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Original Article

FORMULATION OF SELF-NANO EMULSIFYING SYSTEM (SNES) CONTAINING SNAKEHEAD FISH EXTRACT PROTEIN INCORPORATED INTO CORN OIL BY HYDROPHOBIC ION PAIRING METHOD

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ABSTRACT

Objective: The purpose of this study was to determine whether the protein complexation of snakehead fish extract using the hydrophobic ion pairing method with Sodium Dodecyl Sulfate (SDS) as a complexing ligand could be loaded into the corn oil component of the SNES and to explore the ratio of oil, surfactant, and cosurfactant in the Self-Nanoemulsifying System (SNES) formula that can produce good SNES characteristics.

Methods: Snakehead fish proteins were extracted using pressurized hot water and then complexed with SDS at acidic pH. The SDS-protein complex was loaded into the oil component of the SNES and then combined with the surfactant component (Tween 80) and cosurfactant (propylene glycol) in a ratio of 1:9:1, which, based on the preliminary miscibility screening, has the best miscibility among other screening results.

Results: The results of the study were as follows. The binding efficiency of the SDS-protein-complex was 31.74% and the loading efficiency into SNES was 83.17%. The nanoemulsion formed had visible light transmittance of 100.4%, particle size 72.7 nm, zeta potential-53.03 mV, and emulsification time 71.67 seconds. The nanoemulsion was stable at storage and 100 and 1000 times dilution challenges using aqueous media, Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF).

Conclusion: The hydrophobic ion-pair complexation of snakehead fish protein extract with SDS as the complexing ligand was able to load the protein into the oil component of SNES and the ratio of oil, surfactant, and cosurfactant in the SNES formula that produce good SNES characteristics was corn oil, Tween 80, and propylene glycol at a ratio of 1:9:1

Keywords: Snakehead fish, *Canna striata*, Albumin, Proteins, Self-nanoemulsifying system, SNES, Corn oil, Hydrophobic ion pairing

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INTRODUCTION

Snakehead fish (*Channa striata*) is a type of fish that lives in freshwater and is widely consumed by people in Southeast Asia, including Indonesia. Its high protein content, especially albumin, makes it popularly and empirically used in Indonesia as a supplement in wound healing, such as surgical wounds [1, 2]. In surgery patients, adequate protein intake is very important for modulating surgical stress, supporting recovery and accelerating wound healing; however, surgery patients tend to under-consume proteins [3, 4], so that protein supplement is necessary. If the body is deficient in protein, it can lead to a longer inflammatory phase. This is caused by the inhibition of fibroblast formation and collagen synthesis so that the wound healing process is delayed [5, 6].

The challenges in using pharmaceutical preparations containing protein orally are enzymatic degradation, gastric acid environment, and its rapid elimination from the body [7]. One solution to this challenge is that protein is made in the form of a Self-Nanoemulsifying System (SNES). SNES is a water-free mixture of oil, co-surfactant, and surfactant that emulsifies spontaneously in water with light agitation, such as by peristaltic movements in the gastrointestinal tract, with nano-sized droplets [8, 9]. The advantages of SNES over conventional emulsions or preparations with other lipid carriers are its significantly less energy required in the preparation, its physical stability during storage because it does not contain water, its longer protein residence time in the form of nanoemulsion in the stomach, dan its ability to reach a wider area of the mucous membrane [9, 10] so that the absorption of the protein is higher [8, 11].

The challenge in loading protein into SNES is the low solubility of protein in oil, so manipulation is needed to increase protein miscibility with the oil component of the SNES [7]. One method that can be used to increase the lipophilicity of proteins/peptides is the hydrophobic ion pair method, in which the polar ionizable protein groups (OH and NH) are shielded by oppositely charged complexing materials (ligand) such as surfactants or other amphiphilic molecules by complex formation via electrostatic interactions at the appropriate pH [12, 13]. The complex is reversible because it is easily dissociated in the presence of an excess of oppositely charged ion. Previous research showed that Sodium Dodecyl Sulfate (SDS) as an ion pairing agent for forming Hydrophobic Ion Pairing (HIP)-lysozyme complexes as a model protein resulted in an average % encapsulation efficiency of 42.85% [13].

This study aims to determine whether the hydrophobic ion-pair complexation of snakehead fish protein extract with SDS as the complexing ligand can load the protein into the oil component of SNES and to explore the ratio of oil, surfactant, and cosurfactant in the SNES formula that produce good SNES characteristics.

MATERIALS AND METHODS

Material

Snakehead fish purchased from local market in Surakarta Indonesia, bovine serum albumin/BSA (Sigma), distilled water, Na_2CO_3, NaOH, $KNaC_4H_4O_6$, $CuSO_4.5H_2O$ (Merck), Folin Ciocalteu reagent (Merck), Sodium Dodecyl Sulfate (SDS), HCl, Tween 80, Tween 20, Span 80, Span 20, propylene glycol, corn oil (PT Agung Jaya, Surakarta Indonesia).

Methods

Preparation of snakehead fish (*Channa Striata*) extract

Snakehead fishes were filleted, washed using clean water, put in a stainless-steel pressure cooker, added with water in a ratio of 1:4, and cooked on a stove for 2 h. Every 30 min, water was added to

keep a constant volume of the liquid extract. The liquid extract was obtained by filtration and was then stored in the refrigerator until used.

Determination of dissolved protein levels using spectrophotometry

The calibration curve equation was prepared by plotting the concentration series (30, 90, 150, 210, 270, and-300 µg/ml) of bovine serum albumin/BSA solution against its spectrophotometric absorbance measured by spectrophotometer (Shimadzu). The concentration series were prepared by pipetting 60, 180, 300, 420, 540, and 600 µl of stock solution of 5000 µg/ml BSA in distilled water into a 10.0 volumetric flask, followed by addition of 8 ml of Lowry's reagent B [(1 g Na2CO3+50 ml NaOH 0.1 N) in (1 ml of distilled water+10 mg of KNa tartrate+10 mg of CuSO4.5H2O)] and allowed to stand for 10 min, then 1 ml of Lowry's reagent A [Folin Ciocalteu's reagent) in distilled water (1:1)] was added, and the volume was made to 10 ml by the addition of distilled water. The same steps were carried out for the snakehead fish protein extract sample and the blank solutions (300 µL water) and the spectrophotometric analyses were performed accordingly. The maximum wavelength of spectrophotometric analysis was 761.0 nm that resulted from absorbance scanning the BSA solution in a range of 400-800 nm wavelength. The operating time was determined as the time when the solution give stable absorbance at the maximum wavelength and the result was 8 min [14].

Protein-SDS hydrophobic ion-pair (HIP) complex formation

Hydrophobic ion-pairing complexation between snakehead fish protein extract and SDS was carried out at molar ratios of 1:6, 1:12, 1:18, and 1:24, each at pH 1.2; 3.5; and 5.0. The extract solutions were made into the desired pH values, each in quadruples, by dropwise addition of HCl 0.1 N, monitored by pH meter (Ohaus). SDS solution in distilled water (1 mg/ml) was added to each extract solution in the desired molar ratios. The solutions were then centrifuged to separate the precipitate and the supernatant. The precipitate containing the HIP protein-SDS complex was freeze-dried for incorporation into SNES. The levels of free protein in the supernatant were analyzed spectrophotometrically using the Lowry method to determine the binding efficiency of the SDS-protein complex.

Determination of complex binding efficiency

Determination of the efficiency of the formation of complex bonds between protein and SDS was carried out by indirect method. During the reaction, the formation of a white precipitate indicates the formation of a hydrophobic SDS-protein complex and the supernatant would contain the more hydrophilic free protein fraction. Percentage of precipitation as the parameter of complex binding efficiency is defined using the following equation [15]:

$$\text{Precipitation efficiency [100\%]} = 100 - \left(\frac{\text{Protein concentration after HIP}}{\text{Protein concentration before HIP}} \text{ x } 100\right)$$

Screening of SNES components

The screening of the SNES components was carried out by visual observation of the miscibility of binary mixture between corn oil and surfactant (Tween 80, Tween 20, Span 80, or Span 20), surfactant and cosurfactant (propylene glycol), and oil and cosurfactant at ratios of 1:9 to 9:1. This was done to obtain the most homogeneously mixed SNES components. The mixture was homogenized with a vortex for 5 min, then sonicated for 15 min and vortexed again for 5 min. After that, the mixtures were visually observed for the presence of phase separation. Further screening for ternary mixture was done using the most miscible pair of the binary mixtures, with the cosurfactant kept at a constant portion.

Protein extract loading test

Twenty-five mg of HIP protein-SDS complex powder was dissolved in 5 ml of corn oil, vortexed for 5 min, sonicated for 15 min and vortexed again for 5 min, then centrifuged. The pellet (sediment) was separated from the corn oil for assay and the liquid phase of the oil was put aside for use as the oil component of the protein-containing SNES. Protein loaded into oil was calculated as the difference between the amount of protein added and the amount of free protein in the pellet. The drug loading efficiency equation is (7) as follows:

$$\text{Drug loading efficiency} = \frac{\text{Amount of protein added} - \text{Free protein}}{\text{Amount of protein added}} \text{ x } 100\%$$

Formation of SNES containing protein-SDS HIP complex

The corn oil containing the protein-SDS complex was mixed with surfactant and cosurfactant in the selected ratios resulting from the previous screening stage. The mixture was vortexed for 5 min, sonicated for 15 min, and vortexed again for 5 min.

Evaluation and characterization of snakehead fish SNES nanoemulsion

Self-emulsification time

Simulated Gastric Fluid (SGF) solution was made by addition of 200 mg of NaCl and 220 µl of concentrated HCl into distilled water in a 100 ml volumetric flask and the volume was made up into 100 ml by water addition. Emulsification time was observed visually as the SNES was emulsified using a magnetic stirrer in a beaker glass. A hundred microliter of snakehead fish SNES was added to 100 ml of SGF pH 1.2 at 37±1 °Cunder continuous stirring at 100 rpm. The self-emulsification time was recorded as the time for the SNES to form a homogeneous mixture since being drop into the medium [16-18].

Percent of transmittance of the formed nanoemulsion

SNES was emulsified with SGF in 1:1000 ratio and analyzed using a spectrophotometer for its transmittance value at a wavelength of 650 nm [16-18].

Droplet size and zeta potential of the nanoemulsion

Distilled water was added to the snakehead fish protein SNES in a ratio of 1:1000 in a test tube and then vortexed for one minute. The particle size and zeta potential of snakehead fish protein SNES was analyzed at a temperature of 25°Cusing a particle size analyzer (Horiba) [7, 17, 18].

Phase separation and emulsion stability

Simulated Intestinal Fluid (SIF) solution was prepared using 6.8 mg of potassium hydrogen phosphate in 250 ml of distilled water with 190 ml of 0.2 N NaOH and diluted to 400 ml. Then, the pH of the mixture was adjusted to 7.5±0.1 by adding 0.2 N NaOH and distilled water was then added to obtain 1 ml solution. One hundred micro liter BSA SNES was added with distilled water, SGF pH 1.2, or Simulated Intestinal Fluid (SIF) pH 7.5 until each volume reached 5 ml. The solution was left to stand at 37 °Cfor 4 h in a water bath and observed every hour for the presence of precipitation [16].

Dilution power

The snakehead fish protein extract SNES was tested for its resistance to dilution with 100x and 1000x dilutions using distilled water and SGF pH 1.2 to mimic the processes that occur after the formula is given orally. The nanoemulsion was then stored for 24 h and visually observed if separation occured [16].

RESULTS AND DISCUSSION

Snakehead fish (*Channa striata*) protein extract

From 962.2 g of snakehead fish extracted, 3.180 ml liquid extract was obtained. According to [19] liquid extract obtained from snakehead fish contains more albumin than other types of protein, with albumin levels being as much as 64.61% of the total protein.

Dissolved protein levels

The calibration curve obtained was $y = 7.29 \times 10^{-3}x+0.226$, with $r^2 = 0.991$, which is linear ($r^2>0.98$). The level of the protein content in the snakehead fish extract was 5.5983 mg/ml or 1.85 g per 100 g of snakehead fish fillet.

Production of hydrophobic ion-paired protein-SDS complex

The liquid protein extract was prepared in an acidic pH of 1.2; 3.5; and 5.0 because at a pH below its isoelectric point, the protein will be

positively charged. The isoelectric point of protein in general, is in the pH range of 4.5–5.5 [20]. HIP complexes are formed from electrostatic interactions between positively charged protein groups and functional groups of complex-forming materials with opposite (negative) charges, such as SDS surfactants at the appropriate pH [12, 13].

The results of the measurement of free protein level and the HIP complex binding efficiency are shown in table 1. Since at pH 1.2, precipitation can occur due to denaturation of the protein and at 5.0 the precipitation is mostly correlated with the isoelectric point of the protein, the series at pH 3.5 were considered as where the precipitation occur due to the HIP complex formation. In this series, the lowest concentration of the free protein, hence, highest level of HIP complex formed, was in the series F, i.e., 3819.07 µg/ml (1779.23 µg/ml of HIP complexed protein).

Complex binding efficiency

Complex binding efficiency is expressed by the percentage of precipitation since, during the reaction, the formation of a white precipitate indicates the formation of hydrophobic protein complexes because more hydrophilic proteins will dissolve in the hydrophilic supernatant (distilled water) (15). The percentage of precipitation of the F series was 31.74%, which was then chosen to be loaded into the selected SNES components.

Table 1: HIP protein-SDS complex concentration series

pH	Series name	Ratio of protein: SDS	Free protein level (µg/ml)	HIP complex binding efficiency (%)
1.2	A	1:6	3297.63	41.10
	B	1:12	3692.7	34.04
	C	1:18	4159.23	25.71
	D	1:24	5919.83	-5.74
3.5	E	1:6	4240.7	24.25
	F	1:12	3819.07	31.78
	G	1:18	4776.4	14.68
	H	1:24	3926.83	29.86
5.0	I	1:6	2437.33	56.46
	J	1:12	3610.9	35.50
	K	1:18	3070,67	45.15
	L	1:24	2951.27	47.28

Table 2: Screening of SNES components

Volume ratio			Miscibility
Tween 80	Propylene glycol	Corn oil	
1	1	9	Not mixed
2	1	9	Not mixed
9	1	2	Poorly mixed
9	1	1	Mixed

SNES composition

Screening of the ratio of oil, surfactant, and cosurfactant was carried out to find a prospective SNES formula. The most mixed binary mixtures observed in the screening results were Tween 80 (surfactant) with propylene glycol (cosurfactant), Tween 80 with corn oil at 9:1 and propylene glycol with corn oil at 1:9. Furthermore, the results of ternary mixture consisted of Tween 80, propylene glycol, and corn oil can be seen in table 2.

The selected SNES components combination that mixed together, showing no phase separation, was Tween 80, propylene glycol, and corn oil at a ratio of 9:1:1.

Fig. 1: The selected mixture of corn oil, Tween 80 and propylene glycol (1:9:1)

Protein extract loading into oil component of SNES

Loading of freeze-dried protein extract samples into the corn oil resulted in the supernatant and precipitate. The supernatant contains HIP complexed protein, while the precipitate is hydrophilic protein that cannot be dissolved in oil, the level of which was measured. The amount of protein sample after freeze drying with the addition of SDS in triplicate was 24.867 mg. The amount of protein pellet after loading the extract in corn oil in triplicate was 4.185 mg. Thus, the amount of protein contained in SNES is the difference between the two, which was 20.682 mg or 83.17%. These results was not much different from the result of the previous study, which was 83.91±1.73% [16].

Evaluation and characterization of snakehead fish SNES nanoemulsion

The first test is the evaluation of the emulsification time on SNES, which describes whether or not the formation of emulsions is easy in the body. This test is an important parameter to assess the self-emulsification efficiency of the SNES formula. The emulsification time test was carried out at 100 rpm because low energy was required to simulate gastrointestinal peristalsis. The emulsification time is influenced by the speed with which the surfactant and cosurfactants create a layer between oil and water. The snakehead fish SNES formula using corn oil showed the average emulsification time at SGF pH 1.2, which was 71.67 seconds. This shows that SNES is quite good because it is close to a good SNES emulsification time, which is in the range of 20-60 seconds [16, 17].

The second test is the transmittance of the snakehead fish SNES formula using corn oil at SGF pH 1.2 visually, the SNES results look clear with a transmittance of 100.4%. A good SNES looks clear so it has a transmittance>90% [16]. This shows that the SNES research results have been good.

The third test is particle size, where the smaller the particle size, the more easily absorbed the drug is. The results of particle measurement using PSA showed that the average SNES size (Z-Average) of the research results was 72.7 nm. These results indicate a good nano size that is<100 nm. The size of the nanoemulsion will be smaller if the ratio of surfactant and co-surfactant is higher. Oil can increase the ability of SNES to carry drugs, but its composition is usually lower than surfactants because it can increase the size of SNES [16, 17].

The fourth test is the zeta potential, which is influenced by the type of surfactant used. In this study, the nonionic surfactant Tween 80 was used, which has the advantage of not being sensitive to changes in pH. The nanoemulsion is stable against deflocculation if the charge is between-10 to-30 mV. SNES research results have a zeta potential value of-53.03 mV. The results show that SNES is less stable to deflocculation and contains a lot of free fatty acids [16].

The fifth test was the stability of SNES in SGF, SIF, and distilled water. The results of this test based on visual observations showed that SNES was stable in the three media because there was no precipitate. A precipitate can appear due to the rupture of the emulsion so that the oil is no longer encapsulated by surfactants and cosurfactants [16].

The last test was the stability of SNES in 100 times and 1000 times dilutions in SGF, SIF, and aquadest media. The results showed that SNES was stable in dilutions of 100 times and 1000 times in all three media characterized by no physical changes, deposits or lumps formed in SNES [16].

CONCLUSION

The hydrophobic ion-pair complexation of snakehead fish protein extract with SDS as the complexing ligand can load the protein into the oil component of SNES, and the ratio of oil, surfactant, and cosurfactant in the SNES formula that produce good SNES characteristics was Tween 80, propylene glycol, and corn oil at a ratio of 9:1:1.

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AUTHORS CONTRIBUTIONS

Gunawan Setiyadi made contributions to the overall research design and wrote the manuscript. Suprapto and Endang N. Widyaningsih contributed by supervising laboratory activities and supporting the writing of the paper. Amanda A. Salsabila and Izzatul Qudsiyah contributed to data collection and the preparation of the research report.

CONFLICT OF INTERESTS

There is no conflict of interest

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