PRELIMINARY STUDY OF BIOACTIVE PROTEIN HYDROLYSATE FROM COCOA POD HUSK

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ABSTRACT - *This study was carried out to examine the bioactive protein hydrolysate from cocoa pod husk, as a source of value added ingredients. At the initial stage of the study, four clones from two different topographic areas were selected for protein screening analysis. Three of the four clones with the highest protein content, which have the potential to contain bioactive protein compounds, were selected for further analysis. To extract the protein, an alkaline based extraction procedure was performed using a 1:30, ratio of alkaline solution (pH 11), to cocoa pod husk (CPH). The extract was then defatted using petroleum ether and freeze-dried after removing the solvent. The drying method is taken into account to prevent the available proteins from being denatured. The extracted cocoa pod husk (CPHP) contained from 7 to 10% of the crude protein content. Then CPHP was hydrolyzed with enzymatic hydrolysis. To obtain optimal results, alkaline enzymes were more suitable to be used to hydrolyze cocoa pod husk proteins. To determine the antioxidant properties of the resultant hydrolysates, antiradical, DPPH and ABTS, and reducing power, FRAP value assays were carried out. The results of the analysis obtained showed that CPHP from the PBC 159 clone was able to trap free radicals, and it was significantly different ($p < 0.05$) compared to the other two clones. In addition, hydrolysate from clone PBC 159 was also able to lower the oxidation component.*

***Keywords**:* Antimicrobial, Antioxidant, Bioactives peptide, Cocoa pod husk, Protein

INTRODUCTION

Theobroma cacao L. (Sterculiaceae) or cocoa is an economically important crop and grown worldwide with the purpose to gain cocoa beans for chocolate production. In Malaysia, it is an important commodity for the country due to its high economic value (Daud *et al.*, 2013). Cocoa beans were harvested and fermented and become into multiple different products from cocoa butter to chocolate. According to Makinde *et al.*, (2019), in the year 2017 to 2018, cocoa production was estimated at 4.49 million tonne worldwide. However, along with its great economic importance, cocoa production generates substantial quantities of waste. Since 2009, with the large quantity of cocoa pod husk, an estimated 70% of waste was produced (Makinde *et al.*, 2019).

Agriculture waste is defined as remnants, the aftermath of an agriculture activity (Nagendran, 2011; Obi *et al.*, 2016). Cocoa pod husk (CPH), is considered as an agriculture waste as it is the by-product from the fruit. When cocoa beans are harvested from the fruit, the remaining part of the fruit left is the pod itself (Yusof *et al*., 2016). According to Laconi & Jayanegara (2015), the cocoa pod husk held up to 75% of the fruit weight. Meanwhile, according to Kilama *et al.* (2019), the cocoa pod husk held about 81 to 90% of the fruit weight, concluding that only 10% of the fruit is commercialized. This situation represents a serious disposal problem (Figueira *et al*., 1993). Normally, the pods are left to rot on the cocoa plantation, which can cause environmental problems. Besides producing foul odours, rotting cocoa pod husks can propagate diseases, such as black pod rot, when left on the cocoa plantations (Donkoh *et al*., 1991; Barazarte *et al*., 2008). The poorly decomposed cocoa pod husk also caused major problems to the drainage system and can possibly be the cause of water pollution (Kilama et al., 2019).

In Malaysia and other cocoa producing countries, processing this cocoa waste could provide economic advantages and decrease some of the environmental problems. Cocoa pod husks is one example of a naturally available agricultural waste, which have the potential for applications in biotechnological aspect because it is non-toxic, abundant, easily available, totally re-generable, non-exotic, cheap and able to support the rapid growth (Dhanasekaran *et al*., 2011).

Since cocoa pod husks are readily available, it could be used to recover value-added compounds such as bioactive protein. According to Adamafio *et al*. (2004), 15% of the chemical compositions are made up from proteins, which meant that the cocoa pods husk can be a high potential source for obtaining bioactive protein.

Recently, interest has been emerging to produce and characterize bioactive protein from plant sources, due to their availability, safety and nutritional aspects. Proteins extracted from several plants by products are widely used as nutraceuticals and functional food ingredients for health promotion and disease risk reduction (Zarei *et al*., 2014).

Bioactive protein is defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen and Pihlanto, 2006). The beneficial effects are attributed to different properties found in protein such as antimicrobial (Reddy *et al*., 2004; Rajanbabu and Chen, 2011), antioxidant (Sarmadi and Ismail, 2010), antithrombotic (Wang and Ng, 1999), antihypertensive (Erdmann *et al*., 2008) and immunomodulatory activities (Gauthier *et al*., 2006).

Protein hydrolysates, once liberated as independent entities, can act as potential metabolism modulators and regulatory compounds with hormone-like activities. Upon oral administration, bioactive protein may affect the major body systems depending on their amino acid sequence. Many protein hydrolysates are known to reveal multifunctional properties (Meisel and FitzGerald, 2003).

Several synthetic antioxidants have been widely used in the food, pharmaceutical and cosmetic industries (Di Bernardini *et al*., 2011). However the use of these synthetic antioxidants, as radical scavengers possesses a potential health risk to the human body. Therefore, natural antioxidants are in high demand as dietary supplements to reduce the risk of aging, inflammation and cardiovascular diseases consequently improving the human health.

MATERIALS AND METHODS

1) Sampling

Four cocoa clones (PBC 123, PBC 159, KKM 22 and MCB C1) collected from Cocoa Research and Development Centre Jengka and Cocoa Research and Development Bagan Datuk Perak were used for the preliminary study. The purpose of the selection from two different areas was to evaluate the protein value of different topography and soil type. Based on the preliminary study, three of the four clones with the highest protein value in their pod husk were selected.

2) Preparation of Cocoa Pod Husk Powder

The cocoa pod husks (CPH) from these clones were dried using drying methods, oven and freeze dryer. These two methodologies were used to compare whether different drying processes could affect the nutrient composition of cocoa pod husk or not. Prior to drying, all the CPH samples are coded as listed in Table 1.

a) Oven drying

Cocoa pod husk were dried in a Memmert oven at 50°C for 48 hours. Once the cocoa pod husk is dry, it is finely ground and filtered with a 100µm particle size filter.

b) Freeze drying

The cocoa pod husks were put in a freeze drying (Labconco, US). The temperature was adjusted at -50°C. The cocoa pod husks were left to freeze dry for 24 hours. This method was used to allow the sample to be dried without using high temperatures and to prevent the sample from being damaged.
After drying, all the dried CPH was ground to 100µm particle size. All samples of powder were kept in air tight container prior to use.

Table 1**:** Sample codes of Cocoa Pod Husk sample from different drying process.

Sample Code	CPH Clone	Drying processing method
W22	KKM22	Freeze Dry
W159	PBC159	Freeze Dry
W123	PBC123	Freeze Dry
WC1	MCB C1	Freeze Dry
D22	KKM22	Oven Drying
D159	PBC159	Oven Drying
D123	PBC123	Oven Drying
DC1	MCB C1	Oven Drying

3) Protein Extraction from Cocoa Pod Husk (CPH)

CPH powder was defatted using Soxhlet apparatus with petroleum ether for 8 hours. After being defatted, the CPH powder was then dried for overnight in a ventilator at 20°C. Protein obtained from the extraction was then mixed together with NaOH (0.03 N), at a 1:30 ratio, and being extracted in Memmert water bath shaker at 150 rpm for 2

hours. After the filtration process, the pH of the supernatant was adjusted to pH 4.0 using HCl and the precipitation collected by the centrifugation (Centrifuge 5810R Eppendorf, Germany) at 10,000 × g for 10 min will be stored at -80°C for further analysis.

4) Determination of Crude Protein Content

a) Kjeldahl Method

The crude protein content of cocoa beans was determined based on the method of Kjeldahl AOAC (1990) with slight modifications.

b)Protein Analyzer

The samples were weighed into tin boats without pre-treatment and pressed to pellets using the manual pressing tool. Analysis was run with Protein Analyzer (Elementar, Jerman),at Analytical Service Laboratory, CITC Nilai, using a standard method implemented in the instrument software, with a total analysis time of about 5 minutes. A protein factor of 6.25 was applied to calculate the average protein content. All samples have been analysed ten times. The average difference between two successive analyses was calculated to compare to International Standard ISO 16634-2 (diff. N < 0.1%) and the relative standard deviation (RSD) to compare with International Standard AOAC 992.23 (RSD < 2%).

5) Preparation of Cocoa Pod Husk Protein Hydrolysate (CPHPH)

CPHPH was prepared using ezymatic hydrolysis, using Alkalase, Pepsin and Trypsin, separately. The enzymatic hydrolisis was carried out under optimal temperature and pH conditions to ensure the enzyme functioning well. Hydrolysis was stopped by adding 70% methanol. Then, the suspensions were stirred at room temperature for 1 hour and centrifuged at 20,000xg for 30 minutes. The supernatants were collected, and the methanol was removed using rotary evaporator. Finally, the aqueous solutions were stored at -80°C.

6) Proximate Analysis

The proximate analysis of cocoa pod husk powder was carried out in accordance with the Association of Official Analytical Chemists methods (AOAC, 2000). The parameters assayed involve the determination of moisture (Method 925.40), crude protein (Method 955.04), fat (Method 920.39), and crude fibre (Method 935.53) content in each cocoa pod powder. Results obtained were expressed in wet basis.

7) Enzymatic Hydrolysis of CPH Proteins

The process of hydrolysis is particularly important for the release of bioactive proteins (Gillo *et al.*, 1996). Therefore, protein hydrolysates from cocoa pod husk (CPH) were produced by enzymatic hydrolysis. To study the degree of hydrolysis (DH), several enzymes were used. The enzymes are alkalase, trypsin and pepsin. Enzymatic hydrolysis was carried out at the appropriate temperature and pH conditions to ensure that all the enzymes function in optimal conditions and the degree of hydrolysis (DH) of cocoa pod husk protein hydrolysate can be determined.

Table 2: Parameters for enzymatic hydrolysis with three different types of protease enzymes.

Enzymes	Temperature (°C)	pH
Alkalase	55	8.5
Pepsin	40	2.0
Trypsin	40	8.5

8) Determination Degree of Hydrolysis (DH%)

The determination of the degree of hydrolysis, DH (%) was based on the method proposed by Sathivel et al. (2003).

RESULT AND DISCUSSION

Determination of crude protein content

Table 3 shows a comparison of protein content using two different methods,: Kjehdahl Method and the Protein Analyzer equipment. A comparison between these two methods was to see the effectiveness of these two methods in obtaining protein values.

The Kjehdah method is a common method used in determining protein content. This method has been used for decades. The process of sample preparation for this method takes longer time, and also difficult to prepare. Apart from that, this method also uses sulfuric acid and hydrochloric acid chemicals that are harmful to consumers. However, by using a Protein Analyzer, the determination of protein content becomes more easier and the preparation method is also faster, where it uses semi-automated method, we just need to prepare the sample in a simple way, and then the Protein Analyzer instrument will process the data and results.

Table 3: Comparison of protein content using Kjehdahl Method and Protein Analyzer

Sample	Protein, g/100g	
	Kaedah Kjehdahl min±SD	**Protein Analyser min±SD**
PBC 123 Hilir Perak	5.33±0.08[a]	5.37±0.07[a]
PBC 159 Hilir Perak	9.15±0.08[a]	9.09±0.08[a]
KKM 22 Hilir Perak	5.00±0.00[a]	5.01±0.03[a]
MCB C1 Hilir Perak	4.48±0.08[a]	4.17±0.05[a]
PBC 123 Jengka	6.14±0.04[a]	6.24±0.10[a]
PBC 159 Jengka	10.22±0.15[a]	10.18±0.06[a]
KKM 22 Jengka	5.10±0.13[a]	5.37±0.07[a]
MCB C1 Jengka	4.09±0.07[a]	4.76±0.89[a]

In this study, four clones were used~~,~~: PBC 123, PBC 159, MCB C1 and KKM 22. These four clones were taken from two different areas~~,~~: Jengka (hilly and peatland areas) and Hilir Perak (areas near the sea and sandy soils). Based on the study, although we used two different methods, these two methods did not show any significant differences in terms of protein composition (P> 0.05). These two methods give similar results for the protein value determination. Therefore, it is easier to use the latest method as compared to the conventional method, where the latest method is cheaper, easier and faster. Statistical analysis using the T sample test on the results obtained, found that only clones of PBC 123 and PBC 159 had significant differences (p <0.05) between the two areas taken. Where the mean of both clones from Jengka gives a higher value when compared to the mean of clones obtained from Hilir Perak. While clones KKM 22 and MCB C1 did not show any significant difference between the two areas, the mean value for these two areas is the same, with p> 0.05.

Based on the results obtained, although only two clones showed significant differences between the two areas, it can be concluded that topography and soil type are also contributing factors to the protein value available for each clone. With the highest protein results obtained, clones of PBC 123, PBC 159 and KKM 22 from Jengka have the potential to have bioactive protein compounds in them and therefore these three clones were selected for further analysis.

Proximate Analysis

The nutrient composition of cocoa pod husk extract was made to obtain basic information related to the nutrient composition found in each selected clone. The nutrient composition of cocoa pod husk extract was calculated based on different drying methods, the first was using oven drying at 50°C for 48 hours, and the second was using freeze drying at --50°C for 24 hours. This comparison is made to see whether different drying processes can affect the nutrient composition or not.

Based on the results obtained in Table 4, it was found that the moisture for both drying methods showed a significant difference (p> 0.05) for KKM clone 22 and PBC clone 123, with the percentage difference between the two methods was between 17 to 56%. While clone PBC 159 did not show any significant difference for the two drying methods (p> 0.05).

Determination of moisture content plays an important role in knowing the properties of compounds. Based on a study from Nguyen & Nguyen (2017) fresh cocoa beans have a high moisture value of 87%. After undergoing both drying processes, both of these methods showed a decrease in the value of moisture content, as both methods functioned to remove water particles from the sample. Therefore, these two methods, freeze drying and oven drying are seen to reduce the moisture in the cocoa pod husk.

Table 4: Proximate compositions for two different drying methods

Drying method	Sample	Moisture (%) mean±SD	Protein (%) mean±SD	Fat (%) mean±SD	Fiber (%) mean±SD
Freeze dry	KKM 22	15.74 ± 0.05^{b}	7.55 ± 0.35^{c}	0.82 ± 0.02^{a}	32.15 ± 0.18^{b}
	PBC159	13.02 ± 0.10^{d}	10.32 ± 0.04^{a}	0.71 ± 0.04^{b}	27.18 ± 0.97^{c}
	PBC123	18.43 ± 0.02^{a}	9.23 ± 0.12^{b}	0.10 ± 0.00^{c}	35.02 ± 1.04^{a}
Oven drying	KKM 22	6.83 ± 0.10^{f}	7.54 ± 0.85^{c}	0.81 ± 0.02^{a}	30.98 ± 0.23^{b}
	PBC159	12.86 ± 0.13^{e}	9.68 ± 0.02^{a}	0.70 ± 0.02^{b}	27.07 ± 3.53^{c}
	PBC123	15.20 ± 0.03^{c}	9.11 ± 0.25^{b}	0.07 ± 0.01^{c}	34.94 ± 3.45^{a}

Based on a study from Claussen et al. (2007) and Maisnam et al. (2017), the freeze drying method provides a porous structure. The oven drying method, on the other hand, uses direct heating at high temperatures, and this increases the probability for the moisture to change rapidly (Aksoy et al., 2019) and further damage the bioactive content contained in the extract.

Referring to Table 4, shows the sample from PBC 123 clones have high moisture content when compared to other clones, with differences between 14 to 29%. This condition is because the PBC 123 clone has a relatively thick skin when compared to the other clones. Based on statements from Nyadanu et al. (2011), the moisture content of cocoa pod husk is also depend on the size and also the thickness of the cocoa pod itself. Since the PBC 123 clone has a thicker skin, it is able to retain moisture compared to other clones. With this it can be concluded that the differences type of clones for the cocoa pod husk influence the different results on its moisture value. Determination of protein crude content is based on nitrogen content in the sample. Referring to Table 4, for the freeze drying method, the protein content obtained ranged from 7.55% to 10.32%. With PBC 159 recorded the highest reading for protein content ($p < 0.05$) compared to other clones. For oven drying, the highest value recorded was also from PBC 159 clones, followed by PBC 123 and KKM 22. However, the value was slightly lower when compared to the freeze drying method, which was 9.68%. According to DeMan (1999) the heating process can cause protein to be destroyed and lost in the final product, and in addition the protein content is also affected by the nitrogen content found in non-protein materials. However, the crude protein content found in the study samples can be used as a source of bioactive protein, which is good for human diet.

The protein content for oven drying was lower ($p < 0.05$). The disadvantage of this method is that it uses a long time and requires a high temperature. High temperatures and repeated heating cause the protein to denaturate and also cause the heat sensitive skin structure of cocoa beans to be damaged. Based on Aksoy et al. (2017) drying using an oven caused a rate of depletion to the food samples and in turn caused a decrease to the quality of the food.

Fat is one of the sources of energy after carbohydrates. Determination of fat content was done to identify the remaining fat that is still present in the cocoa pod husk extract after defatting process was done. Based on Table 4, shows the remaining fat content found in cocoa pod husk extract, where the fat content is found to be low, which is between 0.10% to 0.82% for freeze drying method, and 0.07% to 0.81% for oven drying method. This low reading indicates the effectiveness of the defatting process performed at the beginning of the study Based on the results, these two methods did not show any significant difference ($p > 0.05$) in the content of crude fat determination. Meanwhile, based on the fat content between clones, KKM 22 clone had the highest fat content ($p < 0.05$) when compared to the other two clones with a difference of 13% when compared to PBC 159 clone and 87.8% with PBC 123 clone.

Based on Table 4,, the fiber content of the two different drying methods, showed a significant difference for KKM 22 only ($p < 0.05$), while the other two clones, clone PBC 159 and clone PBC 123 did not show any significant difference ($p > 0.05$) using both methods. Meanwhile, for clone comparison, clone PBC 123 showed a high fiber value ($p < 0.05$) when compared to the other two clones, followed by clone KKM 22 and PBC 159. The presence of crude fiber is important in the final result of a product, this is because fiber is made up of polysaccharides that cannot be digested by the body and are very beneficial in ensuring healthy intestinal activity.

Based on all the results obtained, it can be confirmed that between freeze drying and oven drying, freeze drying is the most suitable and more effective method of retaining different nutrient elements in the cocoa pod husk, including protein. However, it should be remembered that the diversity of cocoa clones can also affect differently with the two treatments, depending on their physical properties.

Hydrolysis of CPH proteins with different Enzymes

To improve the quality of cocoa pod husk protein (CPHP), the hydrolysis process was carried out enzymatically. In this study, CPHP was hydrolyzed using three different enzymes, alkalase, pepsin and trypsin. Figure 1 and Figure 2, shows the hydrolysis result (DH%) for each enzyme used. This study focuses on 2 main factors of hydrolysis, time of hydrolysis and enzyme concentration.

To study the effect of hydrolysis time, CPHP was hydrolyzed for 0, 6, 12, 24 and 48 hours. Within a 6 hour, it can be seen that all enzymes showed a pattern of increasing degree of hydrolysis (DH) (Fig. 1). This situation is very similar to the concept that has been put forward by Gbogouri et al. (2004) and Kim el al. (1990), in which the DH profile of the enzyme showed an increasing pattern. This is because all protease enzymes provide active properties in the early stages of the hydrolysis process.

Statistical comparisons according to different enzymes, using 2 sample T Test were made between alkalase and trypsin enzymes, along with alkalase and pepsin. Both comparisons showed that alkalase gave very significant values for both enzymes, with a reading value of $p < 0.05$. Where the alkalase enzyme gave the highest DH which is 64% to 72% within 48 hours of hydrolysis. However, when compared the mean of pepsin is not much different from the mean of trypsin with a p value between these two enzymes is 0.257, which is $p > 0.05$. The pattern of hydrolysis shown by pepsin and trypsin is almost the same, with an increase after the first 6 hours up to 24 hours, and then a decrease in the 48 hours.

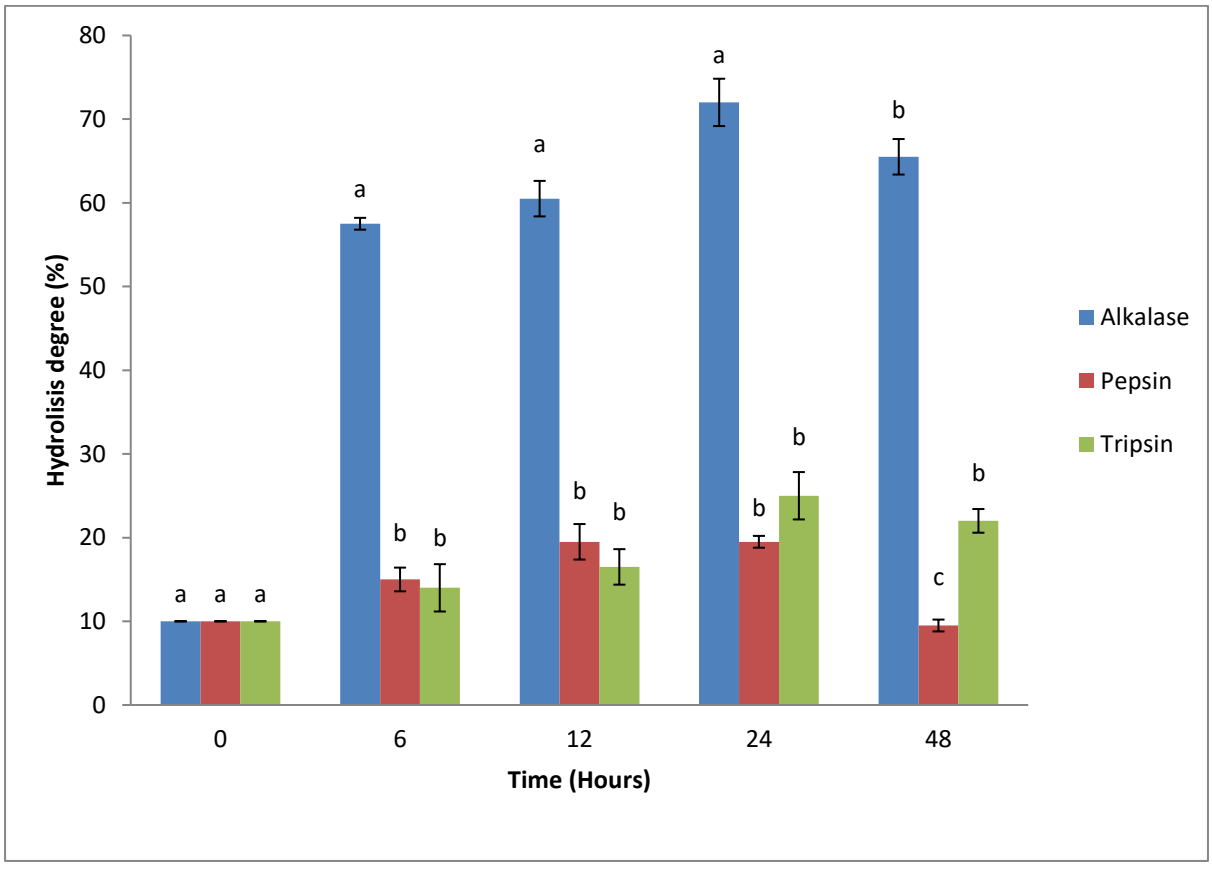

Figure 1: Effect of hydrolysis time on % DH of CPHP using 3 types of enzymes with different enzyme concentrations

As noted in the study of Ng et al., (2013), protease enzymes will act on native protein molecules rapidly to break down polypeptide bonds on the molecular surface and in turn cause the protein molecules to be hydrolyzed. The denser the protein molecule the slower the hydrolysis process takes place. Due to the slow kinetics of the enzyme and the small amount of protein, the time to complete the hydrolysis becomes longer.

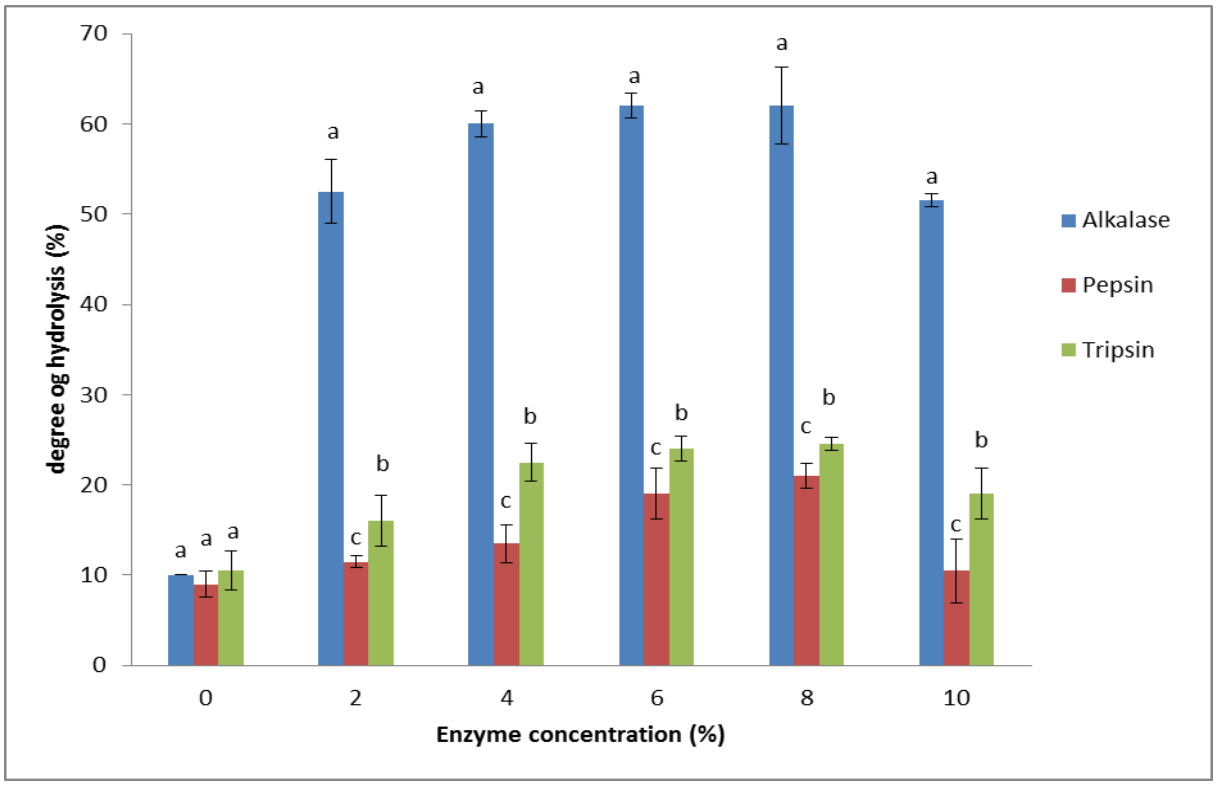

Figure 2: Effect of enzyme concentration on % DH of PCPH hydrolysate for one hour of hydrolysis

Enzymatic activity is directly proportional to its concentration. In Figure 2 it can be observed that when the protease enzyme concentration increased between 0 to 8%, then the % DH of CPHP hydrolysate was also increased during the hydrolysis period carried out, and decreased when it reached a concentration of 10%. Increased in enzyme concentrations mean these enzymes are able to break more CPHP peptide bonds.

Based on the statistics of T test 2 samples, between alkalase and trypsin enzymes, along with alkalase and pepsin, it was found that alkalase enzyme is the most active and effective protease enzyme, which can be seen in Figure 2, % DH increased up to 3 times more than other enzymes ($p < 0.05$). This is due to the specialization properties present in each protease enzyme and acting specifically on peptide bonds (Ng, 2013).

For the effect of enzyme concentration on the Degree of Hydrolysis (DH), the pattern shown by trypsin and pepsin was slightly different. Statistical comparative analysis made between pepsin and trypsin as a whole also showed a significant difference between the two ($p < 0.05$). Within one hour, trypsin was found to provide a more effective enzymatic hydrolysis rate than pepsin enzyme. This is in line with the study by Sweeney and Walker (1993), where trypsin only acts on certain lateral bonds and the rate of hydrolysis slows down when it reacts with acidic residues or it may not react at all if there is a proline residue on the lateral peptide bonds.

In addition, based on Rebeca et al. (1991) stated that alkaline protease enzymes such as alkalase and trypsin are able to hydrolyze more effectively when compared to acidic enzymes, such as pepsin. Therefore, this statement confirms that the results obtained using trypsin enzyme are faster ($p < 0.05$) when compared to pepsin enzyme. However, the trypsin enzyme does not provide a high degree of hydrolysis even though it comes from the same family as alkalase. From the results obtained, it can be said that the enzyme alkalase and trypsin enzyme, which is an enzyme that is alkaline is the most suitable enzyme for hydrolyzing protein from the skin of cocoa beans.

CONCLUSIONS

High demand exists for cocoa beans and their contents, but today's cocoa pod husk is also a lucrative by-product of the food business. The husk from cocoa pods is a good source of bioactive chemicals, protein, and dietary fiber. This finding demonstrates the potential for antioxidant action of cocoa pod husk proteins. Some of the components in the extract may be the cause of the biological activities it exhibits. This research demonstrates the significant value of acquiring cocoa pod husk (CPH) from a plentiful, affordable, renewable, and sustainable source. CPH will be used as a high-value

resource and will benefit to consumers in terms of nutrition and health.

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