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Research Paper

Liposome Photosensitizer with Enzyme from Black Soybean Tempeh: Formula Optimization and In Vitro Thrombolytic Activity Evaluation

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Abstract
Stroke and myocardial infarction contributed significantly as the leading causes of global mortality rate, both commonly caused by thrombosis. Black soybean tempeh (BSBT), a traditional Indonesian food fermented with *Rhizopus oligosporus* fungus is rich in proteolytic enzymes, with potential to be utilized for thrombosis related ailments. Herein, we report the first findings of BSBT enzymatic activity and its subsequent formulation into liposomal system as a thrombolytic. Additionally, we incorporated photosensitizer dyes into the liposomes, phycocyanin and fluoresecein, creating a photothermally active therapeutic delivery system. Liposomes containing BSBT were formulated using soy lecithin and tween 80, which were then subjected to evaluations including size, PDI, zeta potential, morphological, and stability studies. Furthermore, we observed their photothermal efficiency and thrombolytic activity using whole blood clot in vitro model. BSBT crude and purified extract produced satisfactory enzymatic activity, stable at neutral pH (~7) and maintained stable activity at temperatures of ~60°C. Liposome formulation was spherical with a particle size of 607.8 nm; PDI of 0.339; and zeta potential of -24.2 mV. BSBT crude extract and purified enzyme at a concentration of 100% gave 51.28 and 56.05% thrombolytic activity. Based on the test results obtained, the optimum formula of photosensitizer liposomes produced had high encapsulation efficiency, with photothermal efficiency of 57.66 and 44.23% for Lip-Flu and Lip-Phy respectively. The formulations with laser exposure generated good thrombolytic activity (~55-56%) comparable with nattokinase. Based on these findings, liposomal delivery of BSBT enzymes can maintain proteolytic activity, providing the first insights for thrombolytic purposes of BSBT enzymes.

Keywords
Black Soybean Tempeh, Liposome, Photosensitizer, Photothermal Therapy, Thrombolytic

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1. INTRODUCTION

Stroke and myocardial infarction are among the leading causes of mortality and morbidity worldwide. The prevalence of stroke has risen by 70% between 1990 and 2019, while mortality rates have increased by 26.3% from 2011 to 2021. These conditions result from blood vessel blockages due to excessive clot formation, known as thrombosis, which includes arterial thrombosis, deep vein thrombosis (DVT), and pulmonary embolism (PE). If left untreated, DVT can lead to the migration of a thrombus to the lungs, significantly raising the risk of fatality (Martin et al., 2024).

Thrombosis treatment typically involves the use of anticoagulants and antiplatelet drugs for prevention, while thrombolytics are used as a curative approach to dissolve existing blood clots. Commonly administered thrombolytics include recombinant tissue plasminogen activator (rtPA), such as alteplase and tenecteplase, as well as streptokinase and urokinase. These therapeutic protease enzymes function as fibrinolytic agents by converting plasminogen into plasmin, which helps break down thrombi. However, rtPA therapy carries risks, including severe bleeding, intracranial hemorrhage, and the potential for thrombosis recurrence at sites of residual clotting. Thrombolytic agents that mimic plasmin enzymes are being developed as alternatives to rtPA due to their lower bleeding risk and faster thrombin-dissolving action. One such example is nattokinase, a protease enzyme that directly degrades fibrin. Nattokinase is derived from natto, a traditional Japanese fermented food (Dipiro, 2020; Jaffer and Weitz, 2018; Ortel et al., 2020; Sahoo and Sahoo, 2020; Weber et al., 2019).

In Indonesia, tempeh, a functional food made from black soybeans fermented with *Rhizopus oligosporus*, has potential as a natural thrombosis therapy. Tempeh is abundant with nutrition and bioactive compounds, including carbohydrates, flavonoids, fatty acid and protein (Wang et al., 2022). The fungus in tempeh is rich in protease enzymes, which exhibit strong thrombolytic activity. Enzymes derived from food sources offer advantages such as a lower risk of allergic reactions, ease of production, and cost-effectiveness (Nadea et al., 2023; Pandey et al., 2016). The enzyme from black soybean tempeh is delivered to the targeted thrombus site using nanocarrier technology. Liposomes are a suitable choice as carriers due to their vesicular structure, which allows them to encapsulate both hydrophilic and hydrophobic substances with high efficiency.

In this study, natural phospholipids in the form of soy lecithin were selected as the primary component of liposomes. Soy lecithin offers greater stability compared to lecithin derived from egg yolk, marine animals, or milk, as it contains fewer unsaturated fatty acids. To enhance the flexibility and permeability of the liposome membrane, Tween 80 is used as a nonionic surfactant. The presence of Tween 80 in lecithin helps reduce multilamellarity by preventing lipid accumulation, thanks to its large hydrophilic component (Le et al., 2019; Tsengam et al., 2022; Weber et al., 2019).

One of the preferred therapies for managing thrombosis is photothermal therapy. A study by Li et al. (2020) demonstrated that infrared light has strong penetration into biological tissues, making infrared-absorbing materials highly promising for clinical diagnosis and disease treatment. Photothermal therapy offers several advantages, including pain reduction, shorter treatment duration, enhanced therapeutic efficacy, and lower toxicity. Photosensitizing agents such as phycocyanin and fluorescein sodium can be used in this therapy. Proteolytic enzyme (including enzyme from BSBT) can be carried to the location of the thrombus or blood clot within the blood vessel by a liposomal carrier. Enzyme-loaded liposome is triggered for release by incorporating photosensitizers, which convert energy from light (laser) into heat (Figure 1). The heat generated not only ruptured the liposome-carrying proteolytic enzyme but also facilitated the breakdown of the fibrin matrix within the blood clot. This synergistic mechanism enhances the fibrin degradation process hence increases thrombolysis (Fithri et al., 2023; Fithri et al., 2025).

This study focused on formulating and optimizing photosensitizer liposomes encapsulating crude and purified enzymes from black soybean tempeh to assess their effects on thrombosis under 450 nm radiation. Photosensitizers selected for this study have absorption peaks around 450-550 nm, decisively chosen to enhance photothermal effects generated by the exposure of 450 nm laser. Liposomes were generated using thin film hydration method, incorporating two different photosensitizers with good absorption within 450-550 nm, namely fluorescein (Flu) and Phycocyanin (Phy). Evaluation of liposomal formulations was conducted based on key characterization parameters, including pH, viscosity, encapsulation efficiency, and photothermal performance of the liposomes. The optimum formula based on thrombolytic activity, temperature increase, and stability was further characterized through nanoparticle characterizations including particle size, distribution, zeta potential, blood compatibility, and photothermal efficiency. Additionally, the crude extract and purified enzymes from black soybean tempeh were analyzed by measuring their enzymatic activity and protein molecular size.

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used were samples and analytical materials. Sample materials consist of black soybeans (Healthy by Tasty) and tempeh yeast (Raprima) for the manufacture of BSBT. Soy lecithin, tween 80, phycocyanin, chloroform, and n-hexane were purchased from Bratachem and ROPA Chemicals. At the same time, the other analytical materials used including sodium fluorescein, bovine serum albumin, biuret reagent, casein, trichloroacetic acid, tris-HCl, ammonium sulfate, Triton X-100, and calcium chloride were purchased from Merck. Nattokinase was purchased online (Dostor's best) and fresh whole blood was obtained in Klinik Sriwijaya University.

2.2 Preparation of Tempeh

The sample used in this study was black soybean tempeh made from black soybeans fermented with yeast. The making of black soybean tempeh was done by soaking 1 kg of black soybeans for 12 hours in water and then washed thoroughly by separating the skin from the soybeans. Cleaned black soybeans were then boiled for 30 minutes at 110±5°C. The black soybeans were then drained and cooled for 20 minutes, then mixed with 3 g of tempeh yeast evenly and put into a clean plastic bag that had been given small holes with a diameter of 0.5 mm. The fermentation time was conducted for 48 hours at 32±4°C.

2.3 Characterization of Tempeh

Tempeh characterization can be done based on several aspects including organoleptic, ash content, water content, protein content, fat content, metal-arsenic contamination test, and bacterial contamination test. Organoleptic tests were conducted by observing the color, texture, aroma, and taste of fermented black soybean tempeh (Ellent et al., 2022). The observed tempeh was then compared with the organoleptic of tempeh obtained in general and compared with previous studies. The results of tempeh characterization need to comply with the requirements set by SNI 3144:2009 to continue making black soybean tempeh enzymes.

2.4 Crude Enzyme Extraction and Purification of Enzyme Extract of Black Soybean Tempeh

The extraction process of the crude enzyme was carried out by blending 200 g of black soybean tempeh with a blender at 10,000 rpm and adding 500 mL of cold PBS (pH 7.4). The addition of PBS into the blending process of black soybean

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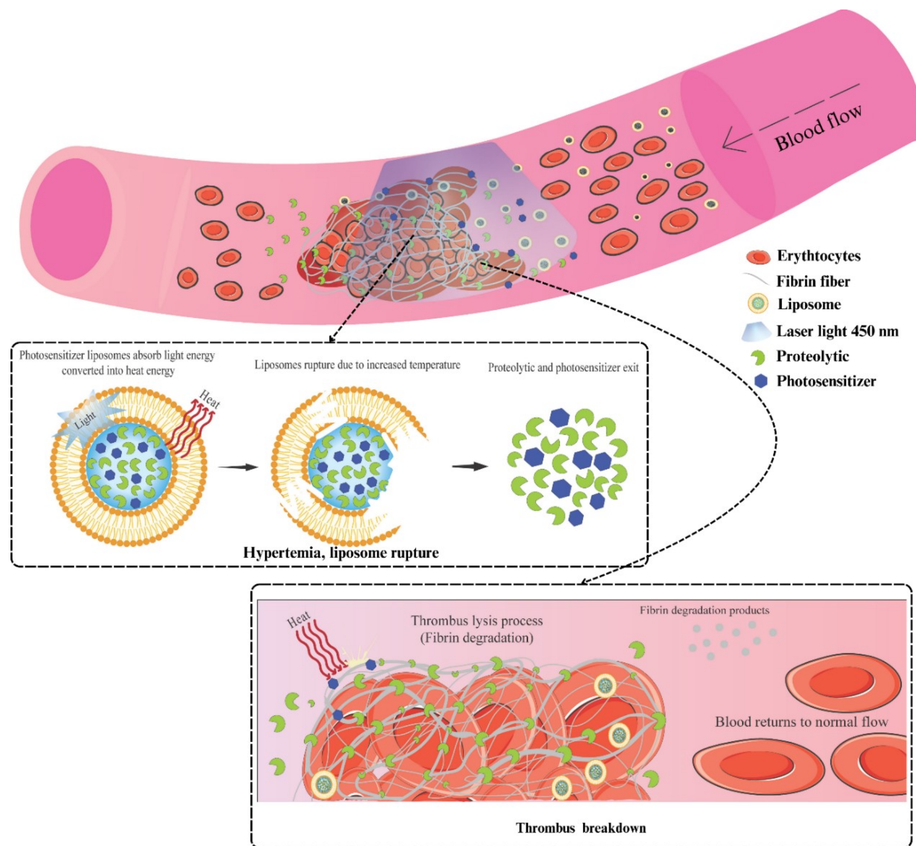

Figure 1. Illustration of Mechanism Describing the Process of Thrombolysis Facilitated by Photosensitisers and Black Soybean Tempeh Extract

Table 1. Formula of BSBT-Photosensitizer Liposomes

Components	Formula			
	Lip-Phy-CEBSBT	Lip-Phy-PEBSBT	Lip-Flu-CEBSBT	Lip-Flu-PEBSBT
BSBT Crude Extract (mL)	25	-	25	-
BSBT Purified Extract (mL)	-	25	-	25
Soya Lecithin (%)	0.605	0.605	0.605	0.605
Tween 80 (%)	0.01	0.01	0.01	0.01
Phycocyanin (mg)	50	50	-	-
Fluorescein (mg)	-	-	50	50
PBS (mL)	25	25	25	25

tempeh was performed slowly. The blended samples were homogenized using a shaker (Biobase) at 150 rpm for 30 minutes in a cool state at 4°C. The samples were then filtered with flannel cloth to collect the filtrate. The centrifugation process was carried out on the filtered sample at a speed of 4,000 rpm (RC-180) at 4°C for 30 minutes. The resulting supernatant is the crude extract that will be used to estimate enzyme activity (Chimbekujwo et al., 2020). Purification of the enzyme crude extract was carried out by adding ammonium sulfate with 80% saturation as much as 103.2 g for 250 mL of extract. Ammonium sulfate was slowly introduced into the soybean tempeh enzyme crude extract by stirring at a speed of 1500 rpm. The mixture was then centrifuged at 10,000 rpm (Thermoscientific) for 20 minutes at 4°C (Nadea et al., 2023).

2.5 Determination of Enzyme Activity, Optimal pH, and Optimal Temperature

Enzyme activity was measured using casein as the substrate. A 0.2 mL enzyme solution was mixed with 1.8 mL of 0.5% casein in 0.1 M phosphate buffer (pH 7.0) and incubated at 37°C for

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Table 2. Characterization of Black Soybean Tempeh and Quality Requirements as Specified by SNI

Test Parameters	Result (Mean ± SD)	SNI 3144:2009
Water content (%)	39.84 ± 4.17	Max. 65
Ash content (%)	1.05 ± 0.09	Max. 1.5
Fat content (%)	12.41 ± 4.57	Max. 0.2
Protein content (%)	24.37± 1.10	Max. 0.25
Cadmium (Cd) metal contamination (mg/kg)	0.05	Max. 40
Lead (Pb) metal contamination (mg/kg)	0.083	Max. 0,2
Tin (Sn) metal contamination (mg/kg)	0.019	Max. 0.25
Arsenic (As) contamination (mg/kg)	0.011	Max. 0.25
Microbial contamination *Coliform* (APM/g)	0	Max. 10
Microbial contamination *Salmonella sp* (CFU/g)	Negative/ 25 gram	Negative/25 gram

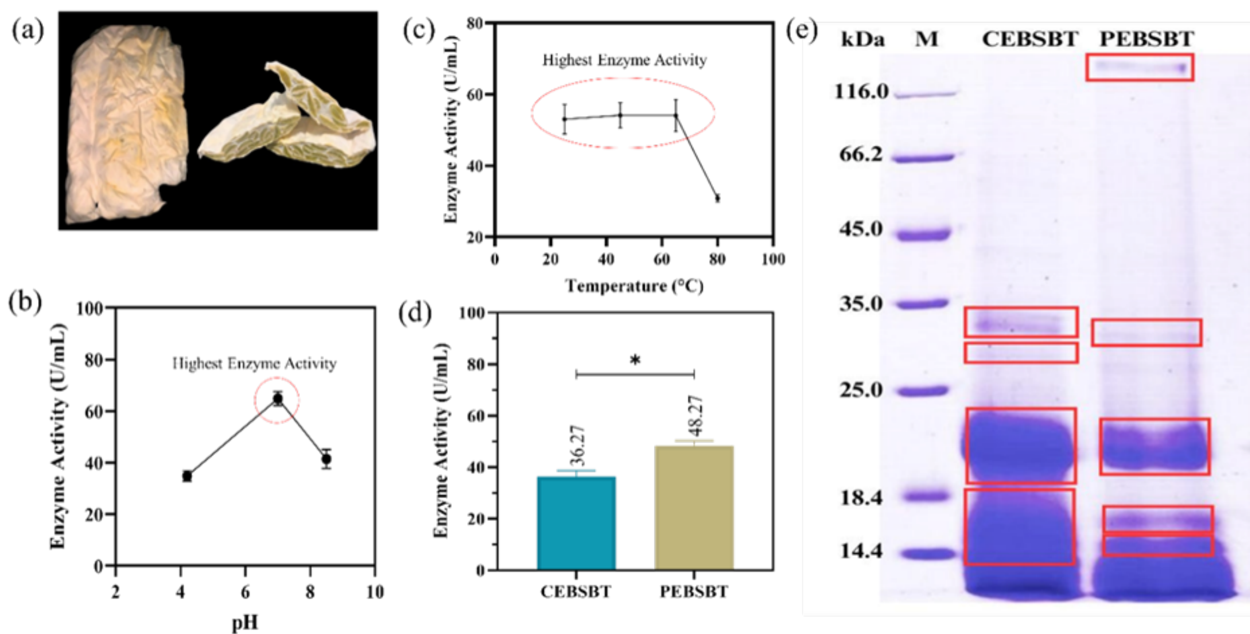

Figure 2. (a) Photograph of the Black Soybean Tempeh (BSBT) Produced (b) Enzyme Activity Graph at Optimum pH Showing Highest Activity at pH ~6 and (c) Enzyme Activity Graph at Optimum Temperature Indicating at Temperature Of >80°C Enzymatic Activity Significantly Reduced. (d) Difference In Enzyme Activity Between Crude (CEBSBT) and Purified Extract (PEBSBT) (e) and Confirmation of Protein Molecular Weight Obtained from the Extracts with SDS-PAGE Method

10 minutes. After adding 2 mL of cold 0.1 M trichloroacetic acid, the mixture was centrifuged at 10,000 rpm for 10 minutes. A blank (distilled water) and a standard (nattokinase) were prepared similarly. Absorbance was measured with a spectrophotometer to calculate enzyme activity. Optimal pH was determined using different buffers: acetic acid (pH 3-5), phosphate (pH 6-7), and Tris-HCl (pH 8-9). The purified enzyme was incubated at 37°C for 15 minutes, then cooled in an ice bath before measuring activity and plotting % enzyme activity vs pH (Alhasani and Al-Younis, 2021). Optimum temperature was assessed by incubating the enzyme at 25, 45, 65, and 80°C for 30 minutes, followed by cooling in an ice bath. Enzyme activity was measured (Biobase spectrophotometer), and the relationship between % enzyme activity and temperature was plotted to determine the optimal temperature (Hmood and Aziz, 2016).

2.6 Analysis of Molecular Weight Size of Enzyme Proteins with SDS-PAGE

Molecular weight measurement of proteins (enzymes) was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-rad). Separating gel was prepared using a 12% acrylamide, mixture was then homogenized quickly before inserting into the glass molds. The hardened gel was poured with isopropanol to prevent bubbles formation followed by rinsing with distilled water until no isopropanol

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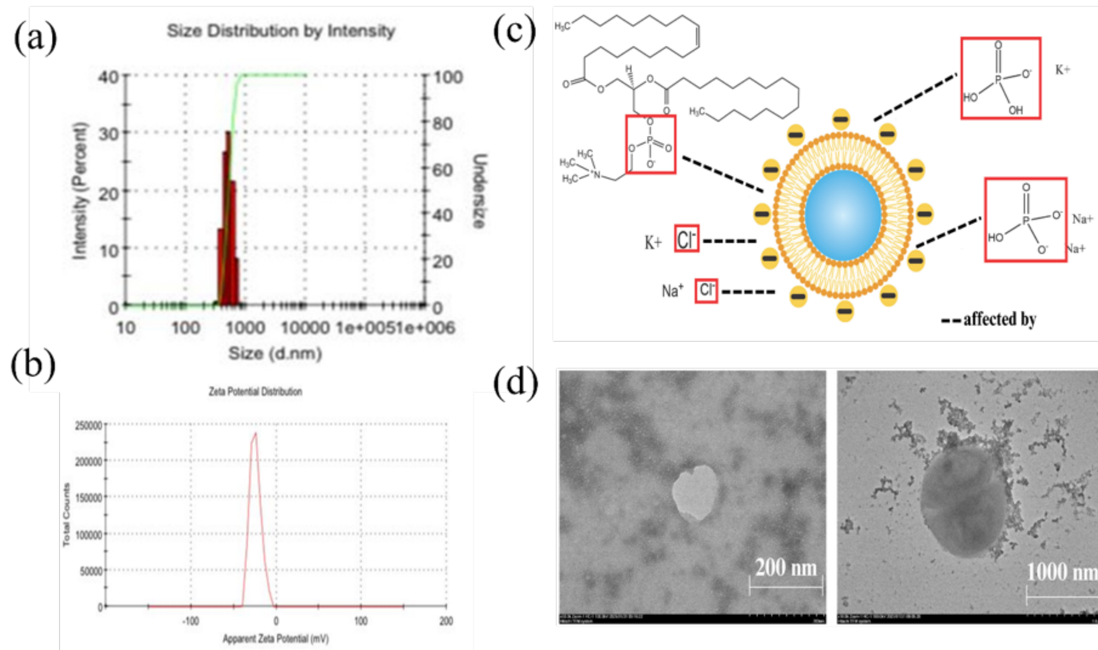

Figure 3. (a) Particle Size Distribution and (b) Zeta Potential Result from Liposome Characterization. (c) Illustration Showcasing Predicted Reasoning of the Negative Charge Presented from Zeta Potential Result Based on Liposome Components. (d) TEM Image of Lip-Phy (Most Optimum Result) with 10000× (Left) and 50000× Magnification (Right)

remained. Preparation of 4% acrylamide stacking gel was prepared similarly with the previous protocol, and a comb to create wells were inserted before the gel hardened. Samples were inserted into the wells created along with a protein marker. Gel was run on 100 V of power until separation was achieved, followed by Coomasie blue staining for observation.

2.7 Liposome Preparation

Liposome were prepared using the thin layer film method by mixing lipid compounds such as soy lecithin and tween 80 in 10 mL of chloroform. The resulting solution was dried in a rotary evaporator (D-Lab) to form a thin lipid film. The process of thin lipid film formation and solvent evaporation was carried out at 73°C, with a rotation speed of 80 rpm for 15 minutes. The thin lipid film formed was then hydrated with 25 mL PBS pH 7.4 which had dissolved 50 mg of phycocyanin in the formula used in determining the optimum formula and 25 mL CEBSBT and PEBSBT which had dissolved the photosensitizer were added to the liposomes of the optimum formula that had been determined. The liposomes were then sonicated using a bath sonicator for 30 minutes (Levine et al., 2013; Lu et al., 2021). The complete formula can be observed in Table 1.

2.8 Liposome Characterization

Liposome characterization includes physical appearance, pH, viscosity, temperature increase, and encapsulation efficiency as responses in determining the optimum formula. Characterization of the optimum formula includes particle size, polydispersity index, zeta potential, particle morphology, encapsulation efficiency, and physical stability.

2.9 Photothermal Efficiency

The ability of nanoparticles to convert light energy into heat can be evaluated by measuring the temperature of liposomes before and after exposure to a 450 nm laser with an intensity of 0.3 W/cm^2 for 300 seconds. After irradiation, the laser was switched off, and the temperature decrease over the next 300 seconds was recorded. Slope between ln θ and time after the laser was turned off was created to calculate the photothermal efficiency (Fithri et al., 2023).

2.10 In Vitro Thrombolytic Test

The thrombolytic activity test was performed following a procedure adapted from Fithri et al by using an in vitro clot lysis method with fresh human blood (Fithri et al., 2023). Clots were formed by mixing 10 μL of 1 M $CaCl_2$ with 380 μL of blood in a 1.5 mL centrifuge tube, incubated at 37°C for 30 minutes, and washed three times with PBS (pH 7.4) before weighing (W_0). Thrombolytic test was conducted with laser irradiation and without laser by incubating the clot and sample. For the thrombolytic test without laser, 500 μL of sample was incubated with the clot at 37°C for 90 minutes. After incubation, clots were reweighed (W_1) to calculate lysis using Equation 1. For the laser-assisted thrombolysis test, clots in 1 mL vials containing 500 μL of the sample were irradiated with a 450 nm laser at a 10 cm distance. The dissolved clot was reweighed (W_1) to determine thrombolysis activity.

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Table 3. Observation Results of Liposomal Formulations

Formula	Responses (n=3) ± SD		
	Organoleptic Evaluation	%EE	pH
Lip-Phy-CEBSBT	Intense blue color, with no visible sedimentation or phase separation	88.20 ±1.76	7.43 ±0.06
Lip-Phy-PEBSBT	Electric blue, with no visible sedimentation or phase separation	90.20 ±0.838	6.41 ±0.01
Lip-Flu-CEBSBT	Yellow, with no visible sedimentation or phase separation	67.87 ±7.253	7.01 ±0.01
Lip-Flu-PEBSBT	Fluorescent yellow with no visible sedimentation or phase separation	88.78 ±4.05	6.66 ±0.03

$$\text{Thrombolytic activity} = \frac{W_0 - W_1}{W_1} \times 100\% \quad (1)$$

2.11 Hemolysis Test

The hemolysis test involved centrifuging blood at 1000 rpm to isolate red blood cells (RBCs) following methods described in Fithri et al. (2023). The RBC pellet (1 mL) was washed twice with 10 mL PBS (pH 7.4) and then resuspended in 10 mL PBS. For the test, 60 μL of the sample was mixed with 540 µL of RBC suspension in a 1.5 mL centrifuge tube and incubated at 37°C for 4 and 24 hours. After incubation, the mixture was centrifuged at 1000 rpm for 10 minutes, and the supernatant was analyzed using a UV-Vis spectrophotometer. The percentage of hemolysis was calculated using Equation 2.

$$\text{Hemolysis}(\%) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative control}}}{\text{Abs}_{\text{positive control}} - \text{Abs}_{\text{negative control}}} \times 100\% \quad (2)$$

Statistical Analysis and Illustration Data analysis was performed using Graphad® Prism 8 software by observing normally distributed data. Statistical processing was conducted using analysis of variance and Tukey's multiple comparison test method. All illustrations were created using Adobe Illustrator.

3. RESULTS AND DISCUSSION

The black soybean tempeh (BSBT) obtained has the appearance of a thick block, each part of the soybean is coated with white hyphae growth due to the fermentation process as in Figure 2a. The aroma produced by tempeh has a distinctive aroma of fermented products. The white surface color of the fungal hyphae can turn black due to the formation of spores or excessive production of ammonia aroma. BSBT obtained has an organoleptic form that is in accordance with the tempeh booklet sourced from the national standards agency (SNI 3144:2009). By maintaining similar quality with the specification from SNI, we can conclude the process of BSBT manufacture has been carried out properly and correctly and has obtained tempeh that has quality according to standards (Table 2).

Enzymes have an optimum pH to perform their activities optimally and at higher or lower pH the activity can decrease or increase. This is because the amino acid side chains in the active site of the protein have the ability to function as weak acids and bases to maintain a certain ionization state depending on the pH and the surrounding environment. Enzyme activity affected by pH shows the highest enzyme activity value when the enzyme is in a neutral pH environment, namely 7.0 with an enzyme activity value of 64.86 U/mL. Enzyme activity decreases when the enzyme is in a basic environment, with an enzyme activity value of 41.46 U/mL. The lowest enzyme activity is shown when the enzyme is in an acidic environment with an enzyme activity value of 34.86 U/mL as in Figure 2b. The enzyme activity value decreases at acidic pH due to potential structural changes in proteins in acidic conditions that interfere with enzyme activity due to disruption of the interaction between the enzyme and the substrate (Gu, 2024). The results of determining the optimum pH show that the enzyme tends to have stable activity when it is at pH 7, so the process of making and recommending that testing with PEBSBT be carried out by maintaining pH conditions within the range of 7.

Enzyme activity can be affected by temperature and enzymes can function optimally at certain temperatures. Enzymes are sensitive and at certain temperatures enzyme activity can decrease drastically. Enzyme activity at temperatures of 25, 45, and 65°C was respectively obtained at 53.00; 54.13; and 54.06 U/mL and experienced a decrease in activity at a temperature of 80°C with enzyme activity of 30.80 U/mL as in Figure 2c. This can be caused by enzyme denaturation (Kabir and Ju, 2023). Various research has noted the significant impact of temperature change on enzyme activity. Research by Mcleod et al. (2024) showed that increasing temperature can cause a shift in the active site configuration of the enzyme to be more active within a specific temperature range. At the highest temperature, the shift in the active site configuration of binding site becomes inactive and causes changes in the structural ensemble of the enzyme before denaturation occurs. This process caused significant effect towards enzymatic activity; thus, enzymes processing is recommended at temperatures below 60°C to prevent protein denaturation.

The crude extract activity of black soybean tempeh enzyme (CEBSBT) obtained was 36.27 U/mL. The purified enzyme activity of black soybean tempeh (PEBSBT) obtained was d48.27 U/mL (Figure 2d). The results of the enzyme activity obtained were compared with other types of crude extract samples of tempeh enzymes because there has been no research on the enzyme activity of crude extracts of BSBT enzymes.

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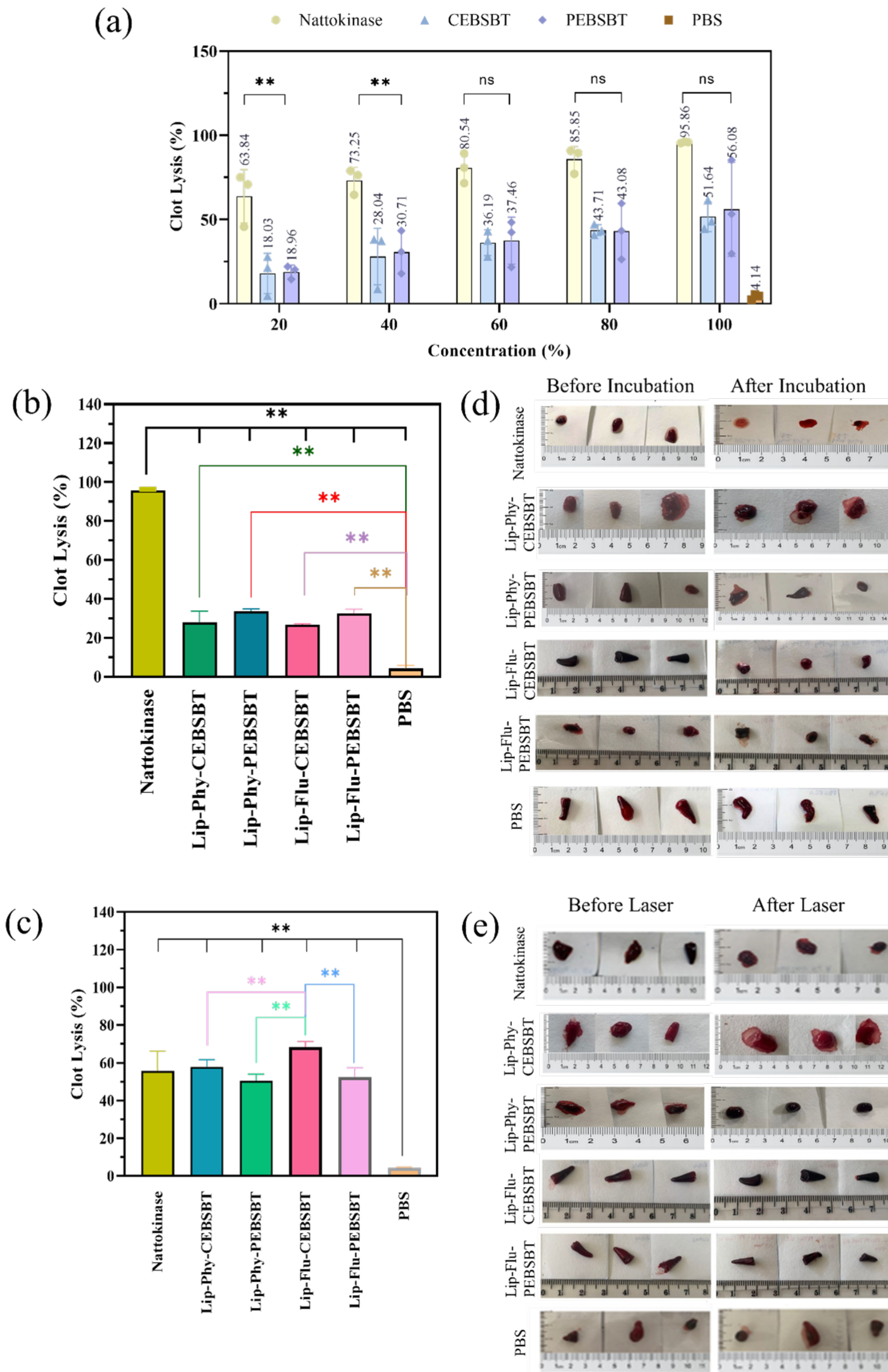

Figure 4. Thrombolytic Test Results Comparing Crude and Purified Extract with Positive Control Nattokinase, (b) Graph of Thrombolytic Activity Test of Liposomal Formulation Without Laser and (c) Thrombolytic Activity Test with Laser. (d) Photographs of Blood Clots Before and After Immersing the Clots in the Indicated Solutions, While (e) Displayed the Effect of Synergism Between Laser Exposure and Liposomal Formulation, Enhancing their Thrombolytic Activity

The results of the CEBSBT obtained had a smaller value when compared to the study by Sasmita et al. (2018) with crude extract samples of bongkrek tempeh enzymes. The enzyme activity obtained with the crude extract sample of bongkrek tempeh enzymes was greater, which can be suspected to be caused by differences in the types of soybeans and the addition

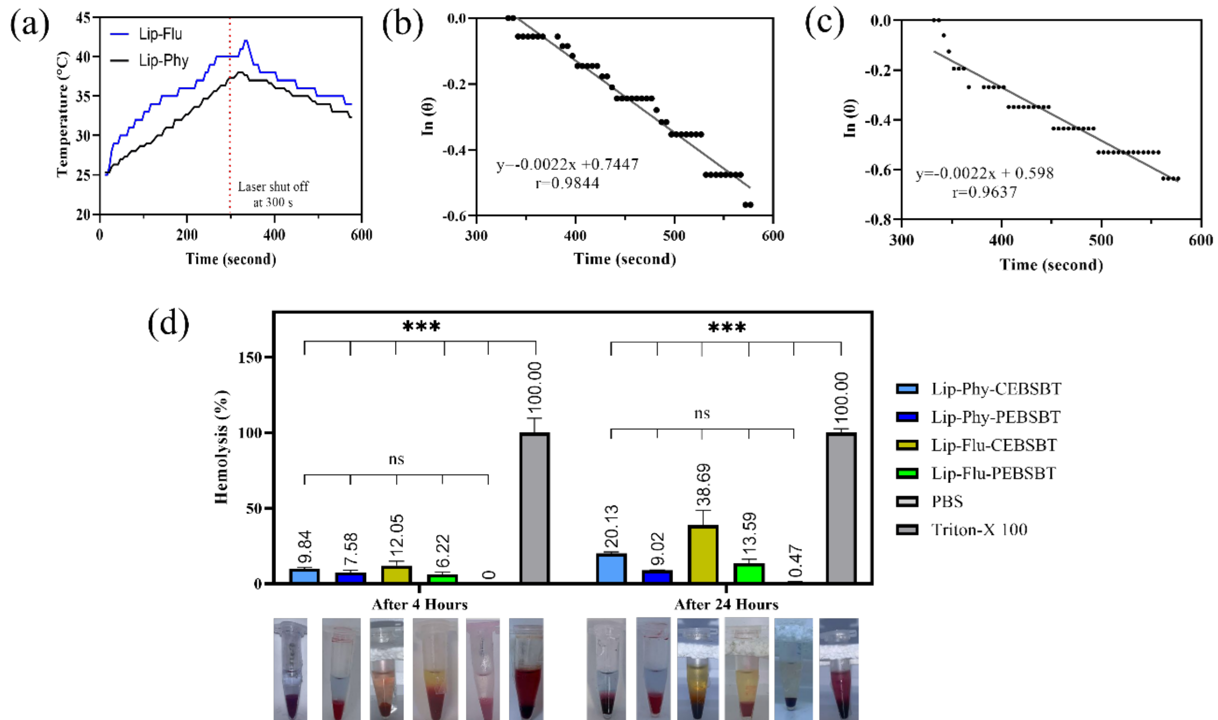

Figure 5. (a) Temperature Increase Observed Using a Thermocouple Probe with Laser Exposure with Subsequent ln θ Results for (b) Lip-Phy and (c) Lip-Flu Calculated Based on Temperature Decrease After Laser was Turned Off (d) Hemolysis Results Indicating Flu Containing Liposomes had the Tendency to Cause Lysis Compared to Phy

of grated coconut dregs during the fermentation process.

Protein size was characterized using SDS-PAGE with the aim of separating proteins in samples according to molecular weight. The SDS-PAGE result displayed that there were several bands visible after the Coomasie blue staining process on the gel. The CEBSBT sample was found to have 4 visibly thick bands (Figure 2e). The thick bands visible in the sample were compared with the marker protein, indicating CEBSBT sample had proteins with molecular weight size within the range of 14-35 kDa as seen in Figure 2d. Compared to results from black bean tempeh obtained by Wang et al. the profile obtained was rather similar (Wang et al., 2022). Based on the research conducted, black bean tempeh proteins were mostly around 14 kDa when exposed to intestinal buffer condition (Wang et al., 2022). As the treatment for crude and purified enzymes during this study was maintained with PBS, this showed similar results with previous research. Additionally, this result was able to demonstrate that purification process conducted within this research was able to maintain similar proteins and did not cause significant dimerization or change to the proteins.

Liposomes were chosen as a form of carrier delivery because liposomes have a phospholipid layer that is effective in delivering vast variety of active substances that are hydrophilic and hydrophobic. Enzymes have sensitive properties that are expected to be protected by liposome vesicles when encapsulated. Liposomes were prepared using the thin layer film method, which was then hydrated with an aqueous phase, in the form of CEBSBT and PEBSBT combined with photosensitizers. To ensure the size and shape of the liposomes were more homogeneous, a sonication process with bath ultrasonicator was carried out to obtain the size of the liposomes on the LUV scale.

Liposomes were formulated by incorporating two different photosensitizers, phycocyanin (Phy) and fluorescein sodium (Flu), as well as different enzyme purity, one with crude extract (CEBSBT) and another with (PEBSBT). As observed (Table 3), the liposomal formulation incorporating Phy produced intense blue color, while Flu liposomes showed a yellowish – fluorescent yellowish color. This indicated the liposome formulation process was able to maintain the stability of the photosensitizers. Additionally, encapsulation efficiency (EE) and pH from all four formulations produced satisfactory results, by maintaining pH >6 and percent encapsulation >60%.

The results of particle size measurements with soya lecithin concentration factor components of 0.605% and 0.01% obtained a size of 607.8 nm (Figure 3a). Liposomes tend to have a larger size due to the presence of amphiphilic phospholipids which cause the formation of a lipid bilayer that encapsulated the active substance inserted with the aqueous phase (Eugster and Luciani, 2024). Tween-80 which acts as a surfactant can help the stability of liposomes, affect lecithin molecules and change the structure and density of lecithin which causes the

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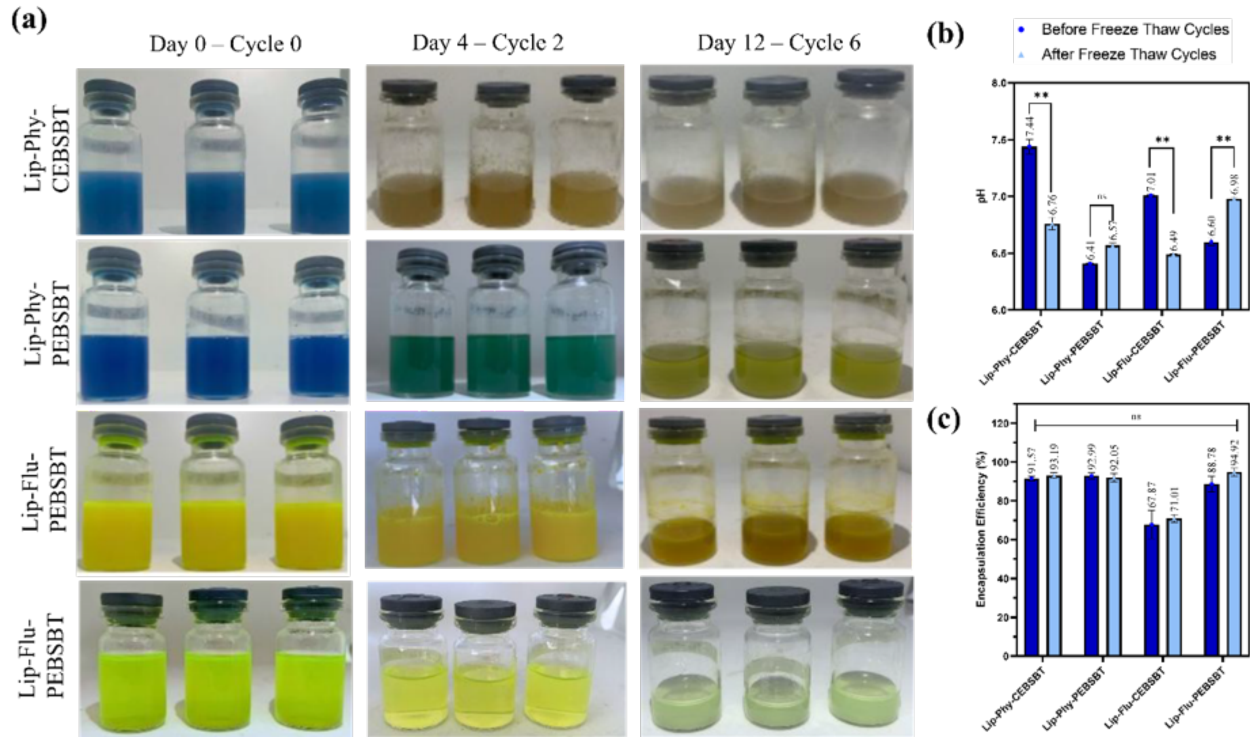

Figure 6. Stability Evaluation of Liposome Formulations of BSBT. (a) Photographs Indicated Freeze-Thawing Cycles Affected the Color Stability of Phycocyanin and Fluorescein As Shown by The Significant Color Intensity Changes. (b) The Freeze-Thawing Cycles Impacted The Ph Of Formulations, (c) However We Found Encapsulation Efficiency of The Enzymes Was Unaffected by the Applied Stress Towards The Liposomal Systems

size of liposomes tend to enlarge (Tsengam et al., 2022). For this study, the reduction of liposome size was only performed with ultrasonicator without heating, to maintain enzymatic and photosensitizers stability. However, as this process was far below the lipid transition temperature (*Tm* of soy lecithin 50-65°C) this caused the liposome size to be larger (Zhou et al., 2024). Further exploration of techniques that are feasible to maintain protein and photosensitizers stability should be considered.

However, the obtained size of the liposomes produced was still acceptable because these liposomes can inhibit platelet activation and aggregation. This aggregation process played a significant role during the thrombolytic process in thrombosis. The size of the liposome can increase the pressure in the area where the thrombus is located and can maintain its presence in the thrombus and increase the thrombolytic effect of liposomes on the target thrombus (Tran et al., 2022). Research by Ta et al. (2018) illustrated that particle size has an influence on its ability to adhere to the walls of blood vessels. Inflammation of the blood vessels that occur can be in the form of an atherosclerotic plaque. Larger particles could adhere more easily to the blood vessel wall than particles with smaller sizes. In laminar flow or disturbed flow, large particles are more efficient at attaching than particles with small sizes. Small particles tend to have difficulty reaching the vessel wall because they are trapped in the red blood cell nucleus (Ta et al., 2018). The obtained liposomes with a size of 607.8 nm are expected to be retained on the target thrombus in the blood vessels. This result is expected to increase the contact time between the liposome and the thrombus, thereby increasing the desired thrombolytic activity.

The results of the polydispersity index (PDI) measurement obtained a value of 0.339. The PDI value is in the range of 0-1, the PDI value that is getting closer to 0 indicates a homogeneous particle size, with a PDI value> 0.5 indicating a non-homogeneous particle size. The PDI value obtained in the optimum formula is <0.5 with a value of 0.339 indicating that the particles tend to be distributed homogeneously. The results of zeta potential measurements using PSA on the optimum formula obtained a value of -24.2 mV as in Figure 3b. Zeta potential is a measure of the repulsive and attractive forces between particles, which play a role in determining the stability of nanoparticles (Fithri et al., 2024). A good zeta potential has a value of +25 mV and -25 mV (Mardiyanto et al., 2024). This indicates that the particles do not collide with each other and can reduce the occurrence of aggregates. Aggregates can form due to the attraction of charges on the particles. The zeta potential value of the optimum formula is still relatively good and has moderate stability (Andra et al., 2022; Enciso et al., 2013). The components in liposomes are soya lecithin,

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tween 80, and PBS 7.4 which consist of NaCl, KCl, Na_2HPO_4, and KH_2PO_4. Some of these components have groups that have a negative charge, so that the zeta potential The resulting negatively charged structure can be seen in Figure 3c.

The morphological results of the optimum liposome formula showed that the liposomes produced are round and regular in shape and the particle sizes are within the range of 200-700 nm, which can be seen in Figure 3d. Based on the TEM results, there are smaller dark spots surrounding the liposome particles. This can be suspected to be an interaction between the photosensitizer compound and the black soybean tempeh enzyme that is not encapsulated by the liposome lipid phase, because the liposome sample that was measured has not undergone re-purification using a spin column. The range of liposome sizes in Figure 3d showed size of liposomes within the range of 200-700 nm. This nanoparticle characterization was produced based on the optimum results of thrombolytic activity performed within this study.

Thrombolytic activity evaluation of each sample group produced different potency (Figure 4a). Nattokinase (as a positive control) was compared with the crude extract of black soybean tempeh enzyme (CEBSBT) and black soybean tempeh purification enzyme (PEBSBT), with nattokinase showcasing superior effect ($p<0.05$). This is largely due to the nattokinase utilized in this study have been purified and quantified (1000 U). Despite that, the thrombolytic activity of CEBSBT and PEBSBT have reached 50% of the activity. The concentration of CEBSBT and PEBSBT used in the thrombolytic test encapsulated in liposomes is 100% due to the results obtained in Figure 4a.

The results of thrombolytic testing using the incubation method showed that there were significant differences in liposomes with positive nattokinase control and negative PBS control 7.4. However, there were no significant differences in Lip-Phy-CEBSBT, Lip-Phy-PEBSBT, Lip-Flu- CEBSBT, and Lip-Flu-PEBSBT. This observation indicated that each liposome has thrombolytic activity that did not differ significantly between photosensitizer content without laser exposure. This supported the theory that photosensitizers could only influence activity when it is external triggered (such as light exposure) (Bartusik-Aebisher et al., 2023; Polat and Kang, 2021). The percentage lysis values obtained in liposomes carrying CEBSBT, PEBSBT, and photosensitizer compounds were still relatively small, below 50% as in Figure 4b and observed with clots sizes (Figure 4d). This can be caused by the active substance still encapsulated in the liposome, causing retention of the proteolytic enzymes that are expected to cause clot lysis.

The same sample was used in the laser thrombolytic test. The clots and liposome samples were exposed to a 450 nm laser with an intensity of 0.3 W/cm^2. The time required for laser exposure was 2 minutes, this indicates a safe time for human body parts to be exposed to laser light without expected damage due too extreme high temperature increase (Fithri et al., 2023). The results of thrombolytic testing using the laser method showed significant differences in liposomes with negative control PBS 7.4. There were no significant differences between Lip-Phy-CEBSBT and Lip-Phy-PEBSBT, Lip-Phy-CEBSBT and Lip-Flu-PEBSBT, and Lip-Phy- PEBSBT and Lip-Flu-PEBSBT. This shows that the percentage lysis value of the three types of liposomes does not have a significantly different percentage lysis value, but the values shown by all three have reached the target 50% expected lysis as in Figures 4c and 4e. The percentage lysis value of Lip-Flu-CEBSBT has a significantly different value compared to the other 3 liposomes.

This value is thought to be caused by the presence of the photosensitizer compound sodium fluorescein, which is a synthetic photothermal sensitizer resulting in a higher increase in heat energy which significantly assisted the clot lysis process. The clot lysis value of liposomes containing CEBSBT tends to have a higher value when compared to liposomes containing PEBSBT. This is thought to be caused by the compounds contained in CEBSBT tending to be more complex due to their unpurified nature, indicating that other unquantified compounds such as flavonoids is suspected of having thrombolytic activity (Wang et al., 2022). This is different from incubated liposomes because the active substance tends to be retained in the liposome. Thrombolytic test using laser has a higher percentage lysis value due to laser light being absorbed by liposomes containing photosensitizer compounds. Light energy was converted into heat energy, the heat produced will rupture the liposome and cause the active substance entrapped to be released. The active substance that was released due to the rupture of the liposome will react with the clot and have a lysis effect on the clot. The increase heat produced assisted the lysis process by disrupting the fibrin network found in the thrombus and causing lysis of the blood clot (Fithri et al., 2025, 2023).

Photothermal efficiency test was conducted to determine how effective liposomes containing photosensitizer compounds were in converting absorbed light into heat energy. The calculation of photothermal efficiency was carried out by observing the maximum temperature obtained during the laser exposure process and the initial temperature of the liposomes before being exposed to the laser (Figure 5a). Photothermal efficiency was calculated following previous method adapted from Fithri et al (Figures 5b and 5c). The heat capacity value of 4.2 J/g.°C corresponds to the heat capacity of water, this value is used because liposomes consist of PBS 7.4 made with distilled water (Fithri et al., 2023). The photothermal efficiency value obtained by liposomes containing sodium fluorescein is 57.56%. This value shows the total light energy absorbed by liposomes when irradiated with a 450 nm laser with a power of 0.3 W/cm^2 is 57.56% and the remaining 42.44% is lost which can be caused by imperfections in energy conversion. Photothermal efficiency obtained liposomes containing phycocyanin of 44.23%. This value indicates the total light energy absorbed by liposomes when irradiated with a 450 nm laser with a power of 0.3 W/cm^2 as much as 44.23%, and the remaining 55.70% was lost which can be caused by imperfections in energy conversion. The value obtained by phycocyanin liposomes was lower than sodium fluorescein liposomes which can

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be explained by knowing that phycocyanin, a natural coloring photosensitizer unlike synthetic sodium fluorescein which is less stable with pH change causing a reduction of absorption at desired wavelengths (Kubrak et al., 2022).

The hemolysis test was analyzed based on the increase of plasma hemoglobin indicating the release of red blood cells due to damage. Hemolysis can be caused by direct contact between liposome materials and blood. The hemolysis test was carried out in vitro by incubating red blood cells with Triton-x100 as positive control and PBS 7.4 as negative control. The assay was carried out for 4 hours and 24 hours to determine the toxicity effects of the sample in the blood during the liposome delivery process in the blood (Figure 5d). Research according to Psimadas et al. (2012) showed the hemolysis percentage value below 10% is considered nonhemolytic and tends to be safe in the blood, while the value above 25% is considered hemolytic and tends to be unsafe for the blood. Liposomes containing CEBSBT tend to have a higher hemolysis percentage compared to liposomes with PEBSBT as in Figure 4(d). This can be caused by other compounds contained in CEBSBT that have not undergone the purification process. Liposomes with photosensitizer compounds in the form of sodium fluorescein after 24 hours of testing gave a % hemolysis of 38.69%. This value is considered hemolytic and tends to be unsafe for blood. Sodium fluorescein is generally considered safe in blood in the process of visualizing blood flow during surgery (Cardali et al., 2022). However, high concentration of fluorescein can ionic imbalance, due to the anionic nature of sodium fluorescein which can affect the osmotic balance of blood. Herein, it is suspected that the amount of fluorescein utilized (500 ppm) was higher than the intended use for fluorescein which was 3-30 mg/kg body weight.

Stability test on optimum formula liposome was conducted by cycling test, heating cooling the liposomes for 6 cycles. One cycle consists of 1 × 24 hours at 4°C and 1 × 24 hours at 40°C for 12 days. Parameters observed during testing stability, namely organoleptic (color, aroma, sediment), pH, and %EE on days 0, 4, 8, and 12. During the testing period, liposomes tend to experience changes in color, aroma, and sediment (Figure 6a) due to extreme temperature fluctuations. This is due to the use of photosensitizer compounds, which could be due to its sensitivity to light and temperature during the stability test process (Zhao et al., 2021).

Cycling test results for Lip-Phy-CEBSBT and Lip-Phy-PEBSBT showed a pH decrease (Figure 6b) in Lip-Phy-CEBSBT, likely due to protein denaturation at high temperatures, exposing acidic groups (Liu et al., 2015; Yu et al., 2021). Meanwhile, Lip-Phy-PEBSBT exhibited a pH increase, attributed to ammonium sulfate degradation into ammonia ions with basic properties (Arvand et al., 2020). However, both formulations remained within the safe blood pH range (6.5–7.5). Statistical analysis using GraphPad® One-Way ANOVA confirmed a significant difference ($p < 0.05$) in pH values across days 0, 4, 8, and 12, while %EE remained stable ($p > 0.05$) despite a slight increase, possibly due to lipid aggregation at low temperatures (Howard and Levin, 2010) (Figure 6c). Sediment formation was observed, likely due to lipid aggregation, and liposome color fading occurred, influenced by temperature instability. A significant color change in Lip-Phy-PEBSBT was noted on days 0, 4, and 8, likely due to ammonium ion degradation, shifting pH towards alkalinity and altering phycocyanin's photon energy absorption (Yamada et al., 2012). Organoleptic observations showed sediment formation due to lipid aggregation, and liposome color fading, likely caused by fluorescein isomerization due to temperature instability.

Compared to the Lip-Phy results, Lip-Flu-CEBSBT and Lip-Flu-PEBSBT showed a pH decrease in Lip-Flu-CEBSBT due to CEBSBT protein denaturation at high temperatures, exposing acidic groups (Liu et al., 2015; Tortajada et al., 2024). In contrast, Lip-Flu-PEBSBT experienced a pH increase, likely due to ammonium sulfate degradation into basic ammonia ions (Arvand et al., 2020). Statistical analysis confirmed a significant difference ($p < 0.05$) in pH values but no significant change was observed in %EE ($p > 0.05$) across days 0, 4, 8, and 12. The slight %EE increase occurred may be due to lipid aggregation at low temperatures (Howard and Levin, 2010). Based on these findings, BSBT and photosensitizers containing liposomes were able to enhance thrombolytic effect compared to crude or purified extracts.

4. CONCLUSIONS

Herein, we have confirmed the proteolytic activity of enzymes produced from black soybean tempeh. The enzyme produced from BSBT both in crude and purified form produced satisfactory enzyme activity and thrombolytic activity, with an optimum pH of 7.0 and a temperature range of 25–65°C. Characterization of the liposomal formulation produced a particle size within sub-micron size, which is acceptable for use within the blood vessel. Liposomes produced showed decent stability with relatively negligible hemolysis for phycocyanin, but fluorescein concentration needs to be reduced due to a higher percentage of hemolysis. The nanoparticle was able to facilitate photothermal increase with a photothermal efficiency of ~40-50%. In vitro thrombolytic activity evaluations demonstrated good thrombolytic activity from liposomal formulations, with lysis of ~55%. Based on these results, we were able to convert enzymes obtained from tempeh into a potential alternative source for thrombolytic. Further evaluations including remaining enzymatic activity after laser exposure and determination of BSBT activity aside from proteolytic could be explored. Furthermore, to complete and enhance the characterization that we have conducted, it is advisable to follow further testing including the determination of other bioactive compounds such as flavonoids.

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