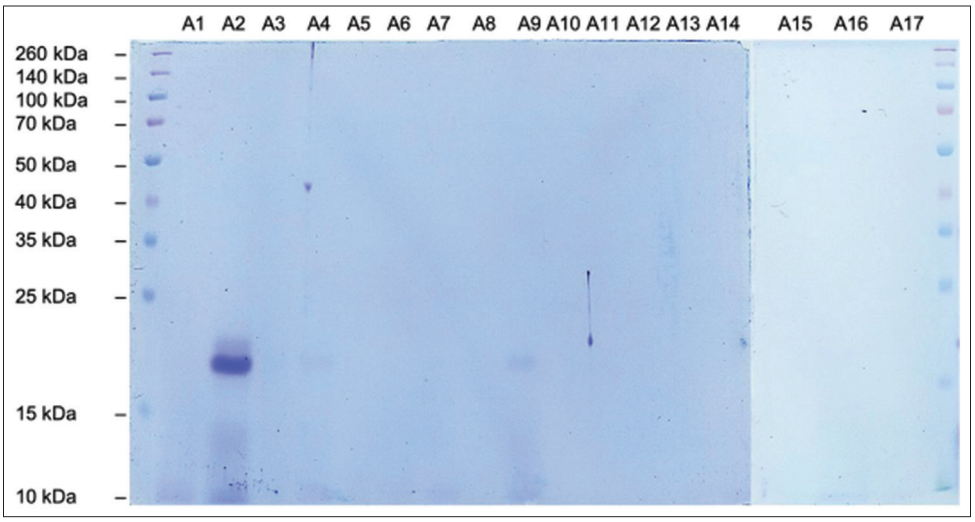


**Figure 7:** SDS-PAGE electropherograms of *Canarium indicum* L. protein hydrolysate produced by papain (Sample Code A1-A17).

protease, flavourzyme is a mixture of exo- and endopeptidase, while pepsin is an aspartic protease (endopeptidase). Papain functions as an endopeptidase, amidase, and esterase enzyme. It tends to break peptide bonds containing leucine, glycine, basic amino acids (particularly arginine lysine), and phenylalanine [58,59]. Furthermore, papain cleaves peptide bonds with hydrophobic side groups (Bulky aromatic residue) [60,61]. The enzyme (aspartic protease), such as pepsin, is the most effective in breaking the peptide bonds between hydrophobic amino acids and, preferably, aromatic amino acids (phenylalanine, tryptophan, and tyrosine) [62]. Meanwhile, flavourzyme, a leucyl

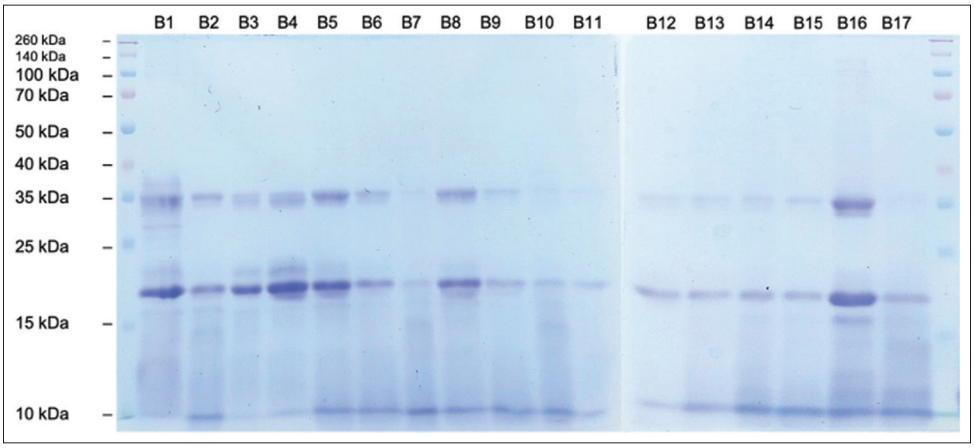


**Figure 8:** SDS-PAGE electropherograms of *Canarium indicum* L. protein hydrolysate produced by flavourzyme (Sample Code B1-B17).

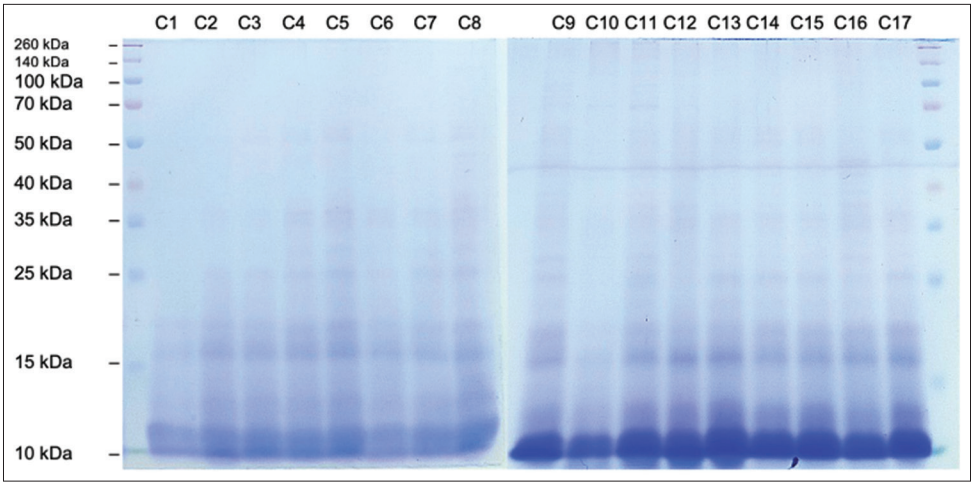


**Figure 9:** SDS-PAGE electropherograms of *Canarium indicum* L. protein hydrolysate produced by pepsin (Sample Code C1-C17).

aminopeptidase, exhibits endo- and exopeptidase activity. Hydrolysis produces N-terminal amino acids, preferentially Leucine, Glutamic acid, and Glycine [63].

## 4. CONCLUSION

Samples A13, B1, and C14 produced the best antioxidant protein hydrolysate using papain, flavourzyme, and pepsin enzymes. A papain concentration of 0.55 % (w/v), at a temperature of 60°C, and a hydrolysis period of 3 h were used to make antioxidative protein hydrolysate. The optimal condition for producing antioxidative protein hydrolysate with flavourzyme was achieved by hydrolysis for 1 h at 50°C with a 0.55% (v/v) enzyme concentration. Meanwhile, pepsin produced protein hydrolysate by hydrolyzing Canarium protein for 3 h at 42°C with 0.1% (w/v) enzyme concentration. The antioxidative activity ($IC_{50}$) values of each hydrolysate were 2.622 ± 0.072 (A13), 0.426 ± 0.008 (B1), and 0.195 ± 0.001 mg/mL (C14).

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## 6. AUTHORS' CONTRIBUTIONS

All authors contributed significantly to the conception and design, data acquisition, analysis, and interpretation, participated in the drafting of the article or critically revised it for significant intellectual content, agreed to submit it to the current journal, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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## 8. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

## 9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## 10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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