OPTIMISATION OF ENZYMATIC HYDROLYSIS CONDITIONS FOR ENHANCED PROTEIN RECOVERY FROM COCOA POD HUSK (*THEOBROMA CACAO L.*)

Hannim Haffeza Haris[1]* and Yusof Maskat[2]
[1] Malaysian Cocoa Board, Nilai, 71800 Negeri Sembilan, Malaysia
[2] Department of Food Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia
**Corresponding author: hannim@koko.gov.my*

Malaysian Cocoa J. 18: 38-41 (2026)

ABSTRACT - *The pursuit of sustainable and functional plant-based proteins has led to the exploration of agro-industrial by-products as potential raw materials. Cocoa pod husk (CPH), an abundant agricultural waste from Theobroma cacao L., is recognised for its potential as a protein-rich source. This study investigates the impact of enzymatic hydrolysis conditions on the degree of hydrolysis (DH) of CPH-derived protein hydrolysates. Protein was extracted from three selected cocoa clones and subjected to enzymatic hydrolysis using Alcalase, Pepsin, and Trypsin under fixed pH and substrate ratios. The study evaluated enzyme type, concentration, and hydrolysis duration. The optimum DH achieved was 72%, using 6% Alkalase over 24 hours. Alcalase resulted in the highest DH yield, whereas Trypsin demonstrated a faster hydrolysis rate. Among the clones, PBC 159 exhibited the highest protein content among the evaluated clones. These findings support the potential use of CPH protein hydrolysates in functional food formulations with prospective bioactivities.*

Keywords: Cocoa pod husk; enzymatic hydrolysis; protein hydrolysate; degree of hydrolysis; Alcalase; Trypsin

INTRODUCTION

Nowadays, the demand for natural and nutrient-dense food products is surging simultaneously with the increment of human consumption behavior. However, the need to use many synthetic antioxidants including butylated hydroxytoluene (BHT) butylated hydroxyanisole, tert-butylhydroquinone, and propyl gallate in food increased frequently in the food industry purposefully to prolong the shelf-life of perishable food. The perpetuating consumption of these compounds has expressed concerns over their linkage to possible health hazards. As such, identifying or finding natural alternatives to replace the synthetic antioxidants is addressed. Various scientific publications have centered on the utilization of animals, plants, or fungal reservoirs for the synthesizing of biologically active peptides and proteins, which in the future may serve as promising replacements for synthetic antioxidants on the markets (Zarei *et al*., 2012).

Theobroma cacao L. or commonly known as cocoa, is one of the economical industrial crops that worldly notable as the main ingredient for chocolate production. Although the cocoa production in Malaysia is considerably low, the production is quite profound globally. In the year 2017 to 2018, cocoa production was estimated at 4.49 million worldwide. However, the production of cacao generates a substantial amount of cocoa pod husk (CPH) approximately 70% of the whole production which helps up to 81 to 90% of the fruit weight (Laconi and Jayanegera, 2015). This biblical waste production poses a serious disposal issue as it feasibly causes environmental problems like water pollution and induces environmental diseases such as black pod rot if left untreated. On the other hand, CPH is seen as a possible natural available agricultural waste to be utilized in biotechnological industries because of its non-toxic, abundancy, total re-generable, non-exotic, and affordable (Dhanasekaran *et al*., 2011). Since CPH is readily available, it could be used to recover value-added compounds such as hydrolysates which contain biologically active proteins. Scientists have studied oligopeptides that reside in cocoa not only limited to generating the chocolate flavor but also potentially exhibiting biological effects. As a result of these avenues, several reports have demonstrated the expression of peptide sequences when released within cocoa beans exerting potential beneficial health effects (Adamafio *et al*. 2004).

By definition, protein hydrolysates are a complex mixture of oligopeptides, peptides, and free amino acids that are produced by partial or extensive hydrolysis, while biopeptides or bioactive peptides are defined as peptides that possess beneficial

pharmacological properties (Korhonen and Pihlanto, 2006). Of all protein extraction techniques, enzymatic hydrolysis is considered the most efficient and useful method due to its effectiveness, easy scaling up, and great predictability (Guo *et al*., 2005). Enzymatic hydrolysis is known to decrease the peptide size, breaking down the peptide structure into available amino acid sources for other uses while improving the functional properties of proteins and maintaining their nutritional value (Meisel and FitzGerald, 2003). The efficiency of this process relies on several factors including enzyme type, substrate, and hydrolysis conditions such as enzyme concentration, temperature, pH, and time. These factors cooperatively affect the enzyme activity and thus make the hydrolysis process more controllable.

The application of enzymatic hydrolysis in extracting proteins from cocoa beans is well employed, especially with commercial enzymes (Adamafio *et al*. 2004). However, the utilization of enzymatic hydrolysis using other enzymes, particularly on cocoa's agricultural waste has not yet been executed. To the best of our knowledge, this is the first study that deals with the preparation of protein hydrolysate from CPH by using other known enzymes. The current study aimed to investigate the effects of the type of enzymes, time, and concentration of enzymes on the degree of the production of protein hydrolysate of CPH.

MATERIALS AND METHODS

Sampling and Preparation of CPH powder
CPH samples from four cocoa clones collected from the Cocoa Research and Development Centres in Jengka (Pahang, Malaysia) and Bagan Datuk (Perak, Malaysia) were used for the preliminary study (PBC 123, PBC 159, KKM 22, and MCB C1).

After the preliminary study, three clones that have highest protein value were selected. Samples were dry using oven drying at 50 °C for 48 hours using a Memmert oven. Once the cocoa pod husk is dry, it is finely ground and filtered with 100µm particle size.

Determination of Crude Protein Content
The crude protein content was determined based on the method of Kjeldahl AOAC, 1990, with slight modifications.

Protein Extraction
Defatting of CPH powder was performed using petroleum ether in a Soxhlet extractor for 8 hours. The powders were then being dried for overnight in a ventilator at 20°C. Protein obtained from the extraction was then mixed with 0.03 N NaOH in a 1:30 ratio. Extraction was carried out in a water bath shaker at 150 rpm for 2 hours. The supernatant was adjusted to pH 4.0 using HCl and centrifuged at 10,000 × g for 10 minutes. The precipitate was stored at –80 °C for further analysis.

Preparation of Protein Hydrolysate
CPH Protein hydrolysate was prepared with variable parameters including enzymes (Alcalase, Pepsin, and Trypsin), time and enzyme concentrations. The enzymatic hydrolysis was carried out under optimal temperature and pH conditions to ensure the enzyme function well. Hydrolysis was stopped by adding 70% methanol. Then the mixture was stirred at room temperature for 1 hour, centrifuged at 20,000×g for 30 minutes. The supernatants were collected, and the methanol was removed using a rotary evaporator. Finally, the hydrolysates were stored at –80 °C.

Degree of Hydrolysis
The degree of hydrolysis, DH (%) was determined using the method proposed by Sathivel *et al*. (2003).

Statistical Analysis
All experiments were performed in triplicate and the results are expressed as mean ± standard error. The data were analysed by one-way ANOVA test using statistical analysis software (version 15.2). The results were considered statistically significant for a P value less than 0.05.

RESULTS AND DISCUSSIONS

Protein content as affected by different area
The results shown in Table 1, revealed that all the clone had no significant difference in terms of protein value. But when comparing the protein value according to the soil area, CPH collected from Jengka Pahang yielded a higher protein value compared to other clones obtained from Hilir Perak. While for clones KKM 22 and MCB C1 did not show any significant difference for the two areas, where the mean value for these two areas is the same with $p > 0.05$.

Based on the results obtained, it can be concluded that topography and soil type are also among the 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 Pahang Malaysia, were selected for further analysis, as they had the highest protein findings and may contain bioactive protein compounds.

Table 1. Protein content using Kjehdahl method

Samples	Protein, g/100g
PBC 123 Hilir Perak	5.33 ± 0.08[a]
PBC 159 Hilir Perak	9.15 ± 0.08[a]
KKM 22 Hilir Perak	5.00 ± 0.00[a]
MCB C1 Hilir Perak	4.48 ± 0.08[a]
PBC 123 Jengka	6.14 ± 0.04[a]
PBC 159 Jengka	10.22 ± 0.15[a]
KKM 22 Jengka	5.10 ± 0.13[a]
MCB C1 Jengka	4.09 ± 0.07[a]

[a] the same letter on the same line indicates no significant difference (p>0.05). All results are presented as mean ± standard deviation (n=3)

Degree of Hydrolysis across Enzymatic Conditions
Figures 1 and 2 showed that the degree of hydrolysis varied according to the type of enzymes, enzyme concentrations, and time respectively.

From Figure 1, it was demonstrated that alkalase gave the highest degree of hydrolysis at a maximum of 70% after 24 hours before reducing substantially after 48 hours. The mean of trypsin and pepsin, when compared, do not differ significantly; the p-value between these two enzymes is 0.257, meaning that 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.

As noted in the study of Ng *et al*. (2016), 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. In addition, based on Rebeca *et al*. (1991) stated that alkaline protease enzymes such as alcalase and trypsin can hydrolyze more effectively when compared to acidic enzymes, such as pepsin. This statement thus verifies that, in comparison to the pepsin enzyme, the outcomes produced with the trypsin enzyme are better and can hydrolyzed more effectively (p <0.05). However, the trypsin enzyme does not provide a high degree of hydrolysis even though it comes from the same family as alcalase. From the results obtained, it can be said that the alcalase and trypsin, which is an alkaline enzyme is the most suitable enzyme for hydrolyzing protein from the cocoa pod husk.

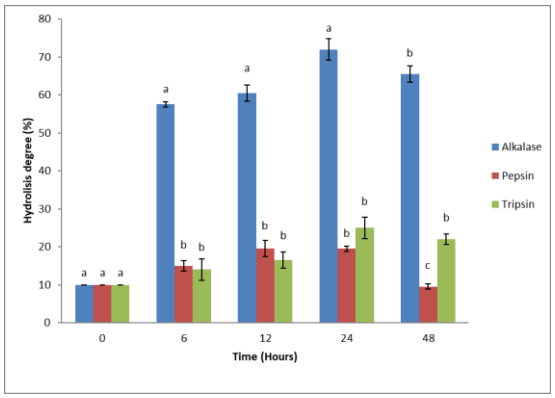


Figure 1: Effect of varying hydrolysis time on CPH protein hydrolysates using three different enzymes.

The results obtained showed that enzymatic activity is directly proportional to enzyme concentration. In Figure 2 it can be observed that when the enzyme concentration increased between 0 to 8%, then the % DH of CPH protein 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 alcalase and trypsin enzymes, along with alcalase and pepsin, it was found that alcalase 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 (Sweeney and Walker, 1993).

For the effect of enzyme concentration on the Degree of Hydrolysis (DH), the pattern shown by trypsin and pepsin were 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.

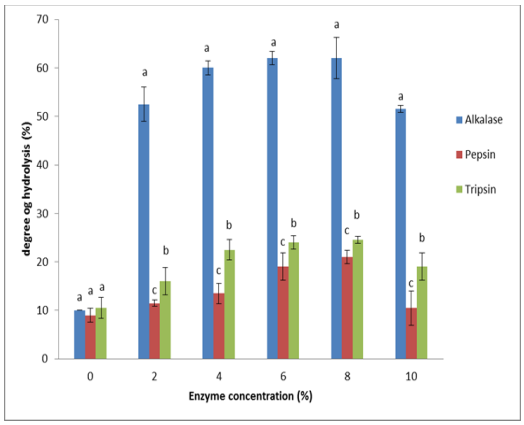


Figure 2: Influence of enzyme concentration on the degree of hydrolysis (%) of protein hydrolysate after one hour.

CONCLUSIONS

This study demonstrates that enzymatic hydrolysis of CPH varies with enzyme type, concentration, and duration. PBC 159 exhibited the highest protein content among the evaluated clones. Alcalase was the most effective enzyme, achieving a DH of 72% under optimal conditions. The findings show that enzymatic hydrolysis can produce protein hydrolysates from cocoa pod husk, with alcalase giving the highest DH. Future studies are needed to evaluate their functional, nutritional, and bioactive properties before they can be used as functional food ingredients.

ACKNOWLEDGEMENTS

The authors acknowledge the Malaysian Cocoa Research Board for financial and technical support.

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