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The effect of Processing Parameters on the Properties of Fish Gelatin Hydrolysate Nanoparticle

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Abstract: Fish gelatin hydrolysate is a well- known fish by-product that is high in protein content. It is produced from by-product waste from the fish processing industry, which includes fish skin, head and bones. Gelatin hydrolysates have recently received much attention due to its high protein content and bioactivity, which includes antioxidant, antimicrobial and antihypertensive activities. The transformation of gelatin hydrolysate into nanoparticles is believed to increase its economic value. Furthermore, reduction into nano-size increases the absorption characteristic of this material. Here, fish gelatin hydrolysate nanoparticles are prepared for the first time using desolvation method. The effects of concentration of gelatin hydrolysate, pH of solution, and acetone concentration on nanoparticle size are determined. The prepared gelatin hydrolysate nanoparticles were found to have spherical shape with sizes varying from 300-400 nm with a mean size of 408 ± 11.4 nm, zeta potential of -16.4 ± 1.2 mV and Polydispersity Index (PDI) 0.203 ± 0.07. This study showed that concentration of gelatin hydrolysate, pH and concentration of solvent have significant effects on nanoparticle size. The gelatin hydrolysate nanoparticles can be applied in the pharmaceutical industry for the encapsulation of drugs to facilitate delivery to target sites.

Keywords: *Fish Gelatin, Gelatin nanoparticles, Halal gelatin, Desolvation, Conceptual model*

Introduction / Pendahuluan

Sub-Heading

The rising concern on Halal foods by Islamic groups has increased interest in marine products as an alternative for future food source material demands. The rising interest in use of marine products is also due to increasing human population and changing food preferences towards protein consumption due to health reasons and nutritional value. In Malaysia alone in 2016, fish production was worth $3 million, with total fish volume of 2 million tonnes [1]. However, the fish industry uses only 40% of fish biomass and almost 60% is considered waste, which includes the skin, head and fins [2]. The volume of this waste is increasing every year and creates serious pollution problems on the environment.

In response to this problem, researchers have looked into processing fishery waste into high-value products such as fish collagen, gelatin and also fish gelatin hydrolysate [3]. These fish by-products are recognized as safe by Food and Drug Administration (FDA) [4]. The fish products also serve as alternatives to markets that are concerned with risk of disease from mammalian sources such as bovine spongiform encephalopathy (BSE) [5].

Following this opportunity, numerous gelatin hydrolysates have been produced from fish waste, such as skin and fin [6]–[8]. Gelatin hydrolysate is produced by the hydrolysis of gelatin. The hydrolysis process breaks the gelatin molecule into small pieces containing approximately 20 amino acids. Gelatin hydrolysate has low molecular weight compared to the precursor of gelatin [7]. Due to its small peptide size, gelatin hydrolysate is a bioavailable amino acid source for the human body. Several applications for gelatin hydrolysates have been reported in pharmaceutical and cosmetic products [8; 9]. However, to date, gelatin hydrolysates have not been used to produce nanoparticles. Converting protein molecules into nano-

sized particles could increase absorption and mobility through the cells [10].

A variety of methods for preparing nanoparticles from protein have been studied, such as coacervation [11], emulsion [12], and desolvation [13]. This study focuses on the desolvation method, since this method is well-described and reviewed. The principle of this method is dehydration of the protein molecule by adding co-solvents, and hardening the resulting nanoparticles using cross-linking agent. Furthermore, the effects of various factors on nanoparticle size have been documented, such as concentration of protein, concentration of co-solvent, and pH condition. Azarmi reported that different types of protein have different factor levels and result in different particle sizes [14]. Thus with this new type of protein, significant factors and levels for production of gelatin hydrolysate nanoparticle need to be looked into.

The aim of this study was to prepare gelatin hydrolysate nanoparticles using desolvation method. In particular, this experiment aimed to explore the effects of several factors such as concentration of gelatin hydrolysate, concentration of acetone as co-solvent, and pH on size of gelatin hydrolysate nanoparticles. One-factor-at-time (OFAT) design was used. This experimental design identified the effects of each factor independently. The nanoparticle size and shape was characterized using scanning electron microscopy (SEM). The results pave way for the design a new type of protein nanoparticle for encapsulation of drugs.

Materials and method

Materials

Tilapia fish gelatin hydrolysates were purchased from Halagel (Malaysia). The gelatin hydrolysate was used without any pretreatment. Glutaraldehyde grade I (25%, v/v aqueous solution), acetone, HCl, and NaOH were purchased from Sigma, Malaysia. All chemicals were of analytical grade and used as received. Double distilled water was used for all the experiments.

Production of Gelatin hydrolysate nanoparticles

Fish gelatin hydrolysate nanoparticle was produced using the desolvation method. About 5% of gelatin hydrolysate solution (10 ml) was prepared under constant stirring and heating (40 °C), until clear gelatin solutions were obtained. The gelatin hydrolysate solution was maintained at pH 3 by addition of 0.5 M HCl or NaOH solution. Acetone (30 ml) was added to the solution drop wise under constant stirring (600 rpm) to form nanoparticles. About 300 µl of glutaraldehyde (25%, grade I) as a crosslinking agent was added to the solution. Then the solutions were stirred for 6 hours at 600 rpm. The nanoparticles were collected using centrifugation at 12000 rpm, and excess glutaraldehyde and acetone was removed by washing the nanoparticles three times.

Experimental design

In order to study the effect of production factors on nanoparticle size, the concentration of gelatin hydrolysate, pH and concentration of acetone was varied. The values were chosen based on previous studies [4], [14]–[16].

The effect of gelatin hydrolysate concentration on nanoparticle size was studied by adding gelatin hydrolysate to distilled water in different amounts. The concentration of gelatin hydrolysate ranged between 1%, 2%, 5%, 10% and 20%. Others factors such as pH, acetone concentration, amount of glutraldehyde and stirring time were maintained at 3, 10, 300 µl and 6 hours of stirring, respectively.

To study of the effect of pH solution on particle size, zeta potential and size distribution, the pH of the solution was set at 2, 3, 4, and 5. Other factors were kept constant (2% of gelatin hydrolysate concentration, 20% of acetone concentration, 300 µl of glutaraldehyde and 6 hours of stirring).

The effect of acetone concentration on particle size and particle distribution was studied by increasing the concentration of acetone from 20% to 70%. Other factors remained unchanged as before. The nanoparticle size and size distribution was measured using zeta sizer (Malvern).

Characterization of fish gelatin hydrolysate nanoparticles

Particle size, particle size distributions and zetapotential

The effects of production factor on nanoparticle size and size distribution were determined and studied using Zeta sizer Malvern (NanoZS, Malvern Instrument Inc., UK). The samples were measured using light scattering method based on laser diffraction at an angle of 135 degrees. Values were measured in triplicates. The surface charge of the fish gelatin hydrolysate nanoparticles (FGHNPs) was measured using Zeta potential (Malvern system 4700, Malvern, UK). About 20

µl of samples were diluted in 2 ml of distilled water and placed in the folded capillary cell. The samples were run in triplicates with three readings recorded for each replicate to calculate the average.

Scanning electron microscopy

Surface morphology of the nanoparticles was determined using field emission scanning electron microscopy (JSM-6700F, JEOL Instrument, Tokyo, Japan). The nanoparticles in suspension were mounted on metal grid with carbon tape and were dried for one day at room temperature. The samples were then sputter-coated with platinum. The samples were observed at an accelerating voltage of 5 kV.

Data Analysis

The samples were analyzed in triplicate and average values were recorded. Data was presented as mean ± standard deviation. Significance analyses were calculated using Minitab. Differences with $P<0.05$ were considered statistically significant.

Results And Discussion

The aim of this experiment was to optimize process parameters for the production of gelatin hydrolysate nanoparticles based on one-factor at a time design of experiment. Desolvation method was used to produce nanoparticles. Concentration of gelatin hydrolysate, pH and solvent concentration were set as independent factors and particle size, dispersity index and zeta potential as response.

The effect of gelatin hydrolysate

Fig. 1a shows the particle size and distribution of nanoparticles for different gelatin hydrolysate concentrations. The result shows that increasing gelatin hydrolysate concentration from 1% to 20% lead to a significant increase in particle size ($p \geq 0.05$). The smallest particles size was 428 ± 15.84 nm (PDI of 0.293 ± 0.07), produced using 2% of gelatin hydrolysate. Larger particle size of 547 ± 46.5 nm (PDI 0.141 ± 0.07) was produced using 20% concentration of gelatin hydrolysate. There was an insignificant difference between 1% and 2% gelatin hydrolysate concentration on particle size. In terms of PDI, different concentrations of gelatin hydrolysate resulted in fluctuations in size distribution of the nanoparticles. The PDI of the nanoparticles was in the range of 0.1 to 0.3 meaning that the particle size distribution had good uniformity, with some larger particles in the product. Broad range of particle distribution was due to non-uniform molecular weight and low purity of the starting material [17].

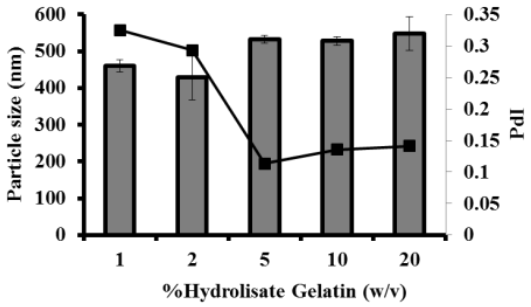
Our results are agreeable with other studies reporting that increasing the concentration of raw material increased viscosity of the solvent phase; hence, diffusion between solvents and non-solvents is slowed down [18], [19].

A significant ($p \geq 0.05$) decrease in zeta potential was observed when concentration of gelatin hydrolysate was increased from 1% to 20% (w/v) (Figure 2a). Highest zeta potential of −16.8 ± 2.1 mV was produced using 1% (w/v) gelatin hydrolysate and decreased to −9.3 ± 0.5 mV using 10% gelatin hydrolysate. However, no significant differences on zeta potential were observed between 1% and 2% gelatin hydrolysate concentration. Zeta potential indicates the ability of particles to avoid agglomeration in the solution. Higher zeta potential is favorable [20]. Based on the obtained particle size and zeta potential, gelatin concentration of 2% was used for the next experiment.

The effect of pH

In figure 1b, the effect of different pH condition during the production process on particle size and PDI is shown. Since the isoelectric point of gelatin hydrolysate is around pH 6 [9], the pH of this experiment was conducted from 2 to 5. The ANOVA test shows that pH had significant effect on the particle size ($p \geq 0.05$), however no significant effects between pH 3 and 4 was found. Besides, PDI value increased from 0.07 ± 0.06 to 0.148 ± 0.10 within the 2 to 5 pH range. This PDI result indicated the distribution of particle size is in the range, but no significant different have been shown, as the *p* value obtained from ANOVA.

(a)



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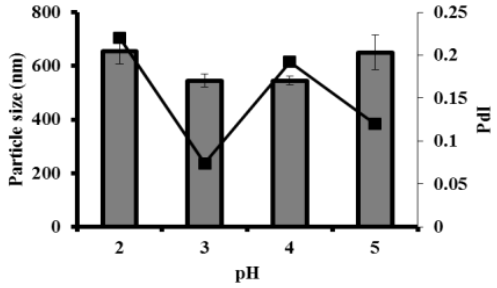
(b)



(c)

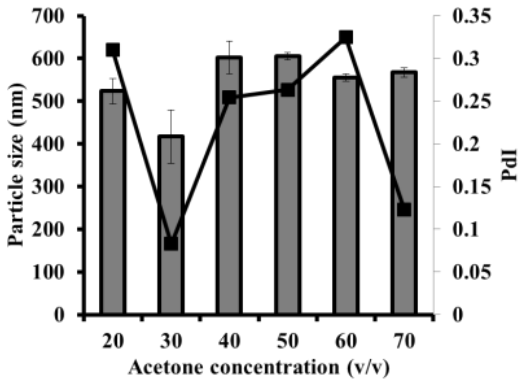


Figure 1. Effect of (a) gelatin hydrolysate concentration, (b) pH and (c) acetone concentration on the particle size and polydispersity index of nanpoparticles. Results are expressed as mean ± SD from three independent experiments

is higher than 0.05. The zeta potential of nanoparticles produced at different pH is shown in figure 2b. Increasing the pH decreases the zeta potential of gelatin hydrolysate particles ($p \geq 0.05$). The zeta potential of nanoparticles produced under pH 2, 3, 4, and 5 are −12.3 ± 0.7, −12.1 ± 0.3, −12.2 ± 0.6 and −9.5

± 0.3 mV respectively.

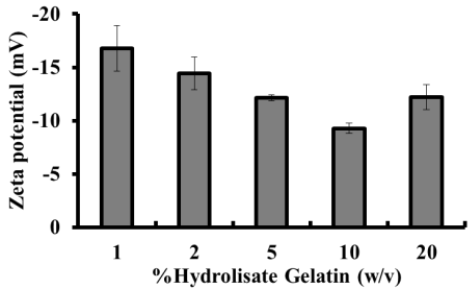
Similar to the gelatin precursor, gelatin hydrolysate is an amphoteric biopolymer that consists of positively and negatively charged amino acid content [21]. Based on the results, changing the pH increases the particle size and lowers the zeta potential. Lowering the pH value far from the isoelectric point will help to denature the triple helical structure of the gelatin hydrolysate [22]. It was reported that pH far from the isoelectric point increased water solubility of gelatin hydrolysate [23]. Hence, the results support previous reports that pH is the major factor determining size of the final product [22], [24]. The fabrication of nanoparticles at isoelectric pH leads to fast aggregation and precipitation since the molecule has no net charge at its isoelectric point. Low zeta potential in nanoparticles is an effects of the increasing particle size [25]. The smallest nanoparticle with highest zeta potential was produced at pH 3. Consequently, pH 3 was selected for further studies.

Effect of acetone concentration

The data shown in Figure 1c shows the effect of acetone concentration on nanoparticle size. This experiment was conducted with various concentrations of acetone varying from 20% to 70% (v/v), while concentration of fish gelatin hydrolysate and pH were kept constant at 2% and pH 3, respectively. ANOVA analysis indicated that acetone concentration had significant effects on particle size and PDI of the fish gelation hydrolysate nanoparticle ($p \geq 0.05$). The nanoparticle size was found to increase as the concentration of acetone was increased from 30% to 70% (v/v). The results indicate that acetone concentration of 30% (v/v) produced the smallest nanoparticle size and PDI of around 416 ± 38.2 nm and 0.083 ± 0.04, respectively. Meanwhile, bigger nanoparticles were produced using 50% acetone; particle size was 605 ± 8.8 nm and PDI 0.264 ± 0.1. Figure 2C depicts the effects of solvent concentration on zeta potential. The zeta potential decreased from −15.1 ± 1.2 to −8.4 ± 0.8 mV with increasing acetone concentrations from 30% to 70% (v/v). Further analysis by ANOVA also indicated that acetone concentration had significant effects on zeta potential ($p \geq 0.05$). Nanoparticles with highest zeta potential of −15.1 ± 1.2 mV were produced using 30% acetone.

Acetone was used as co-solvent since acetone has a high hydrogen bond formation capacity with water and produces smaller nanoparticles compared to ethanol and other organic solvents [14], [26]. Acetone works as a disturbing agent

(a)



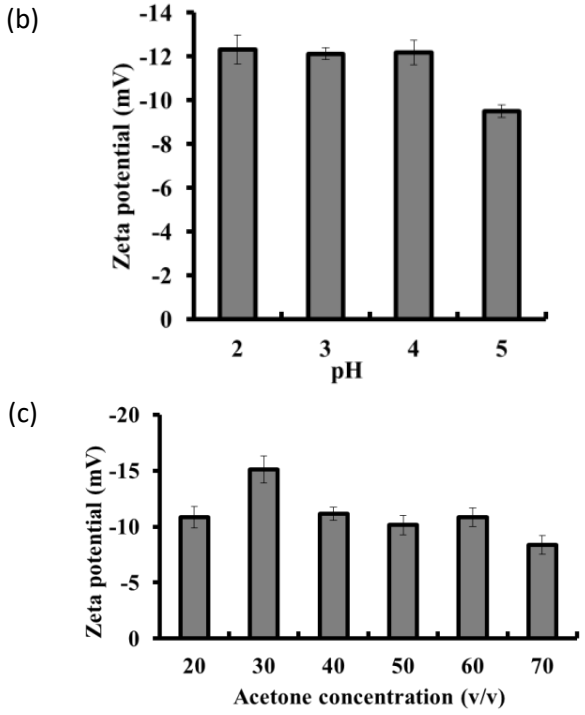


Figure 2. Effect of (a) gelatin hydrolysates concentration, (b) pH, and (c) acetone concentration on the zeta potential of nanoparticles. Results are expressed as mean ± SD from three independent experiments.

between water and amino acids in the gelatin hydrolysate molecule. When acetone was added to the solution, the gelatin hydrolysate dehydrated and created aggregates via amino acid inter- and intra-molecular bonds. The amount of solvent used in production depends on pH. Huge amounts of acetone is needed as the pH is varied away from isoelectric point [24]. In this experiment, 30% of acetone concentration was the optimal concentration to produce small particles with high zeta potential. Further increase of acetone concentration led to an increase in particle size, because the molecule tends to become more dense and precipitates together [24]. The behaviour of this result was characterized by the increased of PDI and lowered the zeta potential. Results from this study was in agreement with previous experiment [15], [27], despite the raw material is different. Based on this result, 30% acetone concentration was chosen for the production of gelatin hydrolysate nanoparticle.

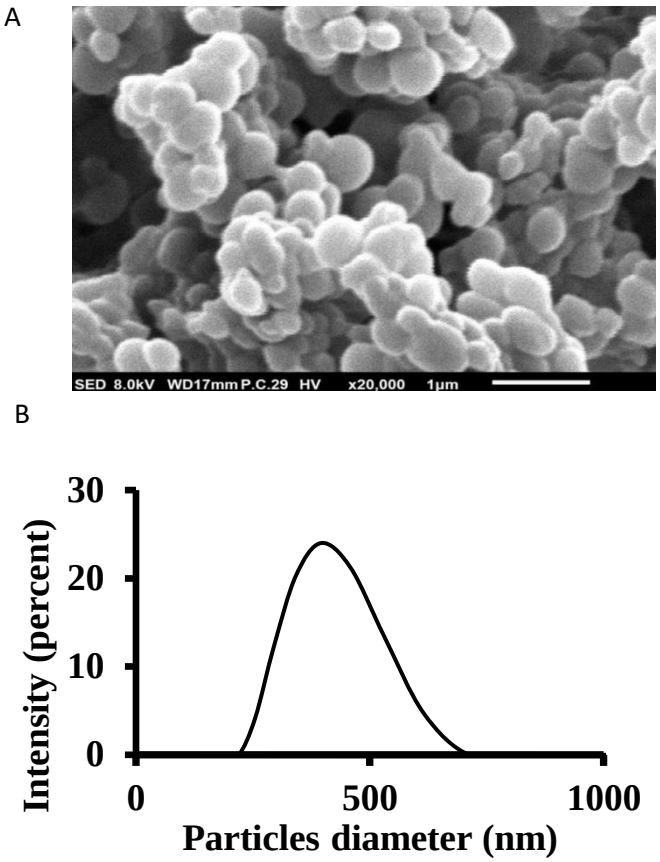


Figure 3. SEM micrograph of gelatin hydrolysates nanoparticles (A), and size distribution of gelatin hydrolysate nanoparticles (B).

Fish gelatin hydrolysate particles size and shape

Gelatin hydrolysate nanoparticles were fabricated using the selected conditions. Gelatin hydrolysate concentration, pH, and acetone concentration were 2% (w/v), 3, and 30% (v/v), respectively. Other factors i.e. amount of glutaraldehyde and stirring speed were set up at 300 µL and 600 rpm. The particle size, shape, and zeta potential of the produced nanoparticles were characterized. Figure 3a and 3b depicts the scanning electron microscopy (SEM) image and size distribution graph of the gelatin hydrolysate nanoparticles. The nanoparticles had a visibly clear round shape and average diameter of 100-300 nm. The average zeta potential and polydispersity index was about 16.4 ± 1.2 mV and 0.203 ± 0.07. Fish gelatin hydrolysate nanoparticles were larger in size compared to mammalian gelatin nanoparticles because the gelatin

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hydrolysate had heterogeneous molecular weight contained impurities such as non-collagenous protein, muco-substance contaminant and inorganic salts [17]. On the other hand, the molecular weight of gelatin hydrolysate is lower than mammalian gelatin, which also affects particle size [14], [28], [29].

Conclusions

The current research presents for the first time, successful preparation of nanoparticles from fish gelatin hydrolysate. Fish gelatin hydrolysate with particle size of 100-300 nm was produced using desolvation method. The gelatin hydrolysate concentration, pH and acetone concentration were found to have significant effects on particle size and zeta potential of the nanoparticle. The selected conditions for the nanoparticle production are: gelatin hydrolysate concentration 2% (w/v), pH 3, and acetone concentration 30% (v/v). The fish gelatin hydrolysate nanoparticles produced were bigger than mammalian or fish gelatin nanoparticles, because the fish gelatin hydrolysate had low purity and heterogeneous molecular weight. However, the nanoparticles obtained from this experiment showed the potential of gelatin hydrolysate to be applied in the field of nanoparticle research for use as a drug delivery system. Successfully converting gelatin hydrolysate to nanoparticles is believed to increase the economic value of the fish product and reduce wastage from marine products.

Conflicts of interest

There are no conflicts to declare.

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