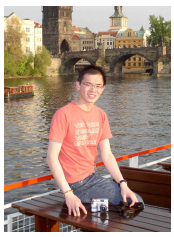
Novel Encapsulation of a Fibrinolytic Enzyme (Nattokinase) by Shellac



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Introduction

Natto is a traditional Japanese food made from fermented soybeans. For centuries, it has been used both as a staple food and a folk remedy for heart and vascular diseases, fatigue and beriberi. However, it was not until the early 1980s that one of the key properties of natto was discovered by Sumi et al. (1987). They found that Nattokinase (NKCP) is a fibrinolytic enzyme, which has similar properties to a subtilisin-like serine protease. It is known to be able to cure and also prevent deep vein thrombosis (DVT) that can lead to serious health problem. However, the activity of enzyme is sensitive to temperature and pH of its environment. It has been reported that the activity of enzyme was unstable below pH 5.0 (Sumi et al., 1987). Commercial NKCP in the form of water-soluble powders may lose its activity via oral administration to the human gastric intestinal tract. In order to stabilise it, direct exposure of the enzyme to the gastric fluid should be avoided.

Enzyme or other active ingredient which is susceptible to acidic medium may be protected by encapsulating them with an enteric material, e.g. shellac. This is normally realised by entrapment of the active ingredient in a solid carrier, followed by fluidised bed coating, as used in pharmaceutical industry (Chang et al., 1990). Shellac is a natural, biodegradable and renewable resin of insect origin (*Kerria lacca*). It has advantages of low water permeability. The aim of this work is to encapsulate Nattokinase using shellac based on a simple extrusion method. The principle of this method is to mix NKCP with aqueous shellac solution, which is dropped to a cross-linking solution of calcium chloride ($CaCl_2$) to form solid shellac particles with the enzyme embedded. The effects of formulation and processing conditions on loading and encapsulation efficiencies of NKCP and its stability in the encapsulation process and simulated gastric fluid were investigated.

Materials and Methods

The schematic diagram of preparing shellac particles with NKCP entrapped is shown in Figure 1. 6g of NKCP powders (Daiwa Pharmaceutical Co Ltd, Japan) were dissolved in 60ml of aqueous shellac solution (Marcoat 125, SYNTAPHARM Harke Group, Germany). The cross-linking solution was prepared by dissolving various amount of calcium chloride into 200ml of solution of ethanol and distilled water in the ratio of 3:1 (v/v). A stabilising medium with 1% (w/v) of calcium chloride and 0.5% (w/v) of NKCP in distilled water was also prepared in order to further strengthen the shellac particles formed. Table 1 shows the formulations investigated in this work.

Entrapment of NKCP in shellac beads

The aqueous solution of shellac with dissolved NKCP was pumped to the cross-linking using a Nisco Encapsulation unit Var J1, SPA-00195, with a coaxial air stream (1.2 L/min), which has a nozzle with internal diameter of 580μm. A peristaltic pump was used to deliver the NKCP-shellac solution to the nozzle, and the flow rate through it was set at 1.2 ml/min. The tip of this nozzle was cut and polished to have a smooth flat end. The distance between the nozzle and surface of cross-linking solution was set at 4 cm in order to produce spherical droplets. The length of the nozzle was kept at 0.5 cm so that all the droplets were produced under similar experimental condition. The beads formed were transferred to the stabilizing solution every 5 minutes. After all the beads were

transferred to the stabilizing solution, they were left to stay for another 15 minutes before being rinsed three times with distilled water. They were then dried in an oven (37^{o}C) overnight.

Measurement of loading and encapsulation efficiencies

A known amount of the formed beads were crushed using pestle and mortar manually until its size was approximately 8.9 ± 0.2 µm measured by an image analyser (Leica Microsystems) and then stirred in 10 ml of distilled water with a magnetic stirrer for 2 hours. The solution was centrifuged followed by filtration using 0.2 µm filter paper. The amount of enzyme encapsulated was detected using BCA assay at the wavelength of 540nm (SLT Spectra, Austria) using NKCP as a reference protein. The loading and encapsulation efficiencies were defined by the following equations respectively

$$Loading\ efficiency = \frac{\text{Actual NKCP content}}{\text{Amount of beads produced}} \times 100\% \quad \text{----------} \quad \textbf{(1)}$$

$$Encapsulation\ efficiency = \frac{\text{Actual NKCP content}}{\text{Actual NKCP used}} \times 100\% \quad \text{----------} \quad \textbf{(2)}$$

Determination of enzyme activity

NKCP activity was determined by an amidolytic method (Fujita, Nomura et al., 1993). The assay started with the incubation of the mixture of 0.1ml Tris buffer (0.5M), pH7.4, containing 17.5mM NaCl with 0.1 ml enzyme solution at 37 °C for 1 minute. Soon after, 0.1ml substrate S-2251 (4mM, CHROMOGENIX) was added to the mixture, which was then incubated in a water bath at 37 °C for 10 minutes. The reaction between NKCP and substrate S-2251 was stopped by adding 1ml citric acid solution (2% w/v) acting as a stopping reagent. Absorbance of the sample was then measured at 405nm wavelength using a spectrophotometer (Cecil CE1020, UK) to quantify the liberation of p-nitroaniline. Blank solution for zero adjustment was made using the same procedure as stated above, but distilled water was used instead of the substrate solution.

Results and Discussion

It was observed that when the shellac solution with NKCP was dropped to $CaCl_2$ solution, solid particles formed almost instantaneously. It is not very clear what the real mechanism of the particle formation is. It had been reported that solutions of the ammonium or alkali salt of some polysaccharides containing carboxyl groups gelatinize with the presence of divalent or trivalent ions (Schweiger, 1962). It is speculated that calcium ions might replace ammonium ions available in the shellac solution to form a cross-linked infinite network of polymer chains by forming either intermolecular or intramolecular linkages until a complete bead was formed. However, since calcium ion has a higher valence number than ammonium ion, the concentration of calcium ion present in the solution should be adequately high for the ion exchange to occur. Therefore the effect of $CaCl_2$ concentration on the loading and encapsulation efficiencies of NKCP and its stability in the encapsulation process and simulated gastric fluid was investigated.

Loading and Encapsulation efficiencies

The loading efficiency and encapsulation efficiency were independent of the $CaCl_2$ concentration (Figure 2). However, it was observed that when the concentration of $CaCl_2$ was equal or lower than

3% (w/v), the beads formed in the cross-linking solution were unstable and tended to stick to each other. The cross-linking solution contained ethanol, which was used to adjust the density of the cross-linking solution in order for the NKCP-shellac droplets to penetrate into the cross-linking solution quickly. There might be a chemical reaction between calcium ion and hydroxyl ion from the ethanol since hydroxyl ion is considered as a strong nucleophile. This might lead to insufficient calcium ion in the solution and hence undesired sharing of calcium ion between interparticles could cause their aggregation. Nevertheless, when the concentration of $CaCl_2$ was greater than 3% (w/v), the loading and encapsulation efficiencies were 24.3 ± 2.2% and 87.2 ± 7.9% respectively.

Activity of enzyme after encapsulation

Figure 3 shows the activity of enzyme after the encapsulation process and being exposed to the simulated gastric fluid (SGF) for 2 hours respectively. As can be seen, they all depended on the concentration of $CaCl_2$ in the cross-linking solution. The activity of enzyme after the encapsulation process decreased with $CaCl_2$ concentration initially, and leveled off at a concentration of 3% (w/v). Correspondingly, the activity of enzyme in SGF increased with $CaCl_2$ concentration, and then reached a plateau. This indicates when $CaCl_2$ concentration was greater than 3% (w/v), there was a loss of enzyme activity by approximately 40%, but there was no significant loss of enzyme activity when the shellac beads were exposed to the SGF for 2 hours. It should be mentioned that the loss of the enzyme activity should be partially attributed to the grinding of shellac particles with the enzyme entrapped since a control experiment also showed that the pure enzyme in powders lost its activity by approximately 20% after they were ground using pestle and mortar, which might distort the molecular structure of the enzyme and denature it. It is speculated that high localized temperature might have been generated and cause the denaturation. Previous work demonstrated that thermo inactivation of enzymes was induced by polymolecular aggregation and monomolecular processes, leading to insolubilisation (Tim and Mark, 1992). This is also in line with an observation of unclear solution when crushed NKCP powders were dissolved in distilled water. Irreversible enzyme inactivation could also result from the shearing condition created during the grinding process, which subsequently provoked its conformation changes (Charm and Wong, 1981).

Further losses of enzyme activity by up to approximately 20% could be due to the detrimental effect of calcium chloride in the cross-linking solution. The elevation in Ca^{2+} ion concentration might lead to dissociation of protein and the loss of activity is likely induced by conformational change of protein structure. However, when the concentration of $CaCl_2$ used in the cross-linking solution was equal or higher than 3% (w/v), the high cross-linking agent concentration is believed to induce a rapid formation of polymer films at the surfaces of beads, thus slowing down the diffusion rate of further calcium ion into films and preventing further direct contact between calcium ion and the enzyme.

Conclusions

NKCP was encapsulated successfully using shellac by a simple extrusion process, and the encapsulation efficiency was 87.2 ± 7.9 %, which was independent of $CaCl_2$ concentration in the cross-linking solution. However, the activity of enzyme decreased with $CaCl_2$ concentration. It was also demonstrated that denaturation of enzyme was partially caused by the grinding process which might distort the molecular structure of the enzyme. Overall, approximately 60% of enzyme activity was maintained after the encapsulation process. There was no significant loss of the enzyme activity when the concentration of $CaCl_2$ in the cross-linking solution was greater than 3% (w/v), which indicates that shellac particles had a very low permeability to acid. Future work includes characterization of the properties of shellac particles and determination of the release rate of the enzyme when the shellac particles are exposed to simulated intestinal fluids.

References

Chang,R.K., Iturrioz,G., Luo,C.W., 1990. Preparation and evaluation of shellac pseudolatex as an aqueous enteric coating system for pellets. International Journal of Pharmaceutics, 60, 171-173.

Charm,S.E., Wong,B.L., 1981. Shear effects on enzymes. Enzyme and Microbial Technology, 3, 111-118.

Fujita,M., Nomura,K., Hong,K., Ito,Y., Asada,A., Nishimuro,S., 1993b. Purification and Characterization of a Strong Fibrinolytic Enzyme (Nattokinase) in the Vegetable Cheese Natto, a Popular Soybean Fermented Food in Japan. Biochemical and Biophysical Research Communications, 197, 1340-1347.

Kaya,F., Heitmann,J., Joyce,T.W., 1994. Cellulase binding to cellulose fibers in high shear fields. Journal of Biotechnology, 36, 1-10.

Schweiger,R.G., 1962. Acetylation of Alginic Acid. II. Reaction of Algin Acetates with Calcium and Other Divalent Ions. Journal of Organic Chemistry, 27, 1789-1791.

Sumi,H., Hamada,H., Tsushima,H., Mihara,H., Muraki,H., 1987. A Novel Fibrinolytic Enzyme (Nattokinase) in the Vegetable Cheese Natto - A Typical and Popular Soybean Food in the Japanese Diet. Experientia, 43, 1110-1111.

Tim,J.A., Mark,C.M., 1992. Stability of Protein Pharmaceuticals Part A Chemical and Physical Pathways of Protein Degradation. Plenum Press New York and London.

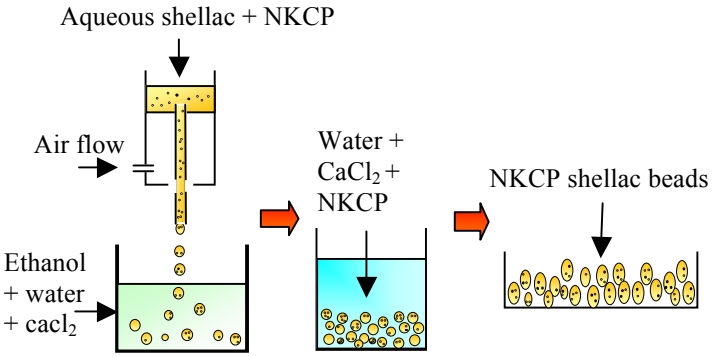

Figure 0: Schematic diagram to illustrate preparation of Shellac beads with an extrusion method.

Formulations	$CaCl_2$ in dispersed solution (%)
F1	1
F2	3
F3	5
F4	10
F5	15

Table 1: Formulations used for the formation of shellac beads

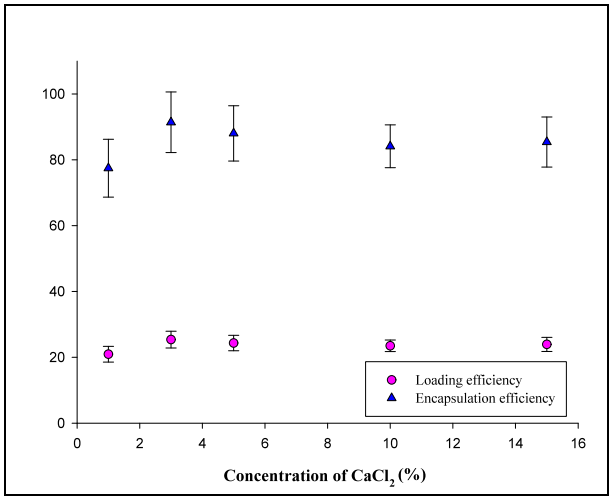

Figure 2: Effect of concentration of $CaCl_2$ in the cross-linking solution on loading and encapsulation efficiencies of NKCP in the shellac beads.

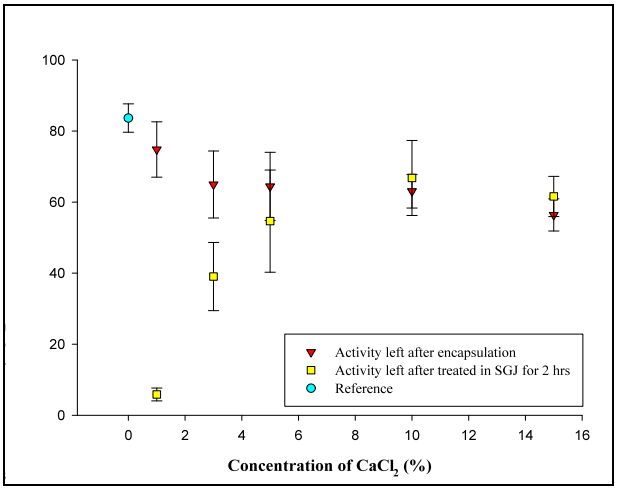

Figure 3: Effect of the concentration of $CaCl_2$ in the cross-linking solution on the activity of NKCP in shellac beads. Reference point indicates the activity of pure NKCP left after the grinding process.