In vitro protein digestibility of enzymatically pre-treated cocoa bean powder using commercial protease

W Haliza\textsuperscript{1}, E Y Purwani\textsuperscript{1}, D Fardiaz\textsuperscript{2}, and M T Suhartono\textsuperscript{2}

\textsuperscript{1} Indonesian Center for Agricultural Postharvest Research and Development. Bogor 16114, West Java. Indonesia
\textsuperscript{2} South East Asian Food and Agricultural Science and Technology, SEAFAST CENTER, Bogor Agriculture University. Bogor 16680, West Java. Indonesia

Email: haliza2013@gmail.com

Abstract. The quality and selling value of cocoa beans could be greatly improved through proper drying of fermented cocoa. Cocoa products are one of the foods that are in great demand by the community, and the fruit could be a source of vegetable protein production. However, the nutritional value of a food is determined by both its total protein content, and the availability of digestible protein. Therefore, the digestibility of this class of food needs to be evaluated. This study was conducted using cocoa beans from the regions of Bali, Jember, Yogyakarta, and Sukabumi, with 2 types of cocoa powder samples namely fat-free, and fat-polyphenols. Furthermore, it used a completely randomized factorial design, consisting of two factors. The first factor was pepsin concentration, comprising of 1000 and 2000 U/ml, while the second was pancreatic activity, consisting of 3 and 8 USP. The protein content of the cocoa was tested and the digestibility was determined. The results show that fat-polyphenol-free cocoa powder with 1000 U/mL pepsin and 3 USP pancreatic and Jember had the highest protein digestibility with a value of about $\pm$ 95\%. Furthermore, the results of protein digestibility showed that variations in the concentration of pepsin and pancreatic enzymes had a significant effect on the protein. Therefore, this research shows that interactions between compounds present in foods, like protein-phenolic, could have a significant effect on protein digestibility.

Keywords: Fermented cocoa beans, protein digestion, digestive enzyme, pepsin, pancreatic

1. Introduction
Indonesia is one of the world’s highest cocoa producing countries, contributing around 13\% of the global production [1]. However, most cocoa beans are produced in smallholder plantations, which have not applied the proper fermentation technology, leading to the production of low quality fermented cocoa beans. In order to improve their quality, these beans need to undergo standard operating procedure for the fermentation of cocoa [2]. Finally, the chemical properties of cocoa beans are important factors in determining their quality [3].

One of the components that determine the nutritional quality of cocoa is its protein and amino acid content [4]. Furthermore, the content and composition of these compounds are strongly influenced by the fermentation process [5]. Before being consumed, cocoa beans are processed into intermediate products such as cocoa powder, liquor, mass, and others. Then they are processed into a chocolate product, which is one of the most demanded foodstuffs by people of all ages. Consequently, the
nutritional properties of this product depend on the composition of the dissolved protein in the cocoa intermediate product [6].

Although the quantity of nitrogen in cocoa and chocolate products are included in the total protein content of foods formulated for labeling purposes, they are usually consumed without much regard for the protein nutritional value. In fact, many studies have shown that the biological value of protein depends on the nitrogen content of cocoa [7] and its digestibility [8]. Furthermore, studies on protein digestion are used to assess bioavailability and increase the health benefits of chocolate by producing new biopeptides [8]. There are indications that levels of undigested protein which cause food allergies can be reduced by increasing its digestibility [9].

The solubility and biological value of proteins are very important to their availability in the body. Therefore, food ingredient assessment can only be carried out by using both protein digestibility and chemical composition or characterization. Previous studies have used digestive enzymes in vitro to test protein digestibility [10-11]. The combination and concentration of the enzymes used will affect the level of protein digestibility [10-12]. Therefore, the aim of this study was to determine the soluble protein content of finely fermented and fat-free cocoa powder, and fat-free polyphenols. It also aims to determine the protein digestibility of cocoa powder by enzymatically using commercial proteases (digestive proteases) with different concentrations and activities.

2. Materials and methods

2.1 Materials
The raw material used was superior fermented bulk cocoa beans which were obtained from four regions in Indonesia, namely Sukabumi, Yogyakarta, Jember, and Bali. All cocoa samples were dried, unroasted, and stored at -20°C. Pepsin and pancreatic was purchase from Sigma-Aldrich, USA. All chemicals used were pro-analytically purchased from Merck Chemical, Germany and Sigma-Aldrich, USA.

2.2 Methods
The research was carried out using a completely randomized factorial design, consisting of two factors and three replications. The first factor was pepsin concentration, which comprised of 1000 U/ml and 2000 U/ml. The second factor was pancreatic activity, consisting of 3USP and 8USP.

2.3 Preparation cocoa powder
Beans from each region were de-shelled manually before grinding. However, the sample was ground using a commercial coffee grinder, dispersed in an n-hexane ratio of 1:5 (w/v) and defatted by using a Rapid Soxtherm Extraction Apparatus. After defatting, the powder was spread on a plate for 2 to 5h at room temperature under a laboratory fume hood to remove the excess solvent. It was then sieved (60 mesh), packed in polyethylene bags, and stored in a refrigerator at -20°C until further analysis. Fat-polyphenols-free cocoa powder was produced by washing three times defatted cocoa powder consecutively with 70%, 80%, and 100% cold aqueous acetone (-20°C). The polyphenol extract was removed through centrifugation (20 min, 10000x g, 4°C) [13].

2.4 Enzyme digestion
In vitro protein digestibility testing of enzymatically pre-treated, cocoa bean powder was performed using the Minekus procedure [12] with slight modifications. It was then briefly suspended in water (1:20 w/v) and then sequentially digested by enzymes such as pepsin/substrate 1:20 (w/w) (pH 2.0) and pancreatic/substrate 1:20 (w/w); pH 7.5 at 37 °C for 2h each. In addition, the hydrolysis was stopped by heating at 100 °C for 20 min, and the resulting cocoa hydrolysates (CH) were centrifuged at 20000x g for 15 min at 4°C. They were then stored at -20 °C until analysis.

2.5. Determination of proteins content and in vitro protein digestibility
Total protein concentration was measured using the Bradford method, [14] with bovine albumin as a standard. The in vitro protein digestibility was evaluated based on total soluble protein content and the protein content after digestion in vitro [15].
PD (%) = 100% - \left( \frac{P_t}{P_r} \times 100\% \right),

PD – in vitro digestibility of protein; P_t – total protein content before in vitro digestion; P_r – the content of proteins after in vitro digestion.

2.6. Statistical Analysis
The research was carried out using three replications for each treatment. Furthermore, the data was analyzed statistically through one-way and variance analysis (ANOVA) at a significance level of 0.05 using SPSS 16. The difference in mean values was determined through Duncan’s Multiple Range Test (DMRT) (p<0.05) to obtain the difference between treatments.

Figure 1. The protein content of cocoa powder, a) untreated, b) fat-free, c) fat-polyphenol-free
3. Result and Discussion

3.1. Protein content
Determining the soluble protein content was done using the Bradford test method, and Coomassie brilliant blue (CBB) dye which binds to proteins in an acidic solution giving a blue color. A bluer color indicates a high soluble protein content, of which the results of the cocoa powder can be seen in Figure 1. The protein content in each sample shows different levels because the cocoa beans were fermented with different periods and different experimental designs. Consequently, the effect of fermentation on proteins has yielded inconsistent results, likely due to different experimental designs, study durations, and variations in the initial protein or amino acid profile of foods. Several studies report an increase [15-17], while others observed a decrease [17] in protein upon fermentation.

Based on these results, an increase in protein content was observed after the hydrolysis of pepsin and pancreatic enzymes. Furthermore, the highest increase in protein content occurred after hydrolysis with pepsin. According to Figure 1, cocoa bean powder untreated (raw) has a protein content smaller than that of the fat-free powder and fat-polyphenols powder. This indicates that cocoa powder without treatment (raw) contains fat and polyphenols in undesirable proportions. Meanwhile, fat-free cocoa powder has a lower soluble protein content compared to the fat-polyphenols-free variety; it occurs because fat-polyphenols-free powder beans do not possess undetected protein-polyphenol complex bonds which make proteins more concentrated. Furthermore, polyphenols with higher molecular weight bind to protein thereby affecting their absorption [18].

3.2 Protein digestibility
Protein digestibility is the ability of a protein to be hydrolyzed into amino acids by digestive enzymes [19]. It can be affected by protein content. The result of the fermentation process will provide a different profile of the resulting soluble protein, whether it is easily digested or not. Furthermore, it increases the digestibility of plant proteins [16, 20-21], which have low digestibility compared to animal protein. This low digestibility may cause gastrointestinal upset which may lead to the fecal excretion of protein. Meanwhile, increased protein digestibility could reduce the levels of undigested proteins which can potentially cause food allergies due to poor absorption in the gut [9].

The results of protein digestibility testing in fat-free cocoa powder (Table 1) show that variations in the concentration of pepsin and pancreatic enzymes were higher when pepsin was used alone for each sample (Bali, Jember, Yogyakarta, and Sukabumi). Furthermore, variance analysis showed that concentration and combination enzymes had significant differences (p<0.05) in protein digestibility. The results also show that Jember cocoa powder has the highest protein digestibility with a range of 95%. Table 2 shows similar results for the fat-polyphenol-free cocoa powder such that variations in the concentration of pepsin and pancreatic enzymes had significant differences (p<0.05).

| Treatment | Fat-free cocoa powder (%) | Bali | Jember | Yogyakarta | Sukabumi |
|-----------|--------------------------|------|--------|------------|----------|
| Pepsin 1000 | 53.55±0.03<sup>d</sup> | 64.32±2.06<sup>c</sup> | 45.44±0.99<sup>c</sup> | 65.84±1.20<sup>c</sup> |
| Pepsin 1000+ Pancreatin 3USP | 72.67±1.31<sup>b</sup> | 86.89±1.09<sup>a</sup> | 45.67±3.47<sup>a</sup> | 72.36±1.00<sup>b</sup> |
| Pepsin 1000+ Pancreatin 8USP | 66.90±3.32<sup>e</sup> | 68.54±1.34<sup>e</sup> | 54.32±0.85<sup>e</sup> | 63.71±0.11<sup>e</sup> |
| Pepsin 2000 | 53.04±0.02<sup>d</sup> | 65.56±0.23<sup>d</sup> | 53.08±0.11<sup>d</sup> | 66.58±0.98<sup>d</sup> |
| Pepsin 2000+ Pancreatin 3USP | 64.54±1.95<sup>c</sup> | 64.69±1.81<sup>c</sup> | 52.51±1.94<sup>d</sup> | 71.34±1.44<sup>b</sup> |
| Pepsin 2000+ Pancreatin 8USP | 65.85±0.22<sup>c</sup> | 64.34±0.18<sup>c</sup> | 28.86±2.01<sup>c</sup> | 47.84±0.18<sup>a</sup> |

Remark: Numbers followed by the different letter were significantly different based on DMRT’s test 5%

Furthermore, the analysis of variance in Table 2, shows that the protein digestibility values of cocoa beans from areas (Bali, Jember, Yogyakarta, and Sukabumi) are high due to the in vitro combination of pepsin and pancreatic 3USP enzyme. Each sample undergoes a different fermentation time, which causes a difference in exposure to some of the hydrophilic groups. Furthermore, changes in the protein's solubility are caused when the molecular weight is reduced as a result of enzymatic modification. The
increased solubility observed in Jember cocoa beans is as a result of smaller peptides being generated by hydrolysis with this protease complex, and the exposure of their hydrophilic groups, which would increase interactions between the hydrophilic amino acids and water molecules. This property makes this sample easy to digest.

### Table 2. Protein digestibility (%) of fat-polyphenol-free cocoa powder

| Treatment                  | Bali          | Jember         | Yogyakarta     | Sukabumi       |
|----------------------------|---------------|----------------|----------------|----------------|
| Pepsin 1000                | 27.62±0.66    | 38.96±0.94     | 6.04±0.10      | 9.62±0.38      |
| Pepsin 1000+ Pancreatin 3USP | 83.75±2.02    | 92.10±1.23     | 38.93±1.10     | 84.41±0.36     |
| Pepsin 1000+ Pancreatin 8USP | 45.51±0.44    | 92.20±0.18     | 42.22±0.43     | 43.05±0.24     |
| Pepsin 2000                | 40.59±0.66    | 56.81±1.29     | 48.87±0.54     | 28.75±0.74     |
| Pepsin 2000+ Pancreatin 3USP | 84.61±1.04    | 92.23±4.74     | 44.67±0.62     | 44.04±3.14     |
| Pepsin 2000+ Pancreatin 8USP | 37.56±0.93    | 92.21±0.02     | 35.60±1.57     | 57.36±4.86     |

Remark: Numbers followed by the different letter were significantly different based on DMRT’s test 5%

Tables 1 and 2 show that Jember cocoa powder has a higher digestibility quality when compared to others. Furthermore, the highest protein digestibility was observed in fat-polyphenol-free cocoa powder after using pepsin 1000 U/mL and pancreatic 3 USP. The authors suggested that the rise in digestibility may be due to a reduction in protein cross-linking. This was caused by of non-nutritive compounds (e.g. phenolics and tannins), which make them more susceptible to proteolytic attack. The increase in protein digestibility after fermentation is due to the partial breakdown of complex storage protein into more soluble forms [22]. Furthermore, combining fermentation with other processing methods gives more advantages, because not all samples of cocoa powder have the same digestive profile.

Phenolics can precipitate proteins; therefore, their bioavailability may be significantly reduced. As a result, it may be speculated that the nutraceutical and nutritional potential of enriched cocoa powder is limited by chemical interactions, which influence bioaccessibility and bioavailability. Therefore, food enrichment is justified only when the bioactive components are bioaccessible and bioavailable. Previous studies state the same thing about flavonoids from onion skin; however, the bioactivities of enriched bread with this ingredient are significantly lower than expected [23]. Consequently, the objectives for the next study are twofold: (a) to reveal and examine potential protein–phenolic interactions, (b) to discuss results concerning phenolic levels, antioxidant activity, and protein digestibility of cocoa powder in the light of in vitro bioaccessibility.

### 4. Conclusions

The enzymatically treated cocoa powder showed a significant increase in soluble protein content and digestibility when compared to the untreated cocoa powder. Furthermore, fat-polyphenol-free cocoa powder has a higher protein digestibility compared to the others. The highest protein digestibility was observed in Jember fat-polyphenol-free cocoa powder which used pepsin 1000 U/mL and pancreatic 3 USP. Polyphenols are secondary metabolites in plant. Furthermore, their interaction with compounds presents in foods, like protein can have a significant effect on its digestibility. The contradictive effect can be minimized as a result of cocoa polyphenols impact on health. However, increasing protein digestibility could reduce the levels of un-digested proteins which can potentially cause food allergies.

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