THE EFFECT OF NANO-POLYPHENOLS COCOA WHEY PROTEIN AS FUNCTIONAL FORTIFICATION MATERIALS IN TERMS OF ANTIOXIDANT ACTIVITY AND PHYSICOCHEMICAL PROPERTIES

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ABSTRACT

The study aims to understand the interaction between cocoa polyphenol nanoparticles with whey protein isolate (WPI) bond between polyphenol cocoa nanoparticles with whey protein isolate (WPI) and the role of the nanoparticles of cocoa polyphenols as bioactive compounds in the nano-polyphenol cocoa whey protein. The research method is performed using laboratory experiments with a completely randomized design (CRD), followed by Duncan analysis (DMRT) when there are significant or highly significant differences in each treatment. This research focused on the nanoparticles of cocoa polyphenols in the formulation of the nano-polyphenol cocoa whey protein with 4 treatments (T0: 0%, T1: 5%, T2: 10%, T3: 15%) and 3 replications. The observed variables include total phenolic, antioxidant activity, particle size, emulsion activity, and emulsion stability. The difference in the concentration of the nanoparticles of cocoa results in highly significant differences (P<0.01) in total phenolic, antioxidant activity, and particle size. Gave a significant difference (P<0.05) in the emulsion activity, but does not show a significant difference in the emulsion stability (P>0.05). It can be concluded that cocoa 15% nanoparticles (T3) are the best treatment from other treatments and is able to synergize with whey protein isolate (WPI), improve emulsion quality and have natural antioxidant content used as an alternative for food fortification ingredients.

Key words: Bioactive compound; emulsion; natural antioxidant; particles size; whey protein isolate
INTRODUCTION

Mixing micronutrients such as polyphenols is often done as a formulation for adding nutrients, especially antioxidants in the food industry to increase the functional value of food. Polyphenols are found in plants such as cocoa (*Theobroma Cacao* L.). According to Hii, et al (2009), that cocoa (*Theobroma cacao* L.) is a rich source of polyphenols and is reported to have higher antioxidant activity than tea and red wine.

Cocoa (*Theobroma Cacao* L.) is a plant rich in benefits. Numerous studies show that consuming cocoa foods can ward off free radicals, lower blood pressure, thereby lowering cardiovascular disease, improving insulin sensitivity through improved cell function, and lower aging (Buijsse et al., 2010). These health benefits are related to the phytochemical content in cocoa (Tomas-barberan et al., 2011). Each part contains bioactive compounds, namely polyphenols such as in the skin, roots, leaves, and seeds. Cocoa beans contain a high concentration of polyphenols (about 12-18% dry weight).

Polyphenolic compounds found in cocoa include hydroxybenzoic acid (vanillic acid, gallic, syringic, protocanetetic), hydroxycinnamic acid (caffeic, ferulic, p-coumaric, phloretic acids, clovamide, dideoxyclovamide), flavonols (quercetin), flavones (uteolin, apigenin), flavanones (naringenin) and flavanols (catechins, epicatechins, procyanidins/oligomers and polymers) (Counet et al., 2004). Of the various polyphenols present in cocoa seeds, “flavanols” were found in the highest amounts compared to other flavonoids (Bernaert et al., 2012). Monomeric flavanol compounds are known as catechins and epicatechins, while oligomeric flavanol compounds are known as procyanidins.

Unfortunately, polyphenolic compounds are easily reduced and degraded due to heating factors. Whey protein isolate can act as a substitute for saturated fat, binding hydrophobic compounds, gelling, emulsifying, and as a nano delivery of polyphenols, especially catechins (Rahayu et al, 2019). Nano size has stable properties and is easily digested by the body. To maintain polyphenol compounds, casein protein is needed to protect polyphenol compounds in cocoa. Polyphenols can interact with proteins to increase the physicochemical properties of proteins, play a role in preventing cavities in foam whey protein because it increases foam and emulsion properties (Rahayu et al., 2015). Catechins in polyphenols interact with proteins through hydrophobic interactions and van der Waals binding and hydrogen bonding (Chanphai, et al., 2018).

This study aimed to determine the percentage of addition of the best cocoa polyphenol nanoparticles when binding to whey protein isolate in terms of antioxidant activity and physical-chemistry properties.

MATERIALS AND METHODS

Materials

Whey protein Isolate (WPI) 95% (Milk Specialities Global, USA), cocoa powder (*Theobroma cocoa* L.) (from kampung coklat, Plosorejo, Blitar, East Java) aquades, maltodextrin, DPPH 0.4 mM (Sigma, Chem.Co), methanol PA (E. Merck), ethanol 96%, folin-ciocalteu reagent (sigma), gallic acid, Na2CO3, aquabides, filter paper, soya oil, SDS,

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microwave (SHARP R-222Y) frequency 2450 MHz, hotplate and magnetic stirrer (SBS-A06), beaker glass 400 ml (Pyrex), Erlenmeyer (Herma), rotary evaporator, centrifuge tube, centrifuge (Universal 32R, Hettich, Tuttlingen, Germany), thermometer, spectrophotometer UV Vis (721-Faithful), cuvette, mini hand mixer (Latina), micropipette (HumaPette), ultrasonic homogenizer sonicator (Biomaisen), and PSA (Particle Size Analyzer) Zetasizer Nano Serines Software Version 7.01 Malvern Instrument.

**Methods**

This research used a completely randomized design (CRD) method using four treatments with different cocoa polyphenol ratios (T0: 0%, T1: 5%, T2: 10%, T3: 15%) and three replications, if there were differences in treatment continued with the Duncan Multiple Range Test (DMRT).

**Nano-polyphenols Cocoa Whey Protein Preparation**

Nano-polyphenols cocoa whey protein is carried out based on the method described by (Sataphaty, et al., 2019) which is given a slight modification. Cocoa powder was dissolved with 100 ml of ethanol 70%. Extracted using the Microwave Assisted Extraction (MAE) method at 70 °C for 10 min (radiation for 1 minute, then turned off for 2 min). After the extraction process is complete, cocoa extract is obtained. Cocoa extract is filtered with filter paper of 0.45 μm. To get cocoa polyphenols, it is necessary to evaporate using an evaporator at a temperature of 50 °C for 20 min, then filtered with Whatman paper no 4.

Homogenize Whey Protein Isolate (WPI) 95 by 10% in 100 ml of distilled water. Then formulated with nano-polyphenol cocoa according to the treatment (0%, 5%, 10%, and 15%) (v / v). Homogenized using the ultrasonication method with an ultrasonic homogenizer for 15 min at a probe temperature of 28°C, a maximum alarm set of 60°C and a 70% power rate.

**Total phenolic**

Gallic acid 0.005 grams dissolved with distilled water to the limit mark in a measuring flask of 100 mL. A gallic acid stock solution with a concentration of 50 ppm (mg / L) was obtained, then the dilution of the gallic acid solution was obtained up to 5 ppm. Next, make dissolved ranging from 5 ppm to 50 ppm, each of which is taken 1 ml put in a vial, added 0.5 ml of Folin-Ciocalteau, and let stand for 5 min. After that, a 10% Na 2 CO₃ solution of 2 ml was added to the sample and let the sample stand at room temperature for 10 min, the absorbance was measured at a wavelength of 770 nm with a UV Vis spectrophotometer at a wavelength of 770 nm with a cuvette of 1 cm³. Results are measured by using gallic acid as standard. The sample is repeated three times. The result is expressed as the acid equivalent of gallic acid (Rahayu, et al., 2019).

**Antioxidant Activity**

First, the preparation of DPPH (2,2′-diphenyl-1-picrylhydrazyl) solution is weighed 4 mg DPPH, then dissolved with methanol PA up to 25 ml. DPPH solution is kept in a dark Vial to protect it from light. Second, by making a blank solution, 1 ml of DPPH solution (0.4 mM) is taken, then 5 ml of methanol PA is added, and homogenized. The two preparations of the Master Solution were taken 10 mg samples of nano-polyphenols cocoa whey protein, dissolved in 10 ml of methanol PA then dilution of 25 μl, 50 μl, 75 μl, 100 μl in a 5 ml flask to obtain concentrations of 5, 10, 15, 20 μl / ml.

Finally, antioxidant activity testing is added 1 ml of 0.4 mM DPPH solution in each parent and
comparison solution, then homogenized. Covered with aluminum foil to avoid incoming light.
Next incubated for 30 min at room temperature. Absorbance was measured using a UV Vis spectrophotometer with wavelength 517 (Faithful®, Huanghua Faithful Instrument Co., Ltd China). The following formula was used to compute the percentage of antioxidant activity (Tambunan dkk, 2019).

\[
\text{Inhibition}\% = \left[ \frac{(A \text{ control} - A \text{ sample})}{A \text{ control}} \right] \times 100
\]

\text{Particle Size}
To determine particle size using the PSA tools from the nano-polyphenol cocoa protein whey formulation. A sample of 1mL was dissolved with 19 mL of aquades in a measuring cup. A total of 4 mL of solution is put into the cuvette with a pipette and put into the sample holder. The tool will read the sample for 15 min. After 15 particle sizes it will be legible (Larasati and Nina, 2020).

\text{Emulsion activity}
15 mL of the sample was homogenized with 5 mL of soybean oil using a mini hand mixer for 1 minute. Then 0.1 mL of homogeneous samples are taken and add 1 mL of 0.1% SDS solution. Next, vortex for 10 seconds. After that 3 mL of the sample is taken and placed on the cuvette. Its absorbance is measured with a wavelength of 500 nm with a spectrophotometer (Meng and Li, 2021).

\text{Emulsion stability}
The emulsion stability test is carried out after performing the emulsion activity test (allowed to stand for 10 min). 3 mL of the sample is taken and placed on the cuvette. Absorbance measured with a wavelength of 500 nm with a spectrophotometer (Rahayu et al, 2019)

\text{RESULT AND DISCUSSION}
\text{Total Phenolic}
The addition of 15% cocoa polyphenol nanoparticles as P3 showed the highest percentage of total phenolic values of 8.34 mg/ml compared to the addition of 0%, 5%, and 10% cocoa polyphenol nanoparticles of 6.01 mg/ml, 6.06 mg/ml and 7.51 mg/ml respectively. This shows that the amount of polyphenols in P3 is higher than P0, P1 and P2. Studies on polyphenols in cocoa reported that flavonols (epicatechin and catechin) are the dominant compounds in cocoa powder (Jalil and Amin, 2008). 60% of cocoa's total phenolics are flavonols monomers (epicatechin and catechin) and procyanidin oligomers (Dreosti, 2000). The chemical structure of flavonols and procyanidins plays a role in antioxidant activity because they have free radical trapping and chelation redox-active metal properties, besides that they can prevent lipid oxidation through interactions between lipid-forming membranes and adsorption to polar lipid head groups (Verstraeten et al., 2005).

It can be seen in Table 1 that the treatment of adding nanoparticles of cocoa polyphenols with different concentrations gave a very significant difference (P<0.01) on the total phenolic of cocoa nanopolyphenol whey protein. Polyphenols especially flavonols (catechins, epicatechins, epigallocatechin, procyanidins) show prominent antioxidant potential and were found to be effective and powerful free radical neutralizers playing a role in maintaining heart health in various in vitro research studies (Amic et al., 2017). Clinical studies reveal that oral consumption of 100 mg for 12 weeks can support the prevention of cardiovascular disease and atherosclerosis through stimulation of endothelial function (Mutha et al., 2021). Consumption of a diet rich in polyphenols is able to protect lymphotic deoxyribonucleic acid (DNA) from oxidative damage and works as an antioxidant. Polyphenols not only protect cells and cellular components
from oxidative damage but also reduce the risk of oxidative stress associated with various degenerative diseases (Luqman and Rizvi, 2006).

The natural polyphenols in cocoa are easily degraded due to sensitivity during processing and storage due to their sensitivity to heat, oxygen, light, and pH. Polyphenols added in whey will be protected from decomposition. Whey protein is encapsulated to protect polyphenolic compounds including flavonoids such as procyanidin, epicatechins and catechins present in cocoa. According to Jovanovic et al. (2022) whey protein is able to protect polyphenolic active compounds such as anthocyanins in blueberries simulated in digestive conditions, protect antioxidant capacity, and increase bioaccessibility in the body. The mechanism of action of casein as an encapsulant of polyphenols according to Rahayu, et al (2021) is casein binding polyphenols through hydrophobic and hydrophobic interactions. Polyphenol protein interactions are dominated by non-covalent interactions, polyphenols are dominated by non-covalent interactions, that is, hydrophobic interactions stabilized by hydrogen bonds.

### Table 1. Data of Total Phenolic and IC50 Nano-polifenol Cocoa Whey Protein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Phenolic (mg/ml GA)</th>
<th>Antioxidant activity (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>6.01 ± 0.09a</td>
<td>125.68 ± 5.13c</td>
</tr>
<tr>
<td>T1</td>
<td>6.06 ± 0.04a</td>
<td>56.78±5.14b</td>
</tr>
<tr>
<td>T2</td>
<td>7.51 ± 0.15b</td>
<td>49.84 ± 6.98b</td>
</tr>
<tr>
<td>T3</td>
<td>8.34 ± 0.03c</td>
<td>38.44 ± 6.75a</td>
</tr>
</tbody>
</table>

**Remarks:** *Mean values within a column followed by the different letters are very significantly different at p < 0.01 according to Duncan’s Multiple Range Test

### Antioxidant Activities

The antioxidant activity of IC50 is defined as the amount concentration of sample solution needed to reduce DPPH free radicals by 50% because antioxidants are compounds that can neutralize or stabilize free radicals by complementing the lack of electrons in free radicals. The higher the percentage of addition of cocoa nanoparticles, the lower the IC50 value.

Table 1 shows that the treatment of adding nanoparticles of cocoa polyphenols with different concentrations gave a very significant difference (P<0.01) to the average value of the antioxidant activity. All treatments showed that nano-polyphenols cocoa whey protein at T2 and T3 had very strong antioxidant activity due to IC50<50 values. The higher the concentration of cocoa polyphenol nanoparticles added (0%-15%), the smaller the IC50 value, and the antioxidant activity increases. This event is due to the smaller concentration (38.44-125.68 μg/ml) required to reduce DPPH to DPPH-H (1,1-diphenyl-2-picrylhydrazine). The hydrogen donation given from antioxidants depends on the number of hydroxyl groups it has. This is reinforced by (Chalamaiah et al., 2015) that if the IC50 value is low, the higher the antioxidant activity. Conversely, a high IC50 value indicates less antioxidant activity.

The addition of whey protein cocoa polyphenol nanoparticles with different concentrations showed that the control treatment (T0) had the lowest antioxidant activity value of several other treatments, while those of P1 to P3 (5%-15%) had the same effectiveness. The greater the percentage of cocoa nanoparticle administration, the greater the antioxidant activity. Meng et al. (2021) explained that cocoa contains antioxidant compounds consisting of polyphenols, including flavonoids such as catechins, epicatechins, and procyanidins that are able to reduce free radicals.

Based on the results of research from Tamrin et al., (2012) that if catechins levels are low, antioxidant activity is also low as well as if catechins levels are high then antioxidant activity is also high.
Antioxidants have an important function in the body to protect tissues exposed to free radicals. Antioxidants inhibit free radical activity by neutralizing unstable charges in reactive oxygen species (ROS) (Santosa and Baharuddin, 2020).

The interaction formed between catechins and proteins is included in weak bonds and consequently has the advantage that the role of catechins as antioxidants in fighting free radicals still functions (Rahayu et al., 2021). The higher the concentration of cocoa nano-polyphenol particles added, the lower the IC50 value obtained and included in the category of strong antioxidants.

Based on the discussion, it can be concluded that the results of the analysis on T3 with the addition of nanoparticles of cocoa polyphenols as much as 15% produce the highest antioxidant levels compared to other treatments.

**Particle Size Analysis**

Based on the results of research on control treatment T1 has a larger particle size of 701.37 nm, compared to T1 and T3 (487.93-519 nm). Supported by research conducted by Calva-Estrada, et al (2019) that the particle size in the encapsulation of cocoa nanoemulsion with whey protein has a diameter of 202.13 nm, the process of adding a stabilizer to the nanoemulsion encapsulation makes it more stable due to the presence of protein and polysaccharide bonds that stabilize the surface of larger casein micelles. Table 2 shows that the treatment of adding different concentrations of cocoa polyphenol nanoparticles to whey protein cocoa nano-polyphenols gave a significant difference (P<0.05) on the emulsion activity.

The average whey protein cocoa nano-polyphenol particle size gets smaller as more and more percentage of cocoa polyphenol nanoparticles are added. This event occurs due to phenolic OH interacting with casein (Mehranfar et al., 2013). Bioactive compounds are able to interact with protein compounds through non-covalent interactions that include hydrogen bonds and hydrophobic bonds (Mootse et al., 2014).

Non-covalent interactions such as hydrogen bonds, hydrophobic bonds and Van der Walls interactions play a role in the formation of bidirectional interactions between polyphenolic compounds and proteins. The existence of two-way or reversible interactions will cause covalent bonds, but non-covalent interactions of proteins with polyphenols are more common than covalent bonds (Wang and Wu, 2021).

Polyphenols have excellent solubility properties making them easier to make in nano-particle size. The interaction between polyphenols and whey proteins can cause changes in their structure, stability and functional properties (Yildrim-Elikoglu and Erdem, 2018). The higher the concentration of nano cocoa polyphenol particles added, the smaller the particle size obtained. Based on the discussion, it can be concluded that the results of the analysis on T3 with the addition of nanoparticles of cocoa polyphenols as much as 15% produce the smallest particle size compared to other treatments.

**Table 2. Data of Particle Size, Emulsion Activity, and Emulsion Stability**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Particle Size (nm)</th>
<th>Emulsion activity (m²/g)</th>
<th>Emulsion Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>701.37±8.33⁹</td>
<td>46.67±9.49⁹</td>
<td>54.36±15.80</td>
</tr>
<tr>
<td>T₁</td>
<td>519.23±4.00⁹</td>
<td>35.54±1.61₁</td>
<td>53.08±8.08</td>
</tr>
<tr>
<td>T₂</td>
<td>513.97±7.64⁹</td>
<td>32.02±1.49⁹</td>
<td>46.49±8.81</td>
</tr>
<tr>
<td>T₃</td>
<td>487.93±4.97⁹</td>
<td>33.93±4.32</td>
<td>58.04±9.85</td>
</tr>
</tbody>
</table>

Remarks:  *Mean values within a column followed by the different letters are significantly difference at p < 0.05 according to Duncan’s Multiple Range Test
**Emulsion Activity**

Emulsion activity is one part of the determinant of emulsion properties, playing a role in identifying the quality of whey protein that binds to polyphenols from cocoa that act as emulsifiers. The emulsion is a liquid disperse system in a liquid whose liquid molecules are not mixed (scattered) but are mutually antagonistic (Rahayu et al., 2022).

It can be seen in Table 2 that the treatment of adding different concentrations of cocoa polyphenol nanoparticles to nano-polyphenols cocoa whey protein gives a significant difference (P<0.05) to emulsion activity. The emulsion activity index (IAE) is an area between stabilized surfaces per unit mass of protein (m²/g). The high emulsification ability of proteins is indicated by the high emulsion activity index (IAE) value (Estiasih and Ahmadi, 2004). The calculation of emulsion activity shows that the addition of nanoparticles of cocoa polyphenols with different percentages can increase the ability of whey protein to form an emulsion. The control treatment or without adding cocoa polyphenol nanoparticles (T0) had an average emulsion activity value of 46.67 m²/g, while the treatment of adding cocoa polyphenol nanoparticles to T1, T2, and T3 gave emulsion activity results of 35.54 each; 32.02 and 33.93 m²/g.

The protein emulsion mechanism will form a cation charge by forming a thick viscoelastic film on the surface of the emulsion droplet. Small-size emulsion droplets will give a better indication of emulsion (Wang and Wu, 2021). The addition of cocoa polyphenol nanoparticles to whey proteins results in interactions due to non-covalent bonds, namely hydrophobic interactions that change the surface and structure of the protein, resulting in a decrease in the ability of emulsion formation. The interaction factor of phenolic compounds and hydrophobic groups in proteins that form hydrogen bonds between the OH groups of phenol compounds with polar groups (NH₂, OH, NH and SH) on the protein surface (Rahayu et al., 2022; Wu, 2013).

Based on the average T0 emulsion activity value of 46.67 m²/g decreased in T1 and T2 treatment due to weak interactions between polyphenols that bind to whey protein to form a more complex protein structure. Previous researchers suggested that polyphenols sourced from green tea extract have weak hydrophobic interactions with β-lactoglobulins in emulsion formation (Rahayu et al., 2022). The decrease in emulsion activity index (IAE) is due to an increase in the size of oil globules in the emulsion.

T3 shows an increase in emulsion activity due to casein having the ability to absorb oil-water rapidly with a thick surface layer. The higher the emulsion activity index (IAE), the smaller the size of the oil globules in the emulsion. This thick layer on the surface provides protection against emulsion formation from coalescence or flocculation, and stabilizes the emulsion. Whey proteins absorbed on the oil-water surface in the formation of emulsions have stable physical and oxidative properties (McClements and Decker, 2018).

**Emulsion Stability**

Emulsion stability is the percentage of emulsion resistance by comparing the absorbance in the 10th minute with the 0th minute. Based on Table 8, it can be known that the amount of emulsion stability ranges from 46.49% to 58.04%. The presence of bonds formed between phenolic compounds and proteins can affect the stability of the emulsion. It can be seen in Table 2 that the treatment of adding different concentrations of cocoa polyphenol nanoparticles to nano-polyphenols cocoa whey protein did not provide a noticeable difference (P>0.05) to the stability of nano-polyphenol cocoa whey protein emulsions.

The addition of cocoa nanoparticles as much as 15% to T3 has the highest emulsion stability value of 58.04% compared to other treatments. The binding of whey protein with polyphenols in
cocoa can improve the stability of the emulsion. This is in accordance with the opinion (Prommajak and Ravivan, 2017) that proteins that interact with phenolic compounds have stable emulsions. This event is caused by several bonds through interacting non-covalent bonds such as hydrogen bonds, hydrophobic interactions and Van der Walls bonds (Rahayu et al., 2022). During the interaction between polyphenols and hydrophobic proteins, the weaker the hydrophobic bonds can remain on the surface of the protein, causing changes in protein folding and function (Li et al., 2021).

The addition of nanoparticles as much as 5% (T1) and 10% (T2) decreased the percentage of emulsion stability, namely 53.08% and 46.49%, allegedly due to the amount of protein that is not completely adsorbed on the oil-water surface so that the protein structure does not change and the hydrophobic side is not exposed. As a result, the ability of molecules to stabilize emulsions is difficult.

β-casein and β-lactoglobulin from whey protein are absorbed on the surface of oil droplets thereby providing emulsion stability against flocculation and coalescence. Particle size also affects the stability of the emulsion. The larger droplet size causes emulsion destabilization, such as flocculation, coalescence, coagulation and creaming due to interactions and density differences. The interaction of oil droplets sticking together is a form of damage so that the protective layer on the surface of the droplet is calculated. The coalescence cannot be re-dispersed even if re-shaken is carried out so that it can cause the emulsion to separate again like the initial phase or referred to as destabilization.

In T3 or the treatment the addition of cocoa polyphenol nanoparticles as much as 15% resulted in the highest percentage of emulsion stability. The complex formed between polyphenols and whey protein when added as an emulsifier will absorb the surface of the droplet to form a solid film, thereby decreasing surface tension and increasing emulsion stability (Li et al., 2021).

CONCLUSIONS

Based on the result of this research, we can be concluded that cocoa polyphenol nanoparticles with the addition of a percentage of 35% (T3) produce nano-polyphenols cocoa whey protein with the best physicochemical properties and antioxidant activity. Nano-polyphenols cocoa whey protein can be used as an alternative natural antioxidant ingredient to fortify in food so that it can increase functional value.

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