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Production of Hydrolysate with Antioxidative Activity and Functional Properties by Enzymatic Hydrolysis of Defatted Sesame (*Sesamum indicum* L.)

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Abstract: Sesame protein isolate is produced from dehulled, defatted sesame seed and used as a starting material to produce protein hydrolysate by trypsin and bromelain. The degree of hydrolysis (DH), molecular weight distribution, DPPH radical-scavenging activity, and fibrinolytic activity of the hydrolysates were investigated. Within 120 and 240 min of hydrolysis, the maximum cleavage of peptide bonds occurred was found for trypsin and bromelain, respectively, as observed from the DH (~ 20% DH). The antioxidant of enzymatically hydrolyzed sesame protein was also studied. For trypsin treatment, the hydroxyl radical-scavenging activity of hydrolysates appeared to reach a maximum level for 120 min of hydrolysis. The molecular weight of the hydrolysates was also reduced significantly during hydrolysis. The hydrolysates treated by trypsin for 30 min (hydrolysis time) showed that more than 95% of < 50 kDa fraction was degraded. The molecular weight of the major band of the hydrolysates by trypsin was centred at 28 kDa. No significant changed were observed when treating with bromelain. The results suggested that the antioxidant activity of defatted sesame protein hydrolysates were related to its DH, hydrolysis time and molecular weight. Furthermore, fibrinolytic activity test demonstrated that the trypsin hydrolysate produced a lysed zone on the thrombin-clotted enzyme-induced fibrin plates. This indicates that trypsin hydrolysate function as a plasmin-like protease which can directly degrade the fibrin, thereby dissolving the thrombi rapidly and completely.

Keywords: Sesame; hydrolysate; degree of hydrolysis; DPPH radical-scavenging activity; Fibrinolytic activity

1. Introduction

Enzymatic hydrolysis has been used for centuries for modification of the functional and nutritional properties of food proteins in the production of traditional foods such as cheeses and fermented plant foods [1]. In recent years, a significant growth of interest in functional protein hydrolysates can be observed. Particular attention has been paid to enzymatic hydrolysis of proteins. Protein hydrolysates are widely used as nutritional supplements, functional ingredients, and flavor enhancers in foods, coffee whiteners, cosmetics, personal care products, and confectionery, and in the fortification of soft drinks and juices. Functional protein hydrolysates can be used in food systems as additives, as food texture enhancers, or as pharmaceutical ingredients [2]. Protein hydrolysates are also

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used in soups, sauces, gravies, snacks, meat products, and other savory applications. Extensive protein hydrolysates can be used in special medical diets such as in the production of hypoallergenic foods for patients with reduced absorption surface or reduced digestive capacity [3]. Sunflower protein hydrolysates are utilized for dietary treatment of patients with liver failure [4].

Protein hydrolysates from different sources, such as milk protein [5], maize zein [6], egg-yolk [7], porcine proteins [8], yellow stripe trevally [9], yellowfin sole frame [10], herring [11], mackerel [12], have been found to possess antioxidant activity. The operational conditions employed in the processing of protein isolates, the type of protease and the degree of hydrolysis affect the antioxidant activity. Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates [12].

The utilization of proteins or their hydrolysates for food and/or cosmetic applications not only presents additional advantages over other antioxidants, but also they confer nutritional and functional properties. However, there is little information regarding the antioxidant effect of protein hydrolysates from the sesame by enzymatic treatment. Moreover, lipid peroxidation leads to the development of undesirable off-favours and potentially toxic reaction products [13]. Many synthetic antioxidants may be used to retard lipid peroxidation in a number of fields. However, the safety and negative consumer perception of synthetic antioxidants restricts their applications in food products. Moreover, reactive radicals are implicated in the ethiology of age-associated chronic diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes, and certain types of cancer [14]. Therefore, there is a growing interest to identify antioxidative properties in many natural sources including some dietary protein compounds.

Sesame is widely used in food, nutraceutical, pharmaceutical and industry in many countries because of its high oil, protein and antioxidant contents. The sesame seed (*Sesamum indicum* L.) contains high level of oil (ca. 50-60%), which has excellent stability due to the presence of natural antioxidants such as sesamolin, sesamin, and sesamol and is widely used as a dietary supplement. Sesame seed contains nearly 25% protein, and after oil extraction, the remaining meal contains 35-50% protein, which is rich in tryptophan and methionine, and it may be an excellent protein source for supplementing soybean, peanut, and other vegetable proteins. Dehulling of sesame seed is necessary as the hull contains a very high amount of oxalic acid (2-3%), which could complex with calcium and reduced its bioavailability [15]. The hull also contains indigestible fiber, which reduces the digestibility of the protein and imparts a dark color to the meal. The undesirable components such as fiber, soluble sugar, phytates, and oxalates can be eliminated to a large extent by producing protein isolates or concentrates from dehulled and defatted seed. Proteolytic enzyme modification of protein is an effective way to improve the various functional properties and to increase the field of application of the protein [16]. The peptides that are produced by partial hydrolysis of proteins have smaller molecular size and less secondary structure than the original proteins. The protein solubility, emulsifying properties, and foaming capacities can be improved with a limited degree of hydrolysis, whereas excessive hydrolysis often causes loss of some of these functionalities [17]. There is controversy in the literature as to whether it is necessary to use native protein to obtain bioactive peptides. Accordingly, the biological activity of a whey protein depends on the preservation of their native structure. On the other hand, other authors, report obtaining bioactive peptides from denaturated protein, albeit without specifying its degree of denaturation [18].

In the present study, protein isolate was produced from dehulled and defatted sesame seed by alkaline extraction followed by acidic

precipitation. Protein hydrolysates, with variable degrees of hydrolysis, were prepared from protein isolate by trypsin or bromelain and characterized by their functional properties to determine the potential application of these hydrolysates in various food products. Additionally, this study was determining the antioxidant activity of the protein hydrolysate by trypsin and bromelain from the defatted sesame seed as well. The understanding of antioxidative properties of sesame seed protein hydrolysate may lead to utilize it as a potent natural antioxidant. Meanwhile, the fibrinolytic activity of hydrolyastes derived from sesame seed was also evaluated.

2. Materials and methods

2.1. Raw materials

Sesame seeds were purchased from a local supermarket. Crude sesame seed was mixed with isopropanol in a ratio of 1:2 (w/v) and heated at 70°C for 30 min. The solvent was drained off and the defatted seed was rinsed twice with five volumes of distilled water and then centrifuged at 8,000×*g* at 4°C for 15 min. The defatted seed was mixed with distilled water in a ratio of 1:2 (w/v) and homogenized at a speed of 8,000×*g* for 3 min. The homogenate seed was immersed in water for 2 h follow by dried and milled. The sesame sample (40 g) was suspended in 600 mL of distilled water and then adjusts the pH to 9.5 with 1 N NaOH solution, and extracted by stirring for 4 h at 50-55°C. After centrifugation at 8,000×*g* for 60 min, the pellet was discarded. The pH of the supernatant was adjusted to the isoelectric point (pH 4.5) and the precipitate formed was recovered by centrifugation as described above. The precipitate was resuspened in 50 mM PBS buffer (pH 7.6) until further use. The pretreatment process (defatted and preliminary isolation) for protein hydrolysate from sesame seed was shown in Figure 1.

2.2. Preparation of protein hydrolysate

The enzymatic hydrolysis was carried out at 25°C, constant pH 6.5 with trypsin (purchased from Sigma, St. Louis, MA) and bromelain (kindly gift from Prof. C. S. Chen of Department of Applied Chemistry, Chaoyang University of Technology, Taichung, Taiwan) to substrate ratio 1:200 (v/v) for 0, 10, 30, 90, 120, 180, and 240 min. The enzyme was inactivated by heating at 100°C for 5 min. The resulting hydrolysate was then rapidly cooled to ambient temperature in the ice bath. Subsequently, the mixture was adjusted the pH to 7.0 with 1 N NaOH. Then the mixture was centrifuged at 8,000×*g* for 20 min at 4°C. The supernatant was stored at -20°C until used.

2.3. Degree of Hydrolysis

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was calculated by the determination of free amino group reaction with 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) as described previously [19]. The TNBS reaction was carried out as follows: 0.25 mL of a sample was mixed in a test tube with 2 mL of phosphate buffer and 2 mL TNBS solution. The mixture is shaken and placed in a water bath at 50±1°C for 60 min. During incubation the test tubes and water bath must be covered with aluminum foil because the blank reaction is accelerated by exposure to light. The reaction was terminated by addition of 4 mL of 0.1 N HCl, and the allowed to stand at room temperature for 30 min before the absorbance is read against water at 340 nm. The amount of free amino group liberated was expressed as a L-leucine equivalent. DH was calculated as follows:

$$\mathrm{DH}\ (\%) = \frac{C_0 - C_t}{C_0} \times 100\%$$

where C_0 is L-leucine equivalent of sample

at time = 0, C_t is L-leucine equivalent of sam- ple at time t.

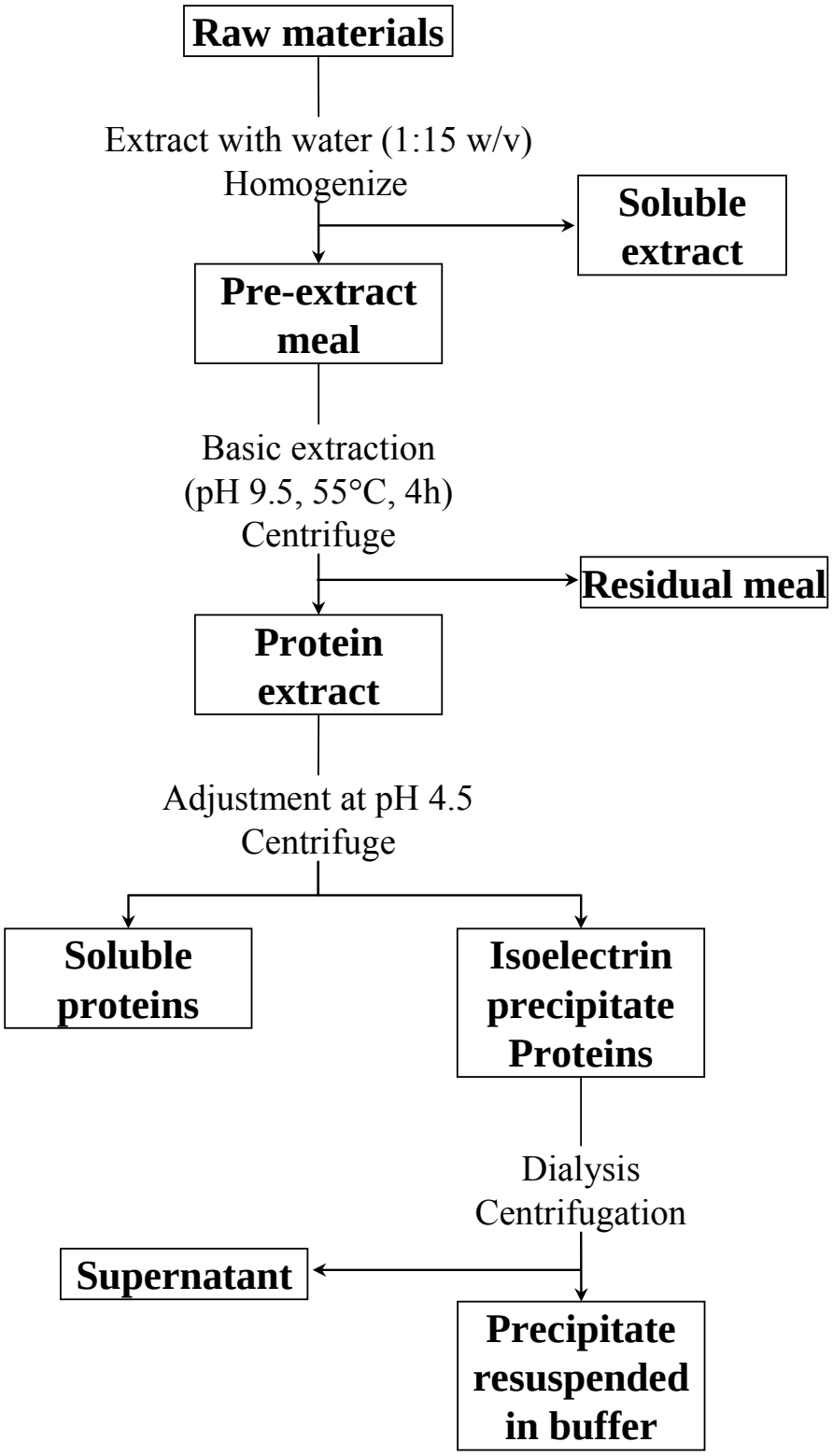

Figure 1. Flow diagram for the preparation of defatted sesame hydrolysates.

2.4. DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined as described by Thiansilakul *et al.* [20] with a slight modification. To diluted sample (0.6 mL), 1.2 mL of 0.6 mM DPPH in 95% ethanol were added. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resultant solution was read at 517 nm using a Cary 50 UV-Vis spectrophotometer (Varian, Victoria, Australia). The blank was prepared in the same manner, except that distilled water was used instead of sample. The scavenging effect was calculated as follows:

Radical-scavenging activity (%) =
$[(B-A)/B]\times 100$

where A is A_{517} of sample and B is A_{517} of the blank.

2.5. SDS-PAGE analysis

Reducing SDS-PAGE (12.5%) analysis was carried out on a Miniprotean II system from Bio-Rad (Hercules, CA) according to the method previously described [21]. For gel electrophoresis 10 μL samples were mixed with 10 μL sample buffer (2% SDS, 350 mM DTT, 25% (v/v) glycerol, 0.01% Bromophenol Blue in 62.4 mM Tris-HCl, pH 6.8) and incubated at 95°C for 5 min before loading. Proteins were separated on a 12.5% SDS-polyacrylamide gel. After the run, the gels were stained with 0.2% Coomassie R-250 (w/v) for 30 min, and destained in a 30% methanol and 10% acetic acid solution (v/v). Protein concentration was determined according to the Bradford method, by measuring absorbance at 595 nm, with bovine serum albumin as a standard.

2.6. Fibrinolytic activity

Fibrinolytic activity was assayed using the fibrin plate methods as previously described with slightly modifications [22]. In a Petri dish, 2.5 mL of 1.2% (w/v) fibrinogen solution in 50 mM sodium phosphate buffer (pH 7.4) was mixed with the 7.4 mL of 1% (w/v) agarose solution along with 0.1 mL of a thrombin solution (10 NIH units/mL). The solution in the Petri dish was left for 1 h at room temperature to form a fibrin clot layer. Ten microliters of the sample solution was then dropped onto filter paper (6-mm diameter) flatted in the fibrin plate. The plate was then incubated for 12 h at 37°C. The enzyme activity was estimated by measuring the dimension of the clear zone.

2.7. Statistical analysis

All the tests were done in triplicate and data were averaged. Standard deviation was also calculated. Duncan's multiple-range test [23] was used to evaluate significant differences ($p < 0.05$) between the means for each sample.

3. Results and discussions

3.1. Hydrolysis of extruded corn gluten

The optimal pH for the protease trypsin and bromelain activity on defatted sesame seed was 7.6 and 7.0, respectively, which fell in the range given by the producer. It was found that the degree of hydrolysis (DH) at pH 7.6 reached maximum at 25°C (180 min) for the trypsin; pH 7.0 at 60°C (240 min) for bromelain. More intensive proteolysis was observed in more diluted suspensions in the present studies. The optimal ratio of E:S was 1:200 (v/v) for both enzymes (data not show). The effect of proteases on protein breakdown in defatted sesame seed at the different hydrolysis time was investigated by DH (Figure 2). It can be noticed in Figure 2 that the protein hydrolysis profile were substantially changed in the both cases of defatted sesame seed. The proteolysis of defatted sesame seed with bromelain was less intensive. Figure 2 also

shows that, under optimal condition, the DH of defatted sesame seed with trypsin was faster than the bromelain perceptibly. After hydrolysis of 180 min, the DH of defatted sesame seed was 20.49% and 16.78% for trypsin and bromelain, respectively.

Analysis of DH of the hydrolysates of defatted sesame seed indicated that the hydrolysate could be prepared more effectively by previous pre-treatments. During pre-treatment, structural changes of protein are caused by drying, and shear forces in homogenizer. Because defatted sesame seed for hydrolysis was previously subjected to pre-treatments, it may be assumed that its structure, consisting of partially denatured protein, is stabilized by weakened hydrogen bonds, electrostatic and hydrophobic interaction, disulfide bonds, and other covalent bonds [24]. Such defatted sesame protein undergoes an enzymatic hydrolysis easily compared to raw materials. The DH was the most intensive at pH 7.6 at ambient temperature for trypsin after 120 min.

Accordingly, protein hydrolysate with a higher functionality and higher nutritional value in a more purified form is favorable. The protein-extraction process to prepare sesame seed hydrolysate is very expensive. The relative high cost can be partly explained by the large amounts of organic solvents used for the extraction of oil and for the removal of the impurities including starch, and for the removal of solvents from the sesame seed in the traditional process. One possible alternative is to apply the process used in this study to remove the oil before hydrolysis. After the separation of oil with NaOH treatment and washing, most of the oil was removed in the present work. Subsequently, the hydrolysis was more efficiently.

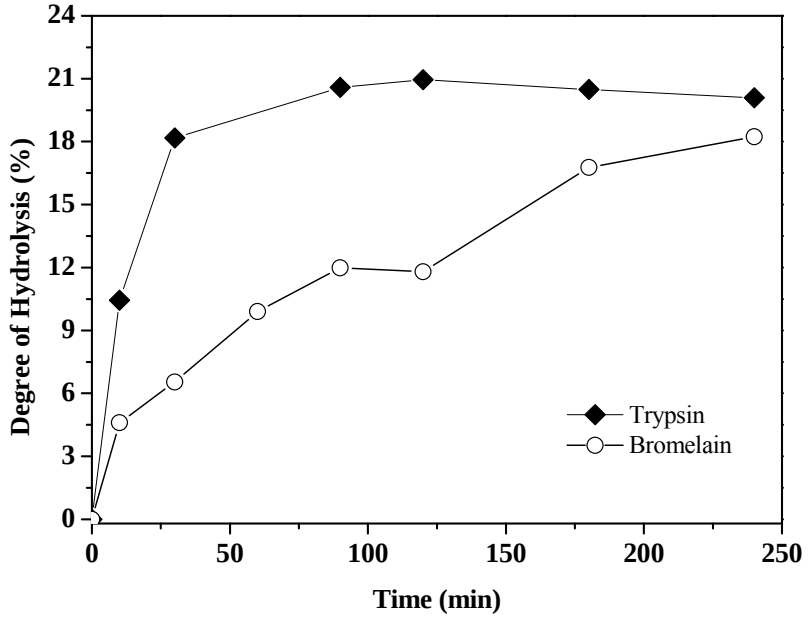

Figure 2. The effects of hydrolysation time on degree of hydrolysis (DH) of defatted sesame hydrolysates.

3.2. Antioxidative activity of the hydrolysate

In order to determine the influence of different treatments on free radical scavenging

activity, a DPPH free radical scavenging assay was carried out. The disappearance rate of DPPH free radicals was used to express the free radical scavenging activity of the defatted sesame seed hydrolysated with trypsin and bromelain during hydrolysis process under optimized condition were investigated. Figure 3 shows the antioxidant activity of sesame seed hydrolysate examined as a function of their hydrolysis time. The radical-scavenging activity (RSA) values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH$^{\bullet}$ solution in the absence of hydrolysate at 517 nm. From the analysis of Figure 3, it could be conclude that the scavenging activity of trypsin treated hydrolysate on DPPH radicals increased with the hydrolysis time. The RSA value of 75.25% was found after 180 min hydrolysis. In contrast, a relatively low RSA value (1.78%) was obtained in the bromelain treated hydrolysate. Yet, the the bromelain treated samples possessed significant free radical scavenging activity in a time-independent manner. Although slightly increasing was found with bromelain treatment by comparison with trypsin treatment samples, implying that the slight changed in free radical scavenging activity may have resulted from lack of the antioxidative fragments in bromelain hydrolysate.

Sesame oil contains sesamin and sesaminol lignans in its nonglycerol fraction, which are known to play an important role in the oxidative stability and antioxidative activity. Different studies have shown that dietary sesame lignans have effects like reducing liver damage and serum cholesterol level, increasing vitamin E activities and α-tocopherol availability and decreasing thiobarbituric acid reactive substance (TBARS) which are important in lipid peroxidation of membranes leading to aging process [22].

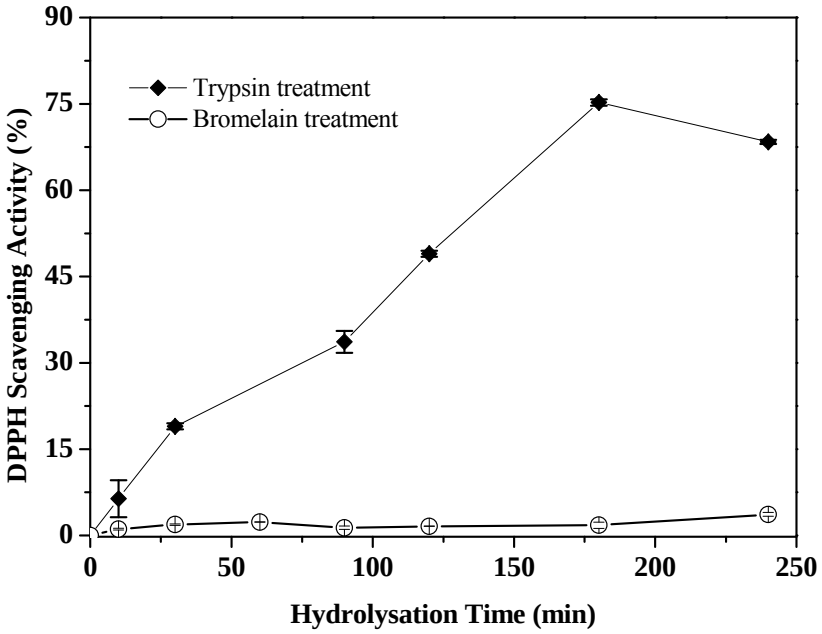

Figure 3. The effects of hydrolysation time on DPPH scavenging activities of defatted sesame hydrolysates.

3.3. Molecular weight distribution of the hydrolysate

Molecular weight distribution of hydrolyzed protein is one of the most important properties in producing protein hydrolysates to be used as functional materials [24], which has a direct impact on their functional properties. Both trypsin and bromelain catalyzes hydrolysis of protein and peptide amides with broad specificity for peptide bonds and is a preference for large uncharged residue. The effect of trypsin and bromelain on protein breakdown in defatted sesame at the different hydrolysis time was analyzed by SDS-PAGE (Figure 4). The results showed that the molecular weight distribution of the peptides of hydrolysates from defatted sesame seed by trypsin were similar when hydrolyzed for 30, 60, and 120 min. High molecular weight fractions were almost disappeared after hydrolysis and the peptides formed were visible in the lower part of gel (Figure 4, top panel). The decrease in molecular weight with time of hydrolysis was very much prominent at the first 30 min and static thereafter. It was found that the sample contains over 95% of 20 and 50 kDa protein at beginning. After 30 min of hydrolysis, about 20% of 28 kDa was observed. A tiny amount of small peptides of 4-6 kDa were also visualized. No significantly difference was found in the case of bromelain (Figure 4, bottom panel).

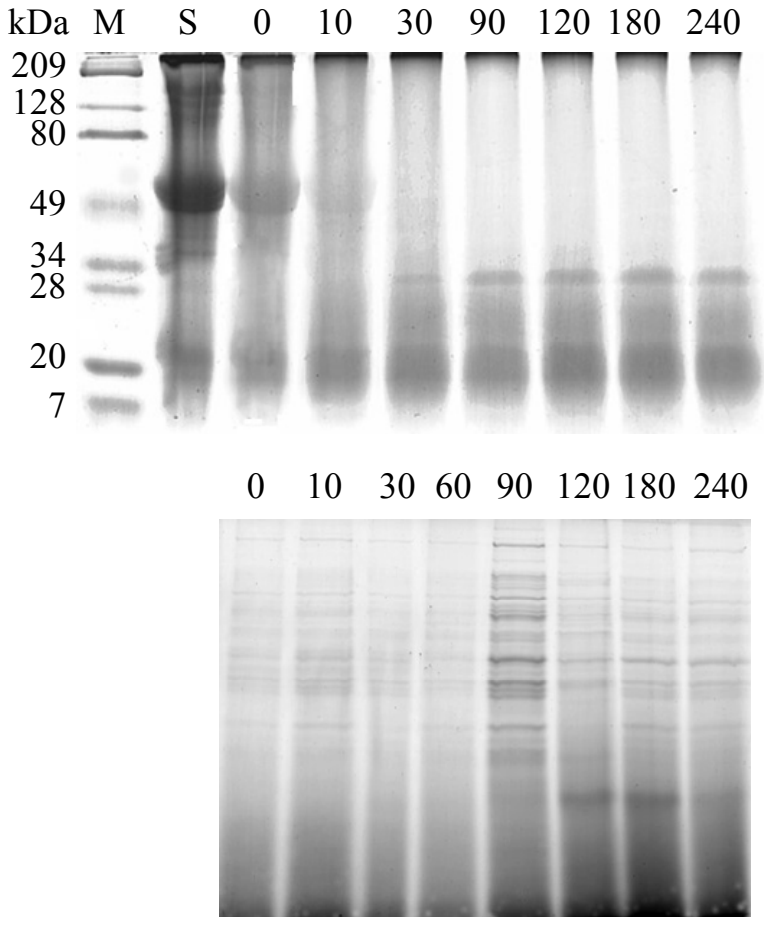

Figure 4. The SDS-PAGE electrophoregrams of trypsin (top) and bromelain (bottom) hydrolysates of defatted sesame. Lane M: Molecular weight standards; Lane S: crude extract; the following lanes shows the samples from different hydrolysis time.

3.4. Fibrinolytic activity

The trypsin hydrolysate sample was shown to have 675 NIH units/mL of clotting activity equivalent as determined by the fibrinogen plate assay. The trypsin hydrolysate produced a lysed zone on the thrombin-clotted enzyme-induced fibrin plates. Basically, the lysed clear zone was found to be increased with the hydrolysis time and reaching the maximum (1.2 cm) at 180 min (Figure 5, dot 3). This value is similar to the positive control (0.5 U/mL plasmin) or trypsin (675 NIH units/mL) on the thrombin-induced clot (Figure 5, dot T). Therefore, it is possible to assume that the defatted sesame seed hydrolysate is able to effectively remove blood fibrinogen by degrading the thrombin-clotted enzyme-induced fibrin clot and consequently reduced the blood viscosity, *in vivo*. Bromelain hydrolysates, however, did not show any fibrinolytic activity against thrombin-induced clot (data not show). This may reflect on its hydrolyzed profiles as found in the SDS-PAGE analysis (Figure 4).

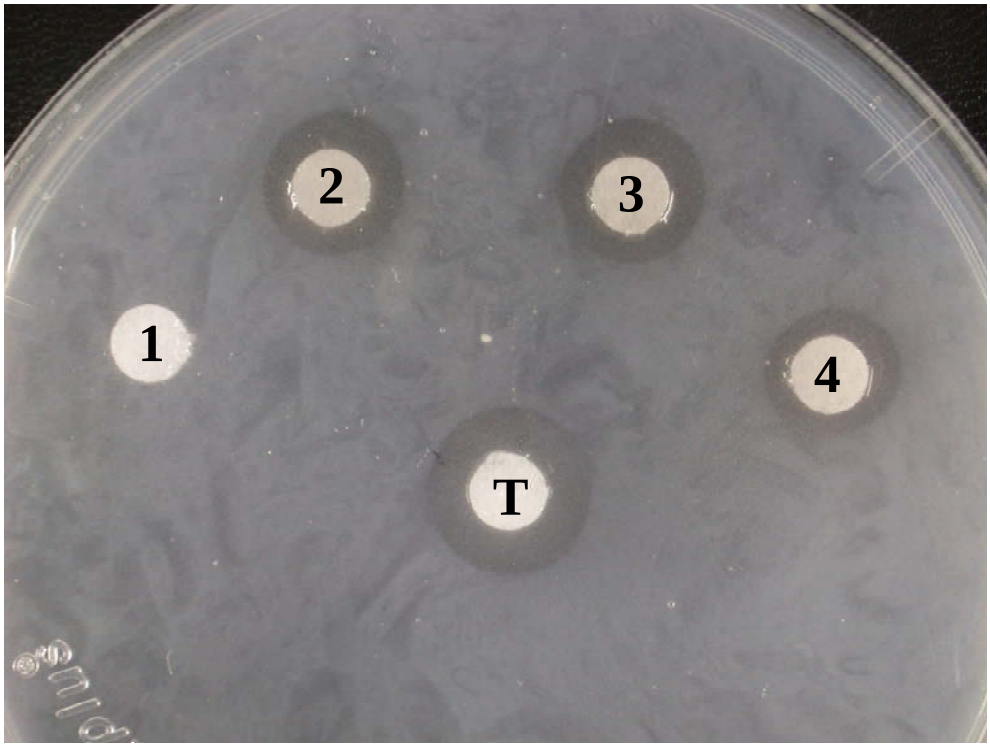

Figure 5. Fibrinolytic activity test of trypsine-treated sesame hydrolysates. Spot 1, 2, 3, and 4 represents the sample after 0, 120, 180, and 240 min hydrolysis, respectively. Trypsin (675 U/mL; spot T) was used as a positive control.

4. Conclusion

With pretreatment of sesame seeds (Figure 1), the crude hydrolysate can be removed from the mass by centrifugation and has a lower amount of fat, as compared to the original material. Hydrolysis of defatted sesame seed using trypsin/bromelain resulted in a DH ~20% under optimized conditions. The DH is significantly influenced by the hydrolysis conditions that included time, temperature, pH of the substrate and the enzyme concentration. The optimumal hydrolysis conditions for trypsin were: temperature of 25°C, time of 180 min, pH of substrate at 7.5 and an optimal ratio of E:S was 1:200 (v/v).

For the bromelain hydrolysation, the optimal conditions were: 60°C, time of 240 min at pH 7.0 under the same E:S ratio.

The profiles of the molecular weight distribution on the SDS-PAGE mainly were 20, 28 kDa, and less, which was similar to that determined for the metalloendopeptidases from other report [16]. Sesame protein peptides produced by trypsin treatment have high DPPH free radical scavenging activity. The high-value protein hydrolysates prepared by trypsin can be used as value-added ingredients in many food formulations for medicinal purpose due to the ability of degrading the fibrin clot. Experimental data obtained in this work will be useful not only for understanding the function of protease hydrolysate from defatted sesame seed, but also for developing an alternating therapeutic agents related to thrombotic disorders such as urokinase or nattokinase. These improved functional properties of different protein hydrolysates would make them useful products, especially in the food, pharmaceutical, and related industries.

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