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Process optimization for the production of fish gelatin nanoparticles

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Abstract

Animal proteins have become an useful source for producing gelatin nanoparticles, due to its application in cosmetics and therapeutics. Gelatin nanoparticle (GNP) is an excellent biodegradable and biocompatible material. Due to its chemical modification potential gelatin nanoparticles are very promising in carrier system for drug delivery. Most of the commercials gelatin are derived from mammalian sources, such as porcine and bovine. Fish gelatin has become a good alternative resource for GNPs production in view of the various religious, safety and economic reasons. In this present work, the tilapia fish gelatin was used as a raw material for the production gelatin nanoparticles via modified two-step desolvation method. In this process, obtaining high molecular weight (HMW) fraction content of fish gelatin is very crucial for the preparation of stable and small size GNPs. Hence the present study was carried out to assess the various formulation parameters in the first step in the two-step desolvation method to produce fish gelatin nanoparticles (FGNPs). The nanoparticles formed were characterized for mean size and size distribution, while the morphology of the particles was evaluated by field emission scanning electron microscope (FESEM). The size of fish gelatin nanoparticles was found to be 254±11 nm which is suitable for drug delivery. The study indicated that a high fraction of HMW in precipitate at the first step desolvation could be obtained by using gelatin concentration 9%, temperature 45°C, centrifugation speed at 12000 x g, and centrifugation time was 5 min. It showed that this method is efficient compared to conventional method.

Introduction

Biodegradable nanoparticles have several advantages, such as better encapsulation, best on bioavailability, less toxicity, controlled and targeted release, for the preparation of drug delivery system gelatin nanoparticles (GNPs) are harmless, biocompatible, recyclable, not-antigenic, available in abundant from renewable sources, and possess extra ordinary binding capacity of various active groups for attaching targeting molecules. These properties make FGNPs as a promising drug carrier (Elzoghby *et al*., 2012). In addition, the high content of amino acids like glycine, proline and alanine in gelatin polymer (Weber *et al*., 2000), provides an added advantage for the use of FGNPs.

Gelatin, the denatured collagen is usually obtained by hydrolysis or by thermal or enzymatic degradation of animal collagen protein. Two different types of gelatin, type A (from porcine) and type B (from Bovine), are available in large amounts. The application of gelatin has been approved GRAS (generally regarded as safe) material by the USA Food and Drug Administration (US FDA), and has been used for a long time in pharmaceuticals, cosmetics, as well as in food products (Elzoghby, 2013).

The emphasis on the application of fish gelatin has shown tremendous growth in the last decade (Karim and Bhat, 2009). This is mainly due to the concerns from a religious group like Judaism, Islam and Hinduism, and the concerns from vegetarians (Karim and Bhat, 2008). In this regard, fish gelatin has been highlighted as a better alternative to mammalian gelatins (Jan Arnesen Arnesen and Gildberg, 2006). The capability of animal tissue-derived collagens and gelatins to transmit the pathogenic vectors such as prions is also a matter of concern (Wilesmith *et al.*, 1991). In addition, Over the last several years, our group research has investigated the potential of fish gelatin for process development (Jaswir *et al*., 2009; Jaswir *et al.*, 2011; Monsur *et al.*, 2014).

Initially, nanoparticles from gelatin were prepared by desolvation method. However, due to the broad molecular weight distribution of gelatin, it has always been a challenging task to prepare stable and mono dispersed nanoparticles (Zwiorek, 2006). Therefore, various investigators have utilized different techniques for the preparation of gelatin

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nanoparticles. These techniques include emulsion/ solvent evaporation (Bajpai and Choubey, 2006) reverse phase preparation (Guptaa *et al*., 2004), inverse mini emulsion (Ethirajan *et al*., 2008), coacervation (Saraogi *et al.*, 2011) gamma irradiation (Furusawa *et al.*, 2004), nanoprecipitation (Khan and Schneider, 2013), and two step desolvation (Azarmia *et al*., 2006).

Two-step desolvation method is the most often used for gelatin nanoparticles preparation. This method produced narrow size distribution of gelatin nanoparticles compared to other methods (Coester *et al*, 2000). In this method, the gelatin will separate based on their molecular weight. It has been shown that the mixings of low molecular and high molecular weight of gelatin effect to the size distribution of gelatin nanoparticles product, because the low molecular weight gelatin has inferior gelling properties compared to high molecular weight gelatin (Arnesen and Gildberg, 2007). The gelatin nanoparticles need to be uniform and have a narrow size distribution to use as drug carrier system (Davda and Labhasetwar, 2002). The decantation of low molecular weight and re dissolving of high molecular weight of gelatin were applied after the precipitation process. However, the characteristic features of fish gelatin have a low gel modulus and gelling temperature (Jamilah and Harvinder, 2002; Avena-Bustillos *et al*., 2006), which made fish gelatin become difficult to separate their HMW from LMW by a conventional method that used by coaster.

Bearing in mind this challenge, our focus is to explore the potential of fish gelatin as the raw material to produce nanoparticulate carrier system, by improving the two-step desolvation method and to define parameters for a high content of HMW gelatin in the precipitate at the first step. Therefore, the effect of various precipitation parameters was investigated allowing us to determine a reproducible formulation of FGNPs with HMW.

Materials and methods

Materials

In the current work, fish gelatin (128.11 Bloom strength), was extracted from Tilapia fish skin (Jamilah and Harvinder, 2002), the fish gelatin then produce was used without any pre-treatment. Glutaraldehyde grade I (25% v/v aqueous solution), acetone were purchased from Sigma Aldrich, Malaysia. Sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), ammonium persulfate, 1.5 M Tris-HCl buffer pH 8.8 and 0.5 M Tris-HCl buffer pH 6.8 were purchased from Bio-Rad (Hercules, CA, USA). Double distilled water was used for all the experiments. All chemicals were of analytical grade and used as received.

Production of gelatin nanoparticle by conventional two-step desolvation method

Two step desolvation method was used for producing gelatin nanoparticles. This process begins with the preparation of 5% gelatin solution (10 ml) under constant heating (40°C) and constant stirring, then continued until clear gelatin solutions were obtained. The clear solution was mixed immediately with acetone as a desolvating agent and the resulting mixture was allowed to separate into two layers for 10 min. The low molecular weight (LMW) fraction was at the top and the high molecular weight fraction is at the bottom layer. The LMW layer was discarded and the HMW layer was dissolved in 10 ml of distilled water and stirred at 600 rpm under constant heating at 40°C. The pH of gelatin solution was maintained at 3. Acetone (30 ml) is added to this solution drop wise under constant stirring, to form nanoparticles. Glutaraldehyde (25%) solution (100 µl) was added as a crosslinking agent. Then the solutions were stirred for 12 hours at 600 rpm. The particles were purified by three-fold centrifugation and redispersed in 30% acetone solution in double distilled water.

Production of fish gelatin nanoparticles by modified two-step desolvation method

Our initial attempts to produce FGNPs using conventional method produced large FGNPs with broad size distribution. Hence, the two step desolvation method was modified by adding centrifugation to facilitate faster and better separation of HMW fish gelatin.

Tilapia fish gelatins (0.9 g) were dissolved in distilled water (10 ml) under constant heating until a clear solution is shown. Acetone (10 ml) was added immediately to the gelatin solution as a desolvating agent. In order to achieve the rapid separation of HMW layer, the solution was centrifuged (Eppendorf, Centrifuge 5810 R, Hamburg, Germany) at 12000 x g for 3 min. The remaining steps were same as the conventional method, as described in the previous section. The schematic of two desolvation method with centrifugation has shown in Figure 1.

Formulation optimization

As it is crucial to obtain fish gelatin with HMW content, we have directed our attention on the process parameters that influence this stage. The concentration of gelatin, temperature, centrifugation speed, and centrifugation time were changed to study

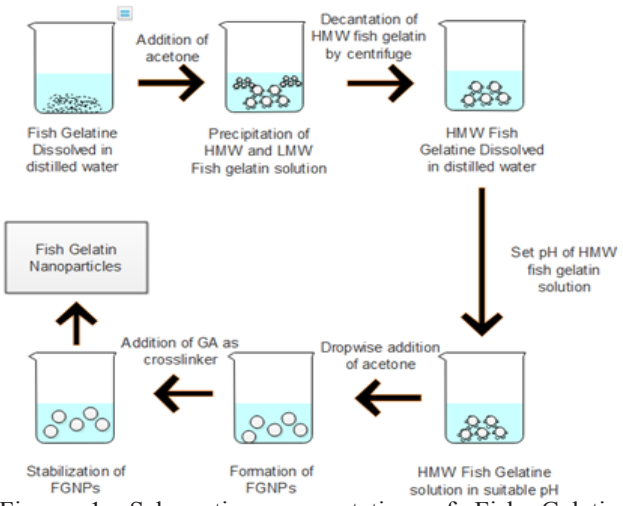

Figure 1. Schematic representation of Fish Gelatin Nanoparticles formation by modified two-step desolvation technique.

their effect on the separation on HMW and LMW fish gelatin fraction. The values of all factors were chosen according to a previous research (Elzoghby, 2013) and several preliminary experiments. SDS-PAGE was used to identify the molecular weight of the HMW gelatin fraction. The intensity and the length of the band are taken as the measure of the concentration and molecular weight distribution of the protein in the HMW fraction.

Gelatin concentration

The concentration of protein in the solution affected the gelatin molecular weight distribution in the precipitation results. It has been found that the aggregation of the protein is usually created at higher protein concentrations (Scopes, 2013). In order to investigate the effect of gelatin concentration on separation, the various gelatin concentrations were studied from 3% (w/v) to 30% (w/v). However, other factors such as temperature (40°C), centrifugation speed (12000 x g), and centrifugation time (3 min) were kept constant.

Temperature

It is important to control the temperature during precipitation stage. The precipitation process at a higher temperature, lead to quick denaturation of the protein. To study the effect of temperature on the separation of gelatin, the temperature for dissolving gelatin was varied from 35°C to 55°C. Furthermore, the gelatin concentration (5% w/v), centrifugation speed (12000 x g), and centrifugation time (3 min) were maintained unchanged.

Centrifugation speed

Since the high molecular weight molecule can be very rapid to be precipitated, the maintaining of the centrifugation speed it is necessary. To study the effect of centrifugation speed on the separation of HMW from LWM, different speed of centrifuge were varied, 6000 x g, 9000 x g, 12000 x g, 15000 x g, and 18000 x g briefly. The gelatin concentration (5% w/v), temperature (40°C), and centrifugation time (3 min) were kept constant.

Centrifugation time

The centrifugation time had effect as well as centrifugation speed, to maintain the centrifugation time is also necessary to prevent of the LMW become precipitated. Scopes (2013) have precipitated the mammalian protein from 5-15 min. In this experiment, the centrifugation time was varied from 1 min to 15 min. Furthermore, gelatin concentration (5% w/v), temperature (40°C), and centrifugation speed (12000 x g) were maintained unchanged.

Electrophoretic analysis of fish gelatin precipitate

SDS-PAGE was used to analyse the HMW content of the precipitate obtained after separation by centrifugation. The precipitates were dissolved to get 5 mg/ml solution in distilled water at 50°C and then a buffer solution containing β-mercaptoethanol (15 mg/ml) was added. Protein samples were heat-denatured for 5 min at 90°C and analyzed by SDS-PAGE using 4% stacking gels and 12% resolving gels. SDS-PAGE was carried out in a Mini Protean II tetra cell (Bio-Rad Laboratories, CA, USA) at a constant voltage of 150 V for 1 hours. The molecular weight of the polypeptide bands were estimated using a standard marker for SDS electrophoresis (GE Healthcare, Buckinghamshire, UK). The resulting bands were stained with Coomassie brilliant Blue R250 and photographed using Alphaimager HP white light and imaging software (Alphaimager HP).

Determination of particles size

The size of nanoparticles was analysed by dynamic light scattering (DLS) with a Malvern (Zen3600, UK) Zetasizer. The mean diameter and polydispersity index (PI) values were obtained at an angle 90° in 10 mm diameter cells. Samples for particle suspension were prepared by diluting with 3 ml of double distilled water and sonicating for the 30 s. Particle size measurements were performed at room temperature with a detection angle 90°. All measurements were performed in triplicate.

Morphologies characterization by scanning electron microscopy

An FESEM (JEOL, JSM 6700F Model) was used to capture images for evaluation of the shape size,

and morphology of the nanoparticles. Briefly, a small amount of lyophilized fish gelatin nanoparticles were mounted on aluminium stubs, pre-pasted with doubled-side copper tapes. The samples were sputter coated with a thin layer of gold and placed the specimen chamber at an accelerating voltage of 10 kV.

Results and discussion

Gelatin concentration

Figure 2 showed the gel electrophoresis of fish gelatin at different gelatin concentrations. It can be seen that the content of HMW in the precipitate increased with the increase of the concentration of gelatin. The result showed that gelatin concentration had an impact to the separation of HMW from LMW in the gelatin solution. Furthermore, the concentration of fish gelatin at 9% was chosen as the optimum concentration that had the amount of HMW and low of LMW in the sediment. It is indicated that precipitation occurs at a higher concentration of protein. This results correlated with Scopes, that the aggregation is caused by interactions between opposite-charged areas on the proteins surfaces. At lowest concentration of protein, the hydrophobic attractions are less involved by an organic solvent. The larger the molecule, the lower percentage of organic solvent required to precipitate (Scopes, 2013). Scopes also mention that, the precipitation methods are suitable only from protein concentration of above 1 mg/ml. In addition, Doucette (2014) used initial 1 g/l of protein concentration to precipitate E. coli membrane proteins.

Temperature

The SDS-PAGE results of gelatin in different temperature are shown in Figure 3. The precipitation of gelatin is conducted at low temperature, because above 60°C, the fish gelatin become denatures due to disruption of the hydrogen bond, however, the preparation of fish gelatin solution at ambient temperature (25°C) is difficult because fish gelatin form a highly viscous gel at this temperature. It showed that, at the temperature 35°C the gelatin precipitate had a broad distribution of molecular weight, and it was decreased by the increase of temperature until they reach to 45°C. Moreover, the content of molecular weight of gelatin in precipitate was increased when the temperature increase from 45°C to 55°C. As a result, the highest content of the HMW with lesser content of LMW in the precipitate were prepared at 45°C. This might be explained by the precipitation process should be carried out at

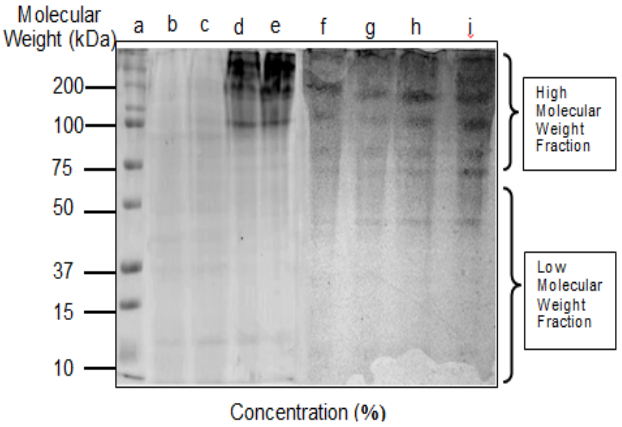

Figure 2. SDS-PAGE gel electrophoresis result of fish gelatin precipitated by difference in the concentration of gelatin (a) marker, (b) fish gelatin solution 3%,(c) 5%, (d) 7%, (e) 9%, (f) 13%, (g) 18%, (h) 23%, and (i) 30% (g/v) respectively. Each experiment was performed in triplicate.

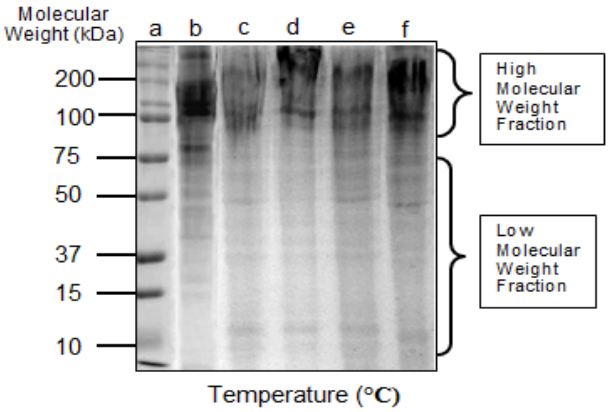

Figure 3. SDS-PAGE gel electrophoresis result of fish gelatin precipitated by difference in the temperature (a) marker, (b) fish gelatin temperature 35°C, (c) 40°C, (d) 45°C, (e) 50°C, and (f) 55°C respectively. Each experiment was performed in triplicate.

low temperature instead at high temperature. Since scopes had suggested at the same condition (Scopes, 2013). This result is consistent with the result obtained in Cuomo *et al.* (2011) and Geethanjali and Subash (2013).Thus, we should keep in mind that, in this experiment, the result with the highest content of HMW and lowest content of LMW were the main target. Its mean that we should keep the temperature had low enough will result in higher HMW with lower LMW content.

Centrifugation speed and centrifugation time

Figure 4A represented the SDS-PAGE results of gelatin separation by different in centrifugation speed. It is shown that the HMW content in the precipitate was increased by increasing of centrifugation speed. The highest HMW content with lowest LMW in the precipitate is shown at centrifugation speed 12000 x

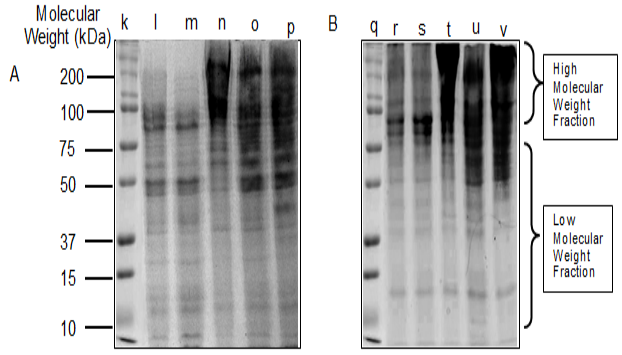

Figure 4. SDS-PAGE gel electrophoresis result of fish gelatin precipitated by difference in the centrifugation speed (A) and different centrifugation time (B);(k and q) marker, (l) speed 6000 x g, (m) speed 9000 x g, (n) speed 12000 x g, (o) speed 15000 x g, (p) 18000 x g, (r) 1 min,(s) 3 min, (t) 5 min, (u) 7 min, and (vu) 15 min respectively. Each experiment was performed in triplicate.

g.

For centrifugation time, a defined centrifugation time from 1 to 15 min was used and the others factor such as gelatin concentration, dissolving temperature, and centrifugation speed were maintained unchanged (Figure 4B). It can be seen that, the content of HMW in the sediment was increased by increasing centrifugation time from 1 to 15 min. The result show 5 min of centrifugation time was the preferred times in the centrifugation process with were generally had the highest content of HMW and lowest content of LMW in the precipitate. These results agree with Scopes (2013) centrifugation process is necessary for term of to get rapid aggregate. The particle size is clearly critical in determining the amount of centrifuging required. Any precipitation process will result in a range of particle sizes. In addition, Crowell (2013) used acetone precipitation followed by centrifuge at 16000 x g to precipitate water-soluble protein.

Fish gelatin nanoparticles particles size and shape

The size distribution of fish gelatin nanoparticles, as shown in Figure 5A, demonstrated that the dimensions of fish gelatin nanoparticles product barely vary (i.e., the size of both is the order 200 nm). It can be seen that, the size distribution of FGNPs produced by acetone precipitation followed by centrifugation had a lower size (254 ± 11 nm) with a narrow size distribution (Figure 5A) compared to the FGNPs produced by the conventional method (mean size 324 ± 14 nm). This results for the size distribution agrees with the past work, that the removal of the LMW gelatin from the precipitate, prevented further secondary aggregation during the process (Coester *et al*., 2000). This result is also indicating this acetone precipitation coupled with

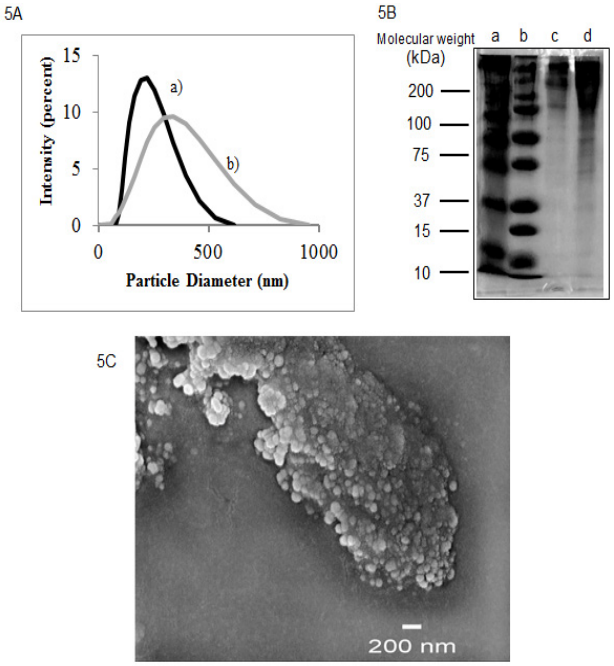

Figure 5. 5A is size distribution of fish gelatin nanoparticles, a) fish gelatin nanoparticles produced by centrifuge method, b) fish gelatin nanoparticles produced by the conventional method. Figure 5B is the SDS-PAGE results of fish gelatin precipitate a and b is marker, c) fish gelatin precipitate prepared with centrifugation, d) fish gelatin precipitate without centrifugation. Figure 5C is Scanning-Electron microscopy (SEM) image of fish gelatin nanoparticles: the particles size was determined about 200 nm.

centrifugation could separate the HMW from LMW of fish gelatin protein solution effectively. Figure 5B provide the SDS-PAGE of fish gelatin precipitate, "c" is the SDS-PAGE results for precipitate prepared by centrifugation, "d" is the SDS-PAGE results for precipitate prepared without centrifugation process. An FE-SEM picture of fish gelatin nanoparticles is shown in Figure 5C, which clearly indicates the spherical nanoparticulate with an average diameter of about 100-300 nm. This result is also indicated that production of fish gelatin nanoparticles by the current method is efficient to produced small fish gelatin nanoparticles compared to a method that introduced by Coester (2000).

Conclusion

Fish gelatin nanoparticles (240-300 nm) could be effectively prepared by modified two-step desolvation method. The precipitation process of fish gelatin could be rapid precipitated by applying with centrifugation process. Thus, the modified two-step desolvation method developed in the present study enabled the production of fish gelatin nanoparticles. By this method, the high molecular weight fractions of fish gelatin are separated following the first desolvation step. The separation of HMW from

fish gelatin was influenced by several process variables including the concentration of fish gelatin, temperature, centrifugation speed, and centrifugation time. The best result (higher content of HMW and lower content of LMW in precipitate) was attained at 9% (g/v) of fish gelatin concentration, 45°C, 12000 x g, and 5 min of centrifugation, by these conditions the fish gelatin nanoparticles diameter of 254 ± 11 nm were achieved. Compared to conventional two-step desolvation method, fish gelatin nanoparticles with diameter 324 ± 14 nm were produced.

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