Original Research Article

Characteristics of whey protein - phylantin as a based for the development of immunomodulatory products

Aims: The purpose of this research was to investigate the characteristic of physicochemical properties of interaction of whey protein with different concentration phylantin sources of the *Phyllantus niruri* L.
Study design: Phylantin was added to whey protein with various concentration treatments (0 (control), 150, 180, 210 and 240 (µg/ml)).
Place and Duration of Study: This study was conducted between June until November 2021 at the Faculty of Animal Science, Universitas Brawijaya.
Methodology: Phylantin was added to whey protein with various concentration treatments (0 (control), 150, 180, 210 and 240 (µg/ml)). An analysis is carried out to determine characteristic physicochemical and functional properties such as emulsion stability, emulsion activity, foaming ability, sedimentation, turbidity, particle size and antioxidant activity.
Results: The characteristic physicochemical of whey protein and phylantin was investigated such as emulsion stability, emulsion activity, foaming ability, sedimentation, turbidity, particle size and antioxidant activity. The addition of phylantin improved the physicochemical characteristic of properties. The addition of phylantin in the right level could increase emulsion stability, activity, and total antioxidant. Beside it, it could decrease the sedimentation and turbidity in the right level. The particle of nano size could be maintained.
Conclusion: As a result, whey protein containing various concentrations of phyllanthin showed that serum interacting with phyllanthin at a dose of 180 µg/ml increased emulsion stability, antioxidant activity, reduced sedimentation, reduced turbidity, and nanoscale has been shown to be able to maintain the particle size.

Keywords: emulsion stability, emulsion activity, foaming ability, sedimentation, turbidity, particle size and antioxidant activity

1. INTRODUCTION

Milk protein is used as an additive because of its properties as a gelling, foaming and emulsifier. Milk protein consists of casein and whey protein, whey protein is a natural carrier that has evolved to deliver essential micronutrients and immune system components (β-lactoglobulin and α-lactalbumin). In particular, whey protein is an important food ingredient in terms of its functional and nutritional properties and is widely used as an emulsifier in food. Recent studies have demonstrated the potential of whey protein as an emulsifier in nanoemulsions specifically designed for food use. β-lactoglobulin, part of whey protein, has the ability to protect labile biologically active compounds from extreme conditions [1].

Phyllantus niruri is a plant that has many properties, one of which is an immunostimulant, an immunomodulator [2,3]. This is because the content of bioactive polyphenolic compounds consisting of flavonoids (quecertin, quercitrin, isoquercitrin, astragalin, rutin, kaempferol-4, rhamnopynoside), lignin (philanthine, hypophilantin, nirantin, lintetratin), alkaloids, triterpenoids, fatty acids (ricinoleic acid, linoleic acid, linolenic acid), vitamin C, potassium, resin, philanthine, tannin and geranin [4].

Polyphenol compounds like those in phylantin are most likely to form complexes with milk protein, especially whey proteins [5]. This binding can affect the electron donation capacity of xanthone by reducing the number of hydroxyl groups available in the solution. Studies in the past have shown the

effects of milk protein on the antioxidant activity of tea polyphenols, whilst the effect of polyphenol complexation on the stability and conformation of milk proteins has not been addressed [6].

The ample proteins in whey milk are α-lactalbumin (α-LA) and β-lactoglobulin (β -LG), which might be of most important in the meals industry. α--Lactalbumin is a 14 kDa protein, the local shape of that's divided into domains: one is basically helical (the α--domain) whilst the alternative has a great content material of b-sheets (the β-domain), and those are related via way of means of a calcium binding loop [7]. β-LG exists as a combination of monomers and dimers, the equilibrium ratio of which relies upon at the affiliation steady of the dimer and at the protein concentration. Each monomer includes 162 amino acid residues and has a molecular mass of 18 kDa [7]. A variety of investigations have discovered that bovine serum albumin is often a ``target`` of therapeutically lively phenolics [8]. Therefore, maximum researchers centered at the interactions among bovine serum albumin and phenolic acids [7]. However, to date, little is thought approximately about the interplay modes of phenolic (chlorogenic acid, caffeic acid, ferulic acid, and coumalic acid) with a-lactalbumin and b-lactoglobulin.

The advantages of nanotechnology in functional foods are that it can efficiently respond to the body's nutritional needs and bioactive nutrients are easily absorbed by the body. One use of nanotechnology in the food sector is to add nanocapsules to easily soluble food material to prevent them from degrading the taste and color. Efforts to make products of this size take into account the materials used and the processes involved in the formation of their interactions. One of the commonly used material is milk protein.

2. MATERIAL AND METHODS

2.1. Process of casein interaction with bioactive compounds source; *Phyllantus niruri*

Whey protein (WP) was dissolved in aqueous solution by adding 5 gr WP in 100 ml phosphate buffer (pH 6,8). WP was homogenized by Ultra-Turrax at the speed of 7,600 rpm for 2 hours, phylantin was added using various concentrations, 0 (control) 150, 180, 210 and 240 (µg/ml)) (3 replications) with temperature heated at 63-65 °C for 15 minutes and homogenized for a minute. The solution was stored in a refrigerator at 4°C. Furthermore, an analysis is carried out to determine physicochemical properties such as emulsion stability, emulsion activity, foaming ability, sedimentation, turbidity, particle size and antioxidant activity.

2.2. Emulsion stability and activity

Emulsion stability and activity was measured according to Rahayu et al. [9] with modifications. Soy bean oil was added to the sample and then using a hand mixer for 1 minute. As plenty as 0.1 mL of the sample and 0.1% of SDS as plenty as 10 mL and stirred the use of vortex for 10 seconds. Afterward, about three mL of sample became taken and put in a cuvette and examined the use of a spectrophotometer with a wavelength of 500 nm and the absorbance value (A0) changed into recorded. After that, the emulsion process waited for 10 min and a comparable test (A10) is performed. The ensuing dispersion is measured through the formula:

$$\text{Emulsion stability}(\%) = \frac{A_{10}}{A_0} x100$$

Description:

A_0 = A_{500} at time of 0 minutes A_{10}= A_{500} at time of 10 minutes

Emulsion activity (g) = I x ϕ x C

Description:

A = A500

ϕ = Oil fraction volume

DF = Dilute factor (100)

C = Sample concentration (gram/m3)
I = Length of cuvette path (m)

2.3. Sedimentation

Sedimentation evaluation was carried out using Airoldi et al. [10] method explaining that the measurement was carried out a filter paper in the oven at a temperature of 105 °C for 1 hour (A gram). Then added sample to filter paper, wrap it and put in to oven at 105°C for three hours then weighed with filter paper (B gram). Calculation of sedimentation makes use of the formula:

$$\text{Sedimentation } (\%) = \frac{A}{B} x100$$

Description:
A = Weight of the filter paper
B = Weight of the filter paper and sample after taken from the oven

2.4. Turbidity

Turbidity measurement analyzed according to Huppertz et al. [11] with modifications. 0.1073522 gram of trisodium citrate was delivered to the 8.5 ml phylantin-whey solution. Incubated at 25°C for 60 minutes. Take 1 ml of casein-catechin solution. Added nine ml of distilled water. Prepare a spectrophotometer. Determined absorbance of 600 nm at a length of 10 mm cuvette. Calculation of turbidity measurement use of the formula:

$$Tr = \frac{1}{10A}$$

$$\tau = -\ln(Tr)$$

Description:
τ = Turbiditas, Tr= Transmisi, A= Absorbansi

2.5. Particle size

The particle size of casein was measured by a static laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada), using deionized water as the dispersion medium (refractive index is 1.465).

2.6. Antioxidant activity

The DPPH activity was measured with the method described by Raghavendra et al.[12] with minor edits. 1 ml of sample (nano phylantin-whey). The solution was composed of 1 ml of 6x105 M DPPH solution. After 20 minutes of incubation at 37° C., the absorbance of the reaction mixture was measured at 515 nm by spectrophotometer (UV Jasco V530, Japan) to give the value **As**. A blank sample was prepared with 33 L of methanol in DPPH solution and measured at the same wavelength (**Ab**). The experiment was carried out in triplicate.The antioxidant activity was calculated from with the following formula:

$$Antioxidant\ activity\ (\%) = \frac{Ab - As}{Ab} x100\%$$

3. RESULTS AND DISCUSSION

Table 1. Physicochemical characteristic of nano-whey-phylantin

Treatment	Emulsion stability (%)	Emulsion activity (m^2/g)	Sedimentation (%)	Turbidity (gr/ml)	Antioxidant activity (%)
P0	83.27±0.49 ^{a}	34.20±0.53 ^{b}	0.19±0.06 ^{a}	0.35±0.01 ^{y}	0.00±0 ^{a}
P1 (150 µg/ml)	85.20±0.18 ^{bc}	30.33±0.47 ^{a}	0.22±0.02 ^{a}	0.27±0.02 ^{x}	66.73±0.64 ^{b}
P2 (180 µg/ml)	88.70±0.02 ^{c}	45.24±0.28 ^{cd}	0.24±0.03 ^{ab}	0.28±0.02 ^{x}	71.17±0.89 ^{c}
P3 (210 µg/ml)	80.34±0.59 ^{ab}	46.22±0.70 ^{d}	0.36±0.03 ^{bc}	0.30±0.02 ^{xy}	75.28±0.82 ^{cd}
P4 (240 µg/ml)	79.10±0.42 ^{a}	44.20±0.21 ^{c}	0.42±0.02 ^{c}	0.27±0.03 ^{x}	77.15±0.78 ^{d}

Note:
^{a,b,c,d} Different uppercase letters in the same column indicated highly significant effect ($P < 0.01$).
^{x,y} Different uppercase letters in the same column indicated significant effect ($P < 0.05$).

3.1 Emulsion stability

The stability of the whey protein with different concentration of phylantin addition is shown in Table 1. The analysis result showed that the addition of phylantine caused a highly significant difference ($P<0.01$) in emulsion stability. The presence of bonds formed between proteins and phenolic compounds affects the stability of the emulsion.

This is in agreement with [13] which explains that emulsion stability is an indication of interaction. This is due to the existence of several bonds that play a role in the interaction, such as hydrogen bonding, hydrophobic interaction, and Van der Walls bond. This is consistent with molecular bonding shows that there are some interaction between whey proteins and phenolic compounds. This is consistent with the findings of the study that proteins interacting with phenolic compounds will produce high emulsion values. This is also consistent with research [14] which showed that proteins that interacted with the phenolic have a stable emulsion.

The control treatment (P0) has an average emulsion index value of 83.27%. This showed that there is an increased need for the size of the hydrophobic globules formed at 83.27%. The treatments P1, P2, P3 and P4 produce emulsion stability at 85.20%, 88.70%, 80.34% and 79.10% respectively. Based on these results whey protein has the ability to increase the overall hydrophobic dimension. The 180 µg phylantin addition was the highest emulsion stability than others. The binding of whey protein-phylantin increased the stability of emulsion. This is due to the existence of several bonds that play a role in the interaction, such as hydrogen bond, hydrophobic interaction, and van der walls bond.

This is indicated via excessive emulsion balance value. A quantity of research defined that the interplay among milk protein and phenolic compounds modifications affect the balance, structure, digestibility and functional properties of the protein. The emulsion stability has a critical role in determining the properties of emulsions in meals emulsion systems [9,15]. The emulsion is an appropriate manner of turning in purposeful elements into meals systems.

3.2 Emulsion activity

Emulsion activity is one of the determinants of emulsion properties that play a critical role in the quality of whey protein. Variance analysis results show that the treatment of phylantin with different concentrations provide a highly significant difference ($P<0.01$) on the emulsion activity statistically. That is because the ability of whey protein and phylantin are capable to stabilize the mixed solution. This statement based on [16], explained that emulsifier molecules are adsorbed at the interface because they orient themselves with the hydrophilic part of water and the hydrophobic part of the oil. An emulsion is a liquid dispersion system in a liquid whose liquid molecules do not intermingle but are antagonistic to each other. In an emulsion there are usually three parts, including the dispersed part in the form of granules consisting of fat, the dispersing media part consisting of water, and the emulsifying part that serves to keep the oil droplets remaining dispersed in water.

Emulsion activity is affiliated with the ability of proteins to cover the oil-water interface [17,18]. Emulsion activity index calculation results show that the addition of phylantin with different concentrations can increase the ability of whey protein to form emulsions. It cause whey protein to emulsify mixed solutions with phylantin concentrations which correspond to the research treatment of 210 µg/ml. The ability of the activity and stability of the emulsion depends on the pattern of distribution of hydrophobic and hydrophilic portions of the protein [19].The treatment without the addition of phylantin solution (P0) had an average emulsion activity index value of 34,20, while the treatment of adding phylantin in the treatments P1, P2, P3, and P4 gave the results of the emulsion activity respectively of 30,33; 45,24; 46,22 and 44,20.
Therefore, it affects the ability of whey protein to form emulsions. It also showed that whey protein is amphipathic and can spontaneously form emulsions. This result is supported by Wu et al. [20] Supported, hydrophobic interactions between phenolic compounds and the hydrophobic groups of proteins form hydrogen bonds. A hydrogen bond was formed between the OH group of the phenol compound and the polar group (NH_2, NH, OH, SH group) on the surface of the protein.
Polyphenols also can engage covalently or non-covalently with protein [21,22]. Murray et al [23] said that non-covalent interactions among phenolic compounds and hydrophobic proteins could be stabilized with the aid of using hydrogen bonds. Interaction among milk protein and phenolic compound forms van der walls bonds in complicated formation, and the protein shape isn`t changed [24].

3.3. Sedimentation

Sedimentation are common in milk and can shorten shelf life. It is caused by the deposition of a layer of proteinaceous material. Factors that affect sedimentation include the density and particle size of the oil and solvent phases in the mixture. The sedimentation of the whey protein with different concentration of phylantin addition is shown in Table 1. The analysis result showed that the addition of phylantine caused a highly significant difference ($P<0.01$) in sedimentation. The presence of bonds formed between proteins and phenolic compounds affects the sedimentation. The more phylantin added, the higher the sedimentation value produced. Stable colloids have several physical properties. That is, it does not form a precipitate and does not separate. The greater the sedimentation produced, the more unstable the colloid. This is according with [25] which states that colloid balance is a crucial parameter in generating a colloid that allows you to make longer shelf life.
Charlton [26] explained that there are three stages in the interaction between polyphenols and milk proteins. The first step is a complex of peptides and polyphenols to form water-soluble aggregates. The second step requires the presence of a polyphenol layer that surrounds the peptide molecule. This phase is supported by weak intermolecular cross-linking interactions carried by polyphenols to form double-sized complexes. As the size increases, the complex can become insoluble in water.
Addition of phenolic to protein can create precipitation in the isoelectric factor [27]. The bond among polyphenols and proteins can purpose conformational adjustments in each protein and philanthine molecules, in order that it is able to have an effect on their solubility. Whey is a protein that has a dense globular structure, so the bigger the whey component, the much less protein molecules might be prompted via way of means of polyphenols. The boom in sedimentation may be resulting from the quantity of phylantin compounds in whey protein.

3.4. Turbidity

The results of the analysis of variance showed that the addition of phylantin had a significant effect ($P<0.05$) on the turbidity value. This is presumably due to the different levels of phylantin addition, so that the interactions that occur between phylantin and whey protein are able to stabilize the protein structure so that with the addition of more phylantin, the solution becomes clearer. This is in accordance with the statement of Gallo et al. [28] which states that polyphenols interact with milk proteins, both whey protein and casein.
The results of the average turbidity of the whey protein solution with the addition of philanthine in Table 1. show that the average tubidity value is at P0; P1; P2; P3 and P4 are 0.35 gr/ml; 0.27 gr/ml; 0.28 gr/ml; 0.30 gr/ml and 0.27 gr/ml while the highest turbidity or the most turbid results were obtained by P0 with a turbidity value of 0.35 without the addition of philanthine to the whey protein solution. It is suspected that the absence of the addition of philanthine caused nothing to bind to the whey protein, so that it was hydrated and the solution became cloudy.

The addition of phylantin tended to reduce the turbidity of the whey nanoprotein solution. Marushin etc. [29] Micelle aggregation or dissociation into submicelle particles alters environmental factors (pH, temperature, ionic strength, etc.) that affect micelle stability due to the lack of strong repetition structure in casein micelles. Casein in milk is in the form of micellar casein and hydrates very easily [30]. P2 and P4 produce the lowest turbidity values, exhibit interaction or cross-linking between whey protein and polyphenols, and maintain colloidal stability between whey protein and phylantin, thereby allowing the solution to be treated with P0.
This is consistent with Hulst [31] that the average particle size was significantly reduced as a result of protein dissociation under the influence of these environmental conditions. Decreasing the particle size of the colloidal component reduces the turbidity. Intermicellar stability refers to the stability of a protein to aggregation [30]. Intermicellar stability refers to the ability of whey protein to maintain its structural integrity under adverse environmental or conditions. Stability increases with the rate of cross-linking. As a result of extensive cross-linking, micelles can be very stable to perturbations.

3.5. Antioxidant activity

In this experiment, the total antioxidant interactionbetween WP and phylantin used 5 treatments of 0; 150 µg/ml; 180 µg/ml; 210 µg/ml; and 240 µg/ml. The different concentrations of phylantin had a highly significant effect ($P<0.01$) on emulsion stability (Table 1). The average antioxidant activity in whey protein-phylantin increased. The highest antioxidant activity was in 240 µg/ml concentration. This means that the concentration produces the antioxidant activity.
Based on the antioxidant activity, it indicates that the way protein could be used as a delivery system for phylantin as based of immunomodulatory products. In accordance with Milani et al., [32] which explains that whey protein is suitable as a transfer system of bioactive compounds. It means that plant based bioactive compounds carried by WP still possess their antioxidant activity. The treatment without the addition of phylantin solution (P0) had an average antioxidant activity of 0.00, while the treatment of adding phylantin in the treatments P1, P2, P3, and P4 gave the results of the antioxidant activity respectively of 66,73; 71,17; 75,28; and 77,15.
Several studies have shown that phylantin source *Phyllantus niruri* have biological activities as good antioxidant activity, along with antibacterial potential, particularly in conditions including diarrhoea, dysentery, dropsy, running nose, winter common colds, blennorrhagia, colic, indigestion, alternating fevers, hepatitis, and malaria [33].

3.6. Particle Size Distribution

The particle size analysis aimed to determine the particle size in nano WP-phylantin complex and their distribution from a representative sample. The aim of this research is to produce nano-sized particles. Particle testing at this stage uses Delsa Nano. The principle of the working system of this tool uses a laser diffraction. Particles will pass through a beam of laser light, then the light is scattered by these particles and collected over a range of direct facing angles. The distribution of the scattered intensity will be analyzed by the computer as a result of the particle size distribution.

Each treatment of different phyantin concentrations produced particle sizes between 923.4-1091 nm (Table 2). This is in accordance with several studies which explain that particles are said to be nano if they have a size of 50-1000 nm [34,35]. These results still reach the size that is in accordance with the study, so it can be concluded that the particle size in protein whey is as expected, which is nano size.

Table 2. Particle size of nano-whey-phylantin

Treatment	Particle size (nm)
P0	1091 nm
P1 (150 µg/ml)	1060 nm
P2 (180 µg/ml)	923.4 nm
P3 (210 µg/ml)	954.9 nm
P4 (240 µg/ml)	1029 nm

The average particle size of nano-whey-phylantin decreases as the concentration of phylantin increases. But in P4 treatment the particle size was increased, it cause the complex in this treatment have the strong interaction then the other. This is due to the interaction of phylantin with OH-phenols that interacts with proteins [36,37]. Proteins that interact with other compounds tend to produce smaller particles. Whey interacts with other compounds to stabilize the surface with larger particles. The addition of dextran has the advantage of being more stable due to the binding between the protein and the polysaccharide that stabilizes the surface of the larger casein micelles. There is a bond between the hydrophilic dextran and casein, forming a bond that is more compact and produces smaller particles of casein micelles. The interaction process for forming nanoparticles requires the correct role of temperature and pH.

It can be concluded that the particle size analysis results for all treatments still give particle sizes classified as nanoparticles. The added higher concentrations give it a smaller size. As a result of the analysis, it can be concluded that the treatment with 180 µg/ml of phylantin has the smallest particle size compared to the other treatments.

4. CONCLUSION

As a result, whey protein containing various concentrations gave the different effect for physicochemical characteristic. The addition 180 µg/ml phylantin is the best treatment because it can increase emulsion stability, antioxidant activity, reduced sedimentation, reduced turbidity, and nanoscale has been shown to be able to maintain.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. The research was funded by the Institute for Research and Community Service Universitas Brawijaya, for the assistance of the Grant Scheme to Hibah Peneliti Pemula 2021.

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