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Nattokinase purification by three phase partitioning and impact of t-butanol on freeze drying

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ABSTRACT

Three phase partitioning (TPP), an efficient bioseparation technique, was used to purify nattokinase from fermentation broth of *Bacillus natto* NRRL-3666. TPP is a relatively recent technique that uses combination of ammonium sulphate and t-butanol to precipitate protein from crude extracts. The precipitated protein forms interface between the lower aqueous layer and the upper organic layer. The key parameters that were studied for efficient purification were temperature, pH, concentration of ammonium sulphate and t-butanol. The pH of 8, temperature of 37 °C, ammonium sulphate 30% (w/v) and ratio of crude extract to t-butanol 1:1.5 gave the most desired results. A single stage TPP led to 5.6-fold purification with an activity recovery of 129.5%. Nattokinase was freeze dried by using different excipients. There was no effect on the secondary structure of nattokinase while using t-butanol (co-solvent) for freeze drying. But there was reduction in drying time as well as the nattokinase cake became more porous.

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1. Introduction

Nattokinase, also named as subtilisin NAT, is primarily isolated from a traditional fermented food "Natto" in Japan [1]. The authors further demonstrated that the oral administration of nattokinase capsules enhances the process of fibrinolysis in canine plasma as observed in their experimental thrombosis model [2]. Later, the fibrinolytic activity was shown to be retained in the blood for more than 3 h [3]. As to the fibrinolytic mechanism of nattokinase, the enzyme was reported not only to possess plasminogen activator activity, but also to directly digest fibrin by limited proteolysis. Nattokinase could cleave plasminogen activator inhibitor-1 into low molecular weight fragments. These preceding studies suggest that the nattokinase could be regarded as a promising agent for thrombosis therapy. Nattokinase (MW = 27.7 kDa, pI = 8.6), a serine protease, belongs to the subtilisin family. It has no disulfide bonds in its secondary structure [4].

In the process of nattokinase purification, most of the techniques involve a combination of two or more steps such as precipitation, membrane filtration, dialysis, ion-exchange, hydrophobic interaction, gel permeation and affinity chromatography [5–8]. Concentration is usually the first downstream processing used to reduce the liquid volume with minimal loss of activity. Although simple to perform, protein concentration by salting out has disadvantages of low process temperature, longer time for protein aggregation and lower product purity. Ultrafiltration, the other most commonly used concentration method, also has certain drawbacks such as extensive prefiltration and membrane fouling. The separation by chromatographic technique is time consuming, require pre-treatment and used mainly for therapeutic proteins. Thus, an alternative method for nattokinase purification is required to solve the aforementioned drawbacks.

Three-phase partitioning (TPP) has been reported as an effective alternative method for concentration and purification of various industrially important enzymes. This technique uses a combination of ammonium sulphate and t-butanol to precipitate proteins from crude extracts. t-butanol binds to the precipitated proteins, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer. Under the optimum conditions of pH, temperature, ammonium sulphate and t-butanol concentrations protein can be selectively precipitated at the interface of the organic and aqueous phases. Kosmotropy, salting out, co-solvent precipitation, isoionic precipitation, osmolytic electrostatic forces, conformation tightening and protein hydration shifts, all contribute to the protein precipitation at the interface [9,10]. In many cases, TPP enhances the activity of various enzymes, resulting in an apparent higher yield in excess of 100% [11]. In addition to the enhanced concentration, purification with TPP was found to be comparable to chromatographic techniques. For example, Saxena et al. have reported 20.1-fold purification of wheat germ

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protease/amylase bifunctional inhibitor using TPP [12]. In comparison, a combination of fractional ammonium sulphate precipitation, affinity, ion-exchange and gel filtration chromatography yielded a maximum 22-fold purification [13]. Thus, the novelty of TPP lies in its ability to concentrate proteins from crude broths with higher purification than conventional concentration methods. The scalability, rapid recovery and requirement of only minimal pre-treatment are some of the additional advantages of TPP. However, no report on nattokinase separation using TPP is available in the open literature. In the proposed study, the influence of various parameters such as pH, temperature, salt concentration, crude to t-butanol ratio on purification of nattokinase has been studied. In the second part, nattokinase was freeze dried to retain its activity over a long time.

In order to preserve the native structure and provide long-term stability and biological activity, various methods have been proposed to mitigate or prevent protein denaturation induced by freezing and drying [14–16]. One of the most widely used strategy is the use of stabilizing additives during the lyophilization process. These stabilizers include sugars, polyols, polymers, amino acids and surfactants. The possible stabilization mechanisms by using these additives have been deeply discussed in detail in the above mentioned studies and other publications [17–19]. For many proteins, the combination of cryo- and lyo-protectants confers suitable chemical and physical stability on freeze-dried molecules.

Although, a vast majority of currently marketed pharmaceutical and biological products are freeze-dried from simple aqueous solutions, many mineral and organic solvents have been shown to possess similar properties to frozen water and can sublime under reduced pressures, and so could be used in freeze-drying [20]. The potential advantages and disadvantages of the use of these organic or organic/water co-solvent systems in lyophilisation have been fully summarized elsewhere [21]. In the promising field of freeze-drying using non-aqueous co-solvent systems, tert-butyl alcohol (TBA)/water systems have been thoroughly evaluated, and used in the manufacture of a marketed pharmaceutical product to be administered by injection [22]. The reason behind the selection of TBA/water co-solvent system is mainly due to its superior physical properties by means of higher vapour pressure, low toxicity and markedly high solubilisation capacity. In addition, TBA can freeze completely in most commercial freeze-dryers, readily accelerating the mass transfer during the primary and secondary drying stages of lyophilisation and thereby increasing the sublimation and desorption rates respectively and decreasing the drying time [23,24]. However, until recently almost all studies published using TBA/water co-solvent systems in freeze-drying were performed on small molecular weight drugs which need not maintain a unique three-dimensional conformation for their biological activity and pharmaceutical functionality [25–27]. The protein lyophilisation in the presence of co-solvent system has hardly been covered in the literature and therefore, the present study was undertaken to gain valuable insight into protein lyophilisation [28].

It is well known that proteins adopt particular secondary and tertiary structures which are extremely closely related to their biological activity. The non-native structures or partly unfolded states often result in protein aggregation, precipitation or accelerated chemical degradation, leading to short shelf life or harmful immunity reactions [18,29]. In the past two decades, these issues have been addressed during the process of formulation, lyophilization and stability for long-term storage [30–33]. In the proposed study, nattokinase was lyophilized in a TBA/water co-solvent system. Trehalose, which is an excellent lyo-protectant agent with a clear stabilizing mechanism of hydrogen bond substitution during drying, was used as a stabilizer [15,19,34]. In the proposed study, effect of co-solvent and excipients on freeze drying, drying time of nattokinase has been studied.

2. Materials and methods

2.1. Chemicals

Chromogenic substrate N-Succnyl-Ala-Ala-Pro-Phe-p-nitroanilide (S-7388), from Sigma Aldrich, CA, USA, 96 Well Clear Polystyrene Microplates from Tarsons were purchased. Ammonium sulphates, sodium chloride, sodium hydroxide, sodium hydrogen phosphate, Tris, sodium dihydrogen phosphate were purchased from HiMedia Limited, Mumbai. Agro wastes such as potato peel, wheat bran were purchased from local markets of Mumbai.

2.2. Microorganism

Standard culture of *Bacillus natto* (NRRL B-3666) for nattokinase production was procured from United States Department of Agriculture (NRRL), Peoria, Illinois, USA.

2.3. Analytical methods

2.3.1. Estimation of amidolytic activity and protein content

The enzyme activity was analysed by chromogenic method [35,36]. Synthetic substrate (S-7388), N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) which is specific for fibrinolytic enzymes nattokinase and subtilisin was used to determine the enzyme activity [6,37]. The activity was colorimetrically estimated using synthetic substrate as previously described by Mahajan et al. [38]. The reaction was carried out in 96 Well Clear Polystyrene Microplate. The mixture containing 16 μL of 3 mM synthetic substrate, 80 μL of 25 mM Tris– HCl buffer (pH 8.0) and 12 μL of enzyme solution was incubated at 37 °C for 10 min. Prior to the addition of substrate, the reaction mixture was pre-incubated at 37 °C for 2 min. The reaction was then stopped by adding 50 μL of 0.2 M glacial acetic acid. The absorbance of released pNA was measured at 405 nm on a MIQ-X200 μQuant Elisa Plate reader. One unit of amidolytic activity was expressed as nmol of p-nitroaniline released due to substrate hydrolysis per min per ml by the enzyme. The experiments were carried out in triplicate and the average value of amidolytic activity was taken as a representative value.

The protein content was estimated by the Folin–Lowry method [39] using bovine serum albumin as the standard protein. The sample (0.1 ml) was added to 1 ml of reagent C. After 10 min, 0.1 ml of Folin reagent (diluted 1:1 with deionized water) was added and mixed. After 30 min, 3.8 ml of distilled water was added and the absorbance was measured at 660 nm. Reagent C was prepared by adding reagent A (2% Na_2CO_3 in 0.1 N NaOH) and reagent B (50 mg CuSO4 and 100 mg potassium sodium tartrate in 10 ml) in a ratio of 50:1. The percentage yield, fold purification were calculated as follows:

$$\%\ \text{Yield} = \frac{\text{Total activity in purified sample} * 100}{\text{Total initial activity}}$$

$$\text{Fold purification} = \frac{\text{Specific activity of purified sample}\left(\frac{U}{\text{mg protein}}\right)}{\text{Specific activity of initial sample}\left(\frac{U}{\text{mg protein}}\right)}$$

2.4. Production condition of nattokinase

Nattokinase was produced from agro waste based media by *B. natto* NRRL 3666. For nattoknase production, the culture was grown at 37 °C at 180 rpm in 250 ml Erlenmeyer flask containing 50 ml of agro waste based media. The agro waste based media consist of 4% (w/v) Potato peel, 3% (w/v) wheat bran, 1 g/l KH_2PO_4,

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