Innovative Food Science and Emerging Technologies 26 (2014) 490–497

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

Processing of soy functional food using high pressure homogenization for improved nutritional and therapeutic benefits

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ARTICLE INFO

Article history:
Received 26 January 2014
Accepted 28 May 2014
Available online 11 June 2014

Editor Proof Received Date 30 June 2014

Keywords:
Nanosuspension
Oral delivery of nattokinase
Tail thrombosis
Ultra high pressure homogenization

ABSTRACT

Nanosuspension as a delivery system for soy nutraceuticals (nattokinase and soy-isoflavones) has been prepared using top-down technique. Ultra high pressure homogenization was used to fabricate fermented soybean powder (FSP) and surfactants into an aqueous nanosuspension. Statistical optimization using Response Surface Methodology showed that 0.24 mg ml-1 of sodium lauryl sulphate, homogenization pressure of 200 MPa and 15 cycles of homogenization produced the nanosuspension with desired particle size of 145.6 ± 1.5 nm and PDI 0.3 ± 1.1. Validation experiments confirmed the reliability of the predicted model for production of nanosuspension. *In-vitro* release of isoflavones from nanosuspension was at a much faster rate than macrosuspension of FSP. Significant prolongation ($p < 0.001$) of both prothrombin time and activated partial thromboplastin time; and decrease in frequency of tail thrombosis and reduced infraction were observed in rats of model groups after oral administration of nanosuspension which suggested the strong anti-thrombotic potential of nanosuspension.
Industrial relevance: Nanosuspension provides a good delivery vehicle for administration of non-water soluble food components. Application of high pressure homogenization results in the development of novel soy functional foods. This process could be used on lab as well as commercial scale for the formulation of nutraceuticals.

1. Introduction

Natto is the traditional fermented food of Japanese people made by steaming soybeans and fermenting them with *Bacillus subtilis* natto (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987). During fermentation various therapeutically active molecules like nattokinase, vitamin K, and gamma polyglutamic acid (Kambourova, Tangney, & Priest, 2001) are produced. Studies have shown that fermented soybeans contain isoflavone aglycone (daidzein, genistein and glycitein) which is said to be absorbed faster and is greater in amount than glucoside present in non-fermented soybeans. These isoflavones are structurally similar to estrogen and exhibit moderate estrogen-like effects (Okabe, Shimazu, & Tanimoto, 2011). Besides having nutritional components, the fermented soybeans are packed with medical benefits as well.

Nattokinase could be safe for long-term intake (Sumi et al., 1987). Nattokinase is stable in the gastrointestinal tract and its oral administration enhanced fibrinolysis in canine plasma in an experimental thrombosis model (Sumi, Hamada, Nakanishi, & Hiratani, 1990). It enhances plasminogen activators and inactivates a plasminogen activator inhibitor (Sumi et al., 1987; Urano et al., 2001). Nattokinase helps in decreasing the rate of progression of plaque formation and reverses evolving atherosclerotic lesions. Nattokinase also possesses anti-hypertensive effect (Fujita et al., 2011) and amyloid degrading ability (Hsu, Lee, Wang, Lee, & Chen, 2009). Because of its convenient oral administration, efficacy, prolonged effects and cost effectiveness, nattokinase is actually superior to conventional fibrinolytic agents. There is very limited literature on the feasible dosage form of crude nattokinase. Nattokinase is isolated from fermented material and supplied in the form of capsules. Effect of nattokinase had been studied in the form of plain drug (Yuan et al., 2012) or coating it with polymers like gamma-polyglutamic acid (Hseih et al., 2009) or microencapsulation of the enzyme extract by alginate (Ko, Koo, & Park, 2008). But, a formulation containing therapeutic benefits of soybeans and nattokinase has never been explored. Herein, an effort has been made to develop the fermented soybeans (containing nattokinase and soy-isoflavones) into a suitable dosage form by formulating it into a nanosuspension.

Nanosuspension is a dispersion of nanosized drug particles which can be prepared by bottom-up or top-down technology and stabilized with suitable stabilizers. Using wet-milling technology, nanodispersion of red mold rice containing higher monacolin K had been prepared

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http://dx.doi.org/10.1016/j.ifset.2014.05.015
1466-8564/

(Yu, Lee, & Pan, 2006). This study showed that nanosuspension or nanodispersion can be employed to formulate functional foods. The stability of nattokinase in acidic conditions was increased when some other additional substances were present with the enzyme in comparison to the conditions when nattokinase alone was present in acidic environment (Sumi et al., 1987). That is why; we have included whole soybeans along with nattokinase to prepare a formulation for nattokinase delivery. Also, soybeans are rich in isoflavones. Soybeans contain glucoside form of isoflavones which are water soluble (Wang & Murphy, 1994). After ingestion isoflavones must be hydrolysed to absorbable aglycone (Piskula, Yamakoshi, & Iwai, 1999) and have higher hydrophobicity. Fermentation of soybeans also increases the amount of aglycone isoflavones in soybeans. So, to make these isoflavones highly bio-available, the fermented soybeans have been crushed to reduce their size. This fermented and powdered soybean was then formulated into a nanosuspension by using high pressure homogenization method. Nanosuspension is a cost effective and technically simple procedure to formulate bioactive molecules present in food. Also, nanosuspension has the advantage of enhancing saturation solubility with improved dissolution characteristics (Talekar, Kendall, Denny, Jamieson, & Garg, 2012). The prepared nanosuspension was rich in nattokinase and provides higher bioavailability of aglycone-rich isoflavones. Because of the presence of fibrinolytic enzyme (i.e., nattokinase), the nanosuspension has anti-thrombotic potential which was studied in rat tail model of carrageenan induced thrombosis.

2. Materials and methods

2.1. Microbes and materials

Soybean variety DS 9814 was procured from Pulse Laboratory of Genetic division of IARI (Indian Agricultural Research Institute), PUSA, Delhi, India; as gift sample. *B. subtilis* MTCC 2616 was obtained from MTCC (Microbial Type Culture Collection), IMTECH (Institute of Microbial Technology), Chandigarh, India. All chemicals and reagents used were of analytical grade. Thrombin and fibrinogen were purchased from MP Biomedicals (USA) and Hi Media (India), respectively. κ-carrageenan, daidzin, genistin and glycitin were procured from Sigma-Aldrich (USA).

2.2. Production and processing of fermented soybeans

Fermentation of soybean variety (DS 9814) with *B. subtilis* MTCC 2616 was carried out in a rotating type solid state fermenter as described in our previous manuscript (Kapoor & Panda, 2013). Briefly, the optimized medium contained glucose 2 g, casein 1 g, K_2HPO_4 0.30 g, $FeSO_4$ 0.01 g and hypoxanthine 1 mg for 100 g of soybean seeds. The fermentation process was carried out at 37 °C for 24 h.

The fermented soybean material was grinded to obtain a uniform mass. This triturated mass was dried in a hot air oven under different temperatures ranging from 40 °C to 60 °C for14 to 16 h. The dried mass that contained the maximum amount of metabolites was selected and grinded to obtain particles of smaller size. The grinded material was passed through sieve no 44 to obtain moderately fine powder of fermented soybeans (FSP).

2.3. Food composition analysis

Food composition analysis of FSP was performed according to the standard procedures of AOAC for the determination of ash value, moisture, lipid, protein and content. The carbohydrate content was calculated by eliminating the total ash, moisture, lipid and protein content.

2.4. Analysis of nattokinase using fibrinolytic assay (in-vitro)

Fibrinolytic activity assay of FSP was determined by fibrin degradation assay (Yin, Lin, & Jiang, 2010). Briefly, 0.4 ml of 0.75% fibrinogen and 0.1 ml of phosphate buffer (0.2 M, pH 7) were placed in a test tube and incubated at 37 °C for 5 min. Then, 0.1 ml of thrombin solution (20 U) was added and again incubated at 37 °C for 15 min, and 0.1 ml of enzyme solution was added, and incubation was continued for 60 min. After 60 min, the reaction was stopped by adding 2 ml of 0.2 M trichloroacetic acid (TCA). Samples were kept for 20 min and then centrifuged at 5000 *g* for 5 min. The absorbency of supernatant was measured at 275 nm. One unit of fibrinolytic activity (FU) was defined as the amount of enzyme that caused an increase of 0.01 in the absorbance at 275 nm in 60 min at 37 °C.

2.5. Analysis of isoflavone content

The isoflavones present in FSP were extracted using acidic 80% acetonitrile. Acidic 80% acetonitrile was added to weighed amount (2 g) of FSP and incubated for 30 min at 30 °C. After centrifugation for 10 min at 3000 *g*, the supernatant was taken and concentrated by heating at 50 °C. The concentrated extracts were re-dissolved in 80% methanol and filtered prior to high performance thin layer chromatographic analysis. For the estimation of isoflavones, toluene:ethyl acetate:formic acid:acetic acid were used in the ratio of 1:8:1:0.5 as mobile phase. The R_f of the isoflavones was measured at 260 nm. The amount of isoflavones present in FSP was calculated as per the formula given by Prasad & Shah, 2012.

2.6. Formulation of suspension and effect of formulating parameters

Fermented soybean powder or FSP (200 mg), sodium lauryl sulfate or SLS (2 mg) and Tween 80 (25 μl) were dispersed in Milli Q water (10 ml) and the resulting coarse pre-dispersion was comminuted using hand homogenizer (Heidolph Diax 900, Germany) for 5 min. The dispersion obtained was homogenized using laboratory scale ultra high pressure homogenizer or UHPH (Stansted Fluid Power Ltd., United Kingdom) for 10–20 cycles with 100 to 200 MPa pressure. The suspension temperature was maintained between 4 and 8 °C by cooling via heat exchanger.

The effect of SLS concentration; pressure and number of cycles of UHPH on particle size and PDI of suspension was studied using 3 factorial Box–Behnken Design (BBD) (Li, Long, Peng, Ming, & Zhao, 2012). The optimization of various independent variables was performed using Design Expert Software 8.0.7.1. A total of 17 experimental runs were performed (Table 1). Each batch was analyzed for particle size, poly-dispersity index (PDI); and nattokinase, daidzin, genistin and glycitin content, of the suspension.

2.7. Verification of optimized model

Nanosuspension was prepared under optimized conditions suggested by the BBD. The experimental values of responses obtained from the independent set of nanosuspension were compared with the predicted values of responses from the optimized model to verify the response surface model.

2.8. Determination of particle characters of the suspension

The mean particle size and polydispersity index (PDI) of the homogenized FSP nano suspension were determined using Malvern Zetasizer Nano ZS (Malvern instruments, UK). Before measurement the samples were diluted 10-fold with Milli Q water. All the measurements were taken in triplicates and average particle size and PDI were considered.