Potency of Protein Cocoa Beans as Food Bioactive Precursor to Prevent Hypertension

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Abstract. Cocoa beans are superior plantation commodity in Indonesia. The fermentation practice on cocoa beans process has a very important role not only to produce quality seeds but also bioactive peptides. Proteolysis that occurs during cocoa fermentation can produce peptide fragments that have health benefits, such as antihypertension. Hypertension is a major risk factor for cardiovascular diseases and has an important impact on public health. Scientific research gathered strong evidence about the role of cocoa beans peptide involved in biochemical pathways in the control of blood pressure that is endothelin converting enzyme systems. This paper describes the current literature that explain potency of protein cocoa beans in action on angiotensin converting enzyme inhibition, that might antihypertensive effects of biologically active peptides from cocoa beans.

1. Introduction
Cocoa beans (Theobroma cacao L.) are the main raw material in chocolate and a major flavouring ingredient in the preparation of beverages, confectionery, ice creams, baked products and other general products. The trading volume of cocoa futures is close to 4.95 million metric tons for 2014/2015 with Ivory Coast, Ghana and Indonesia as the major producing countries. Nowadays, world cocoa production is shared between bulk cocoa (standard quality) and fine or flavour cocoa (highly aromatic cocoa), which accounts for about 95% and 5% of production [1]. In Indonesia, cocoa is important for the national economy, especially as providers of employment, sources of income and foreign exchange.

Cocoa bean quality is linked to the cocoa variety, soil, climate, crop management and mainly to postharvest processing. Postharvest processing, which includes fermentation and drying, that two major steps on cocoa process. Fermentation is a key step in obtaining the characteristic flavour and taste of chocolate [2] and develop bioactive compound [3]. Beside polyphenol, proteins are the cocoa fraction that undergoes the most intensive modification during fermentation, where microbiological and enzymatic reactions lead to extensive breakdown of cocoa seed proteins, yielding oligopeptides and amino acids which are the important flavour precursors [4]. This process generating the characteristic chocolate aroma, the potential biological activities of oligopeptides found in the cocoa samples are of interest, though they are under-investigated in cocoa literature. Potency of cocoa protein as food bioactive precursor to inhibit angiotensin converting enzyme /prevent hypertension is quite elucidating in Table 1.
### Table 1. Bioactive peptides derived from cocoa beans protein

| Protein Source            | Preparation                                                                 | Peptide from            | Bioactivity                                   | Ref    |
|---------------------------|------------------------------------------------------------------------------|-------------------------|-----------------------------------------------|--------|
| Defatted Fermented       | Ultrasonic, microwave10 min Termamyl and Alcalase                            | Alcohol extract         | ACE inhibitor (IC₅₀: 0.9 mg/ml)               | [5]    |
| Cocoa                     |                                                                             |                         | PEP: 42.8% (IC₅₀: 0.19 mg/ml)                |        |
| Barquillo (Cocoa butter) | Termamyl, Alcalase, Neutrase, Ultrafo, Flavourzyme                           | 21 kDa albumin          | IC₅₀: 0.4039 mg/ml (ACE: 73.9%)              | [6]    |
| Unfermented cocoa         | Termamyl, Alcalase, Neutrase, Ultrafo, Flavourzyme                           | 21 kDa albumin          | IC₅₀: 0.09 mg/ml (PEP: 74.8%)                | [7]    |
| Unfermented cocoa         | Autolysis pH 3,5                                                             | Cocoa autolysate        | IC₅₀: 3 mg/ml (ACE: 44.2%)                   | [8]    |
| Unfermented cocoa         | Autolysis pH 3,5                                                             | Cocoa autolysate        | IC₅₀: 2.5 mg/ml (α-amylase: 50.6%)           | [9]    |
| Fermented cocoa           | Tris-HCl 10 mM                                                                | 21 kDa albumin          | Antitumor, antioxidant (IC₅₀: 1.51 mg/ml)     | [10]   |
| Fermented cocoa           | NaOH 0,1N                                                                    | Glutelin                | Antioxidant (IC₅₀: 0.22 mg/ml)               | [10]   |
| Unfermented cocoa         | NaOH 0,1N                                                                    | Glutelin                | Antioxidant (IC₅₀: 0.58 mg/ml)               | [10]   |
| Cocoa protein             | In silico                                                                    | Storage Protein         | ACEInhibitor                                 | [11]   |
| BIOPEP Database           |                                                                             |                         | IC₅₀: 0.351 mg/ml                            | [12]   |
| Fermented cocoa           | NaOH 0,1N                                                                    | Glutelin                | ACE inhibitor                                |        |
|                           |                                                                             |                         | IC₅₀: 0.24 mg/ml                            |        |

Antihypertension peptides were intensive reported because hypertension is a major health problem of epidemic proportions which affects about 30% of all adults worldwide. It is a serious medical condition, caused by high blood flow pressure through blood vessels [13]. This condition can cause damage to the muscles of the blood vessels and the heart leading to a stroke and eventually death, if untreated. Untreated hypertension and an increase blood pressure in progressively are usually used as the most common cause of morbidity in chronic metabolic disorders in the world. However, treating hypertension has been associated with about 40% reduction in the risk of stroke and about 15% reduction in the risk of myocardial infarction [14].

This article summarizes and describes how bioactive peptide could be release through cocoa fermentation process. It could be one way to increase the added value of cocoa or its derivative products. On the other hand, increasing consumer knowledge about the link between diet and health increased the awareness and demand for functional food ingredients to avoid undesirable side effects of chemical drugs and also to avoid the increasing cost of drug therapy.

2. Production of protein cocoa as bioactive precursor

2.1. Cocoa cotyledon storage protein

Cocoa cotyledon protein represents between 10-16% of dry weight of cocoa [15,16]. Cocoa cotyledon protein has been fractionated and classified as either albumin, vicilin (7S-8S)-class globulin, prolamin or glutelin, according to the extent of their solubility in water or low salt buffer, diluted salt solution, aqueous alcohol and diluted alkali or acid, respectively [17,18,19,20]. The common method used to prepare the cocoa protein fraction describe by Voigt et al. [18].

Albumin is abundantly present in cocoa cotyledon and comprises about 14-52% of total cotyledon protein [18]. It has been reported that the biological activity of albumin fraction is similar to protease inhibitor [19]. Moreover, albumin not significantly degraded during cocoa fermentation [21,22,23]. The
vicilin (7S-8S)-class globulin (VCG) of cocoa cotyledon represented 23-43% of dry weight of cocoa cotyledon [18,24]. It was degraded during cocoa fermentation to produce the specific cocoa aroma precursors [24,25]. VCG has predominant polypeptide with apparent molecular weights at range 45-49 kDa and 31-37 kDa [18,23]. The VCG subunits revealed at least 8 multiple forms had pls in the range of 6.0 to 6.8 [18]. This multiple forms may indicate that the VCG occurs in the cocoa cotyledon was not of single gene origin.

In contrast to the major albumin and the vicilin-class globulin, there are, however, no data concerning the prolamin which have been found in the cocoa seeds [18]. Furthermore, the proportion of the glutelin fraction was considerably lower in previously report [17]. Comparative SDS-PAGE analysis of the different protein fractions revealed that the polypeptide patterns of the globulin and the glutelin fractions were rather similar. Therefore, the glutelin fraction of cacao seeds consisted of residual globulin. Glutelin fraction from cacao cotyledon had proteins with molecular mass >200 kDa and others of low molecular weight, 19.7 and 14.4 kDa, while the glutelin from fermented cacao fraction presented only high molecular mass proteins (> 200 kDa) that were slightly higher than those reported previously. This difference in the content of the several protein fractions is influenced by numerous factors, such as seed variety, fractionation method, batch size, fermentation, and storage conditions [10-11].

In addition, the peptide can be extracted from cocoa protein by using solvent and/or proteolytic enzymes [5,6] before or after fractionation cocoa protein to be albumin, globulin, prolamin or glutelin [10]. Moreover, to avoid an irreversible denaturation of storage proteins by oxidation products of polyphenols during extraction, the acetone dry powder (AcDP) should be prepared. AcDP method can be produce extract storage proteins without irreversible denaturation of proteins from oxidation of polyphenols during extraction. AcDP should contain the total protein cotyledons and proteases. The most frequently chosen method before extracting protein cocoa is the method of Kirchoff et al. [26].

### 2.2 The release of bioactive peptide cocoa through fermentation process

There are different methods to obtain bioactive precursor from cocoa protein that are fermentation, digestion, and enzymatic hydrolyses [27]. The bioactive peptide can extract after fermentation process cocoa bean. Fermentation represents a crucial stage in the development of the aromatic precursors and bioactive compounds characteristic of chocolate and cocoa products [28,29]. Fermentation usually lasts 5–7 days, except in some varieties/hybrids. Cocoa fermentation is generally a spontaneous phenomenon. It is performed by a microbial succession of a wide range of yeasts (Kloeckera and Saccharomyces spp.) and lactic-acid and acetic-acid bacteria (Lactobacillus, Bacillus, Pediococcus, Acetobacter and Gluconobacter), producing a wide range of metabolic end products, in particular alcohols and organic acids [29]. Organic acids, acetic acid in particular, diffuse through the beans lowering the internal pH and causing a breakdown of the cell membranes (death bean). The cell contents become mixed, allowing various enzymatic reactions to take place. For example, oxidation of polyphenols and their precipitation together with proteins are responsible for the removal of the bitter taste and the change of bean color, from violet to brown [29,30]. Consequently, fully fermented cocoa beans have a brown color. Unfermented and under fermented cocoa beans appear respectively salty and violet in color, and represent beans dried without fermentation, and those partly fermented, or fermented using improper procedures.

The most important is biochemical change of cocoa cotyledon storage protein during fermentation. Several studies have reported different proportions of protein fractions after fermentation, with an increase or decrease on the protein fractions ratio. The increase in albumin and decrease globulin was due to the proteolysis of complex protein during fermentation. At 72 h fermentation, when the pH was in the vicinity of 4.6, the proteolytic activity was higher. This may be due to the aspartic endoprotease activity which has a pH optimum at pH 4.5. At the end of fermentation, when pH was approximately 5.8, the activity of carboxypeptidase was optimum. According to Voigt et al. [31,32] the optimum levels of hydrophobic oligopeptides were produced by the action of aspartic endoprotease on vicilin-class globulins at pH 4.5, while the formation of hydrophilic oligopeptides and hydrophobic free amino acids was obtain by the action of carboxypeptidase on hydrophobic oligopeptides at pH 5.8.
Hydrophilic peptides and free amino acids generated from the vicilin-class globulin storage protein of the cocoa beans are essential precursors for the cocoa-specific aroma components [31,32,33,34,35]. After fermentation, percentage distribution of albumin, globulin, and glutelin will change. Several studies performed in fermented cocoa seed shown a different distribution of protein fractions. Lerceteau et al. [23] was reported a higher content of albumin and globulin (14-52%). Voigt et al. [33] presenting albumin being predominant protein fraction with total content of protein was 79 %. Others, Preza et al. [10] and Tovar-Pérez et al. [11] have reported a higher content of glutelin with the following percentage 52.8% and 81.88%.

Study of oligopeptides or peptides cocoa beans have been reported by Marseglia et al. [36] and Caligiani et al.[4], was identified 35-44 peptides, most of them belonging to vicilin and 21 kDa cocoa seed albumin that gave the most intense signals. The oligopeptides were characterized on the basis of their MW and in source fragmentation detectable with the single-quadrupole mass analyser by ESI-MS and ESI-MS/MS. Among the peptides identified in the LC-MS chromatograms, fermentation strongly influenced the formation of peptides in cocoa beans. The total peptide content was very low in fresh beans (10.19 mg/Kg) and under-fermented dried beans compared to the corresponding well-fermented beans (74.5 mg/Kg versus 246.13 mg/Kg). Considering the distribution of peptides, total peptide was higher in fermented beans.

The potential biological activities of oligopeptides found in the cocoa samples are of interest. The recent papers report the physiological effects (antioxidant, ACE inhibitor and hypoglycemic activities) of cocoa protein, containing peptides and amino acids that responsible for the activities [8-10]. Future more, glutelin fraction from under-fermented cocoa bean showed the highest antioxidant and albumin fraction from under-fermented cocoa bean shown the highest antitumor [12]. According to these results, degradation of these proteins occurred during fermentation obtain peptides and amino acids are responsible for the activities.

3. Cocoa bioactive precursor to inhibit angiotensin converting enzyme (ACE)

ACE plays a central role in the control of blood pressure through its regulatory actions on various vasoactive peptides. The decapeptide Angiotensin I, is acted upon by ACE to produce the potent vasoconstractor octapeptide Angiotensin II. A derivative of Angiotensin I, is acted upon by ACE to produce Angiotensin III, a vasoconstrictor. In addition, ACE degrades the vasodilator peptides kallidin and bradykinin to inactive peptides. ACE has a large variety of functions within the body but its main role is the control of blood pressure due to the production of Angiotensin II, a potent vasoconstrictor, from Angiotensin I and the inactivation of bradykinin, a vasodilator [37].

Protease activity of two enzymes (aspartic endoprotease and carboxypeptidase) could accumulate oligopeptides and amino acid that could be inhibiting ACE activity. Potency of protein cocoa as bioactive compound to this activity was first published by Iwaniak and Dziuba [11], with their in silico study of protein as precursor of ACE inhibitor peptide. Cocoa storage proteins have better ability and higher prediction to release from protein fragments than soybean protein.

Fermentation of cocoa prefers to cleave hydrophobic residues protein of cocoa cotyledon at their C-terminal ends [35]. Therefore, mostly hydrophilic peptides and hydrophobic amino acids were produced. Leucine, alanine, phenylalanine, and valine were the predominant free amino acids accumulated during fermentation. Proteolysis of food proteins results in production of a broad variety of peptides and free amino acids. During proteolysis, enzyme specificity affects the size and amino acid sequence of peptides as well as level and composition of free amino acids which subsequently could influence ACE inhibitor activity of protein [38,39,40,41]. ACE inhibitor activities of cocoa may be related to the high amount of their hydrophobic amino acids.

Other amino acids in cocoa peptide include acidic and basic amino acids which exist in high amounts followed by aromatic amino acids, while carboxyl and amino groups in the side chains act as chelator of metal ions [42]. ACE substrate or its competitive inhibitors with C-terminal dicarboxylic amino acids, like Glu, have little affinity for the enzyme [43]. It has also been reported that the peptide with comparatively low IC₅₀ value contains a high content of branched and aromatic amino acids such
as Ile, Val, Phe, and Tyr in its peptide sequence [42,43,44]. Thus, it is very likely that aromatic amino acids along with hydrophobic amino acids contribute to ACE inhibition activity of cocoa. In addition, not only amino acid composition of peptides but their structure and amino acid sequence are also responsible for their activity [43].

The majority of ACE inhibitor peptides are relatively short sequences with low molecular masses. This is in agreement with the results of Natesh et al. [38], which demonstrated from crystallography studies, that the active site of ACE cannot accommodate large peptide molecules. This can be seen on elongating a potent peptide at the N-terminus which results in a decrease in ACE inhibitor activity. Although the structure-activity relationship of food derived ACE inhibitor peptides has not yet been fully established, several structural features have been identified which appear to influence the ultimate potency of an ACE inhibitor peptide [43]. Binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate or inhibitor. ACE prefers substrates or inhibitors containing hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions. Many naturally occurring ACE inhibitor peptides contain Tyr, Phe, Trp, Pro or Lys at the C-terminal end especially the di-peptide and tri-peptide inhibitors. Pro, Trp and Lys seem to be most effective in increasing the ACE inhibitor activity.

4. Potency cocoa bean of Indonesia
Fermentation is a central theme in increasing the competitiveness of Indonesian cocoa beans. Until now, the quality of Indonesian cocoa beans is still very diverse. The weakness of Indonesian cocoa in market competition mainly occurs due to of the low quality of cocoa beans, which is caused by high levels of non-fermented beans (> 3%) and impurity content (> 2%). In addition, the competitiveness of cocoa beans is also determined by the functional characteristics and preferences of consumers. Consumers of cocoa beans or producers of cocoa products often mention that Indonesian cocoa beans have a poor quality flavour compared to cocoa beans from Ghana and Ivory Coast. Indonesian cocoa beans can only be used as fillers for cocoa products, while the typical cocoa flavour and determines the taste of cocoa products obtained from cocoa beans from other countries [45].

Model of Good Handling Practices (GHP) and fermentation of cocoa beans is one way that can be done by Indonesia's farmer or all cocoa business operators to improve the quality of cocoa beans. In addition, PERMENTAN No. 67/2014 is a concrete step from the Ministry of Agriculture in fostering farmers to implement the fermentation process and improving the quality of cocoa beans. However, these policies need to be complemented with a price incentive policies and guidance trader of cocoa beans that be traded cocoa beans only fermented cocoa beans and qualify SNI requirements.

Moreover, there are potential health-promoting benefits from fermented cocoa beans besides taste and aroma precursors of chocolate as described in the previous section. Fermented cocoa is rich in peptides and amino acids that reported to possess antihypertensive effects. Study by Haliza and others [2018], protein fractions from fermented bulk cacao from different origin in Indonesia have more than 80% of ACE inhibitor activity based on the total protein present in beans. Fermented cocoa is a good raw material for the production of peptide fraction with ACE inhibitor activity. In addition, there is need to increase the knowledge of each consumer that are more benefits from fermentation process carried out, namely the potential biological activities of oligopeptides and amino acids found in the fermented cocoa beans.

5. Conclusion
Hypertension is called the silent killer because it is plays a major etiologic role in the development of cardiovascular diseases, ischemic heart disease, cardiac failure, and renal failure. It has been responsible for 62% of cases of cardiovascular diseases, 49% of cases of ischemic heart disease, and 7.2 million deaths per year. The high prevalence of hypertension in the human population requires a combination of controls, including changes in lifestyle, diet and pharmacological treatment. All individuals should adopt appropriate lifestyle modifications including consume functional food that contain bioactive
compound. Food-derived peptides from fermented cocoa beans opens new frontier on the health benefits of peptides in cocoa and cocoa-based products. Health benefits of these components could be explored in short- and long-term studies and among healthy and disease-state subjects. Functional effect of fermented cocoa beans can effort all Indonesia’s cocoa business operators to improve the quality of cocoa beans by Good Handling Practices.

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