MICRO/NANOENCAPSULATION OF PROTEINS WITHIN ALGINATE/CHITOSAN MATRIX BY SPRAY DRYING

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ABSTRACT

Currently, therapeutic proteins and peptides are delivered subcutaneously, as they are readily denatured in the acidic, protease rich environment of the stomach or gastrointestinal track and low bioavailability results from poor intestinal absorption through the paracellular route. Encapsulation of therapeutic peptides and proteins into polymeric micro- and nano- particle systems has been proposed as a possible strategy to overcome limitations to oral protein administration. Furthermore, it was shown that nanoparticles having diameters less than 5μm are able to be taken up by the M cells of Peyer's patches found in intestinal mucosa . However, the current methodologies to produce particles within desired range involves organic solvents and several steps. In this study, spray drying was investigated as a microencapsulation alternative, as it offers the potential for single step operation, producing dry particles, with the potential for extending the microparticle size into the nano-range. The particles were produced by spray drying of alginate/protein solutions. The effect of spray drying operational parameters on particle properties such as recovery, residual activity and particle size was studied. Particle recovery depended on the inlet temperature of the drying air, whereas the particle size was affected by the feed rate and the alginate concentration of the feed solution. Increase in alginate:protein ratio increased protein stability during the process and shelf live experiments. Presence of 0.2 g trehalose/g particle increased the residual activity up to 90%. The resulting spherical micro and nanoparticles had smooth surfaces. Stable glycol-chitosan-ca-alginate particles were produced with single step operation. The resulting particles had mean diameter around 3.5μm and released 35% of the initial protein content to the simulated stomach environment within 2 hours. The protein

distribution within the particle was studied by confocal laser scanning microscope with florescent labeled protein. The image showed protein deposition toward the surface of the particles. Total drying time and Peclet number was calculated for the particles and found to be 8.5 ms and 240, which indicates that particle formation was governed mainly by convection, which resulted in a hollow central region and protein distribution toward the particle surface. This study shows that stable alginate particles containing proteins can be produced in a single step by spray drying, where the particles had a mean size lower than the critical diameter necessary to be orally absorbed by M cell's of the Peyer's patches in the gastrointestinal tract and thus can be considered as a promising technology for oral peptide and protein delivery.

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NOMENCLATURE

η	Viscosity of the droplet, cp
D	Diffusion rate of BSA molecule within droplet, μm^2/ s
D_{av}	Average droplet diameter, μm
D_d	Initial droplet diameter during constant rate period, μm
D_h	Diameter of the hollow region in the particle
D_p	Droplet and particle diameter during falling rate period, μm
k_d	Thermal conductivity of the water vapor in the stagnant layer around the droplet, kcal/m h ^oC
k_d a	Thermal conductivity of the air in the stagnant layer around the particle , kcal/m h ^oC
k_B	Boltzman constant, $1.3806503 \times 10^{-23}$ m^2 kg / s^2 K
P	Pressure of the atomizing air, psi
Pe	Peclet number, dimensionless
Q_{aa}	Atomizing air feed rate, L/h
Q_{da}	Drying air flow rate, m^3/h
Q_{lf}	Feed solution flow rate, mL/min
R_H	Hydrodynamic radius of BSA molecule, nm
R	Radius of the droplet, μm
t_{cr}	Drying time during critical period, ms
t_d	Drying time of constant plus falling rate,ms
T_{outlet}	Outlet temperature of the drying air, ^oC
T_{inlet}	Inlet temperature of the drying air, ^oC
T_{wb}^{inlet}	Wet bulb temperature of the drying inlet air, ^oC
T_{wb}^{outlet}	Wet bulb temperature of the drying outlet air, ^oC
ΔW_{cr}	Total mass loss during constant rate period, kg
ΔW_{fr}	Total mass loss during falling rate period, kg
W_{in}	Initial weight of the droplet, kg
W_p	Weight of the particle during falling rate, kg
λ	Latent heat of vaporization of water, kcal/kg
ρ_a	Bulk density of alginate, kg/ m^3
ρ_s	Average density of the dry particle, kg/ m^3

CHAPTER 1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Protein Microencapsulation

Microencapsulation is a process, where active biologicals such as enzymes, cells or therapeutics such as antibiotics or vitamins, are entrapped within a semi-permeable matrix. The resulting capsules or particles generally range from micrometers to millimeters in size (Thies,2005). Applications of microencapsulation include controlled release of the active components, particle coating, flavor stabilization, taste masking, physical/chemical stabilization, improvement of shelf life and prevention of exposure of the active material to the surroundings (Benita, 1996). Many microencapsulation techniques have provided important innovations to the pharmaceutical, agricultural, cosmetics, medical, biotechnology, food, paper and textile industries.

A number of microencapsulation strategies have been described in the literature, which result in wet suspensions of microparticles, and often involve several processing steps including the use of toxic solvents. The goal of the present study was to examine spray drying as a microencapsulation alternative, as it offers the potential for single step operation, producing dry particles, with the potential for extending the microparticle size into the nano-range. Nanoparticles are becoming increasingly important in the pharmaceutical field, such as toward the oral dosage of peptide or protein based therapeutics. A variety of model proteins were nano/microencapsulated in the present investigation, using alginate polysaccharide as a biodegradable matrix material.

1.2 Microencapsulation of Proteins by Spray Drying

Currently, therapeutic proteins and peptides are delivered subcutaneously, as they are readily denatured in the acidic, protease rich environment of the stomach or gastrointestinal track and low bioavailability results from poor intestinal absorption through the paracellular route. Encapsulation of therapeutic peptides and proteins into polymeric micro- and nano- particle systems has been proposed as a possible strategy to overcome limitations to oral protein administration (Reis *et al.*, 2006). Furthermore, it was shown that nanoparticles having diameters less than 5μm are able to be taken up by the M cells of Peyer's patches found in intestinal mucosa (Hussein *et al*., 2001).

Several formulation techniques have been investigated previously to produce nano particles (<5μm), including nanoemulsion dispersion (Reis *et al.*, 2004), ionotropic pre-gelation (Sarmento *et al.*, 2006) and spray drying (Coppi *et al.*, 2001). Although, the desired particle sizes were achieved through nanoemulsion-dispersion and ionotropic pre-gelation techniques, they have the drawback of requiring organic solvents and multiple steps. However spray drying is a single step process, which can be operated continuously. Spray drying utilizes heat from a hot gas stream to evaporate micro-dispersed droplets created by atomization of continuous polymer/protein feed, thus the encapsulation of the desired drug is achieved.

During the spray drying process, proteins can unfold due to dehydration stress (Carpenter and Manning, 2002) although the droplets reach only the relatively low wet bulb temperature by high rates of moisture evaporation (Broadhead *et al*., 1992). This protein stability problem is prevented by using an additive such as sucrose, trehalose, dextran or maltodextrin, which remain in the amorphous phase with the protein and/or

hydrogen bond to the protein in the place of water during drying, such as is the case with sucrose or trehalose (DePaz *et al.,* 2002). In addition to dehydration, there are also stresses that lead to protein denaturation, such as shear in the spray nozzle, and protein adsorption at the water/air interface (Adler and Lee, 1998). However, shearing stress occurring during pumping, flow and atomization, do not appear to cause major damage to proteins. For example, Maa and Hsu (1997) studied the high shear and adsorption to the air/liquid interface. For recombinant human growth hormone (rhGH), shearing stress occurring during pumping, flow and atomization, did not appear to cause major damage. It was concluded that protein adsorption at the air/liquid interface is the primary cause of the observed denaturation.

Spray drying was studied previously to produce protein loaded polymer particles with diameters ranging from nanometers to several microns. In this context, several polymeric matrices have been studied such as poly (D-lactide) (Tanaka, 1994), poly(lactide-co-glycolide) (Blanco *et al.*, 2005, Wang and Wang, 2003, Pavenetto *et al.*,1993, Wageneer,1994), and poly (ε-caprolactone) (Blanco *et al*.,2003). However these polymers all require organic solvents such as dichloromethane to formulate the spray drying feed solution. Usage of hydrogels such as alginate as an encapsulation matrix is favorable, since they form aqueous solutions, are biocompatible, highly inert toward protein drugs and are bioadhesive, increasing drug residence time at the site of intestinal absorption (Gombotz and Wee, 1998, Tønnesen and Karlsen, 2002). Alginates are known to sustain release due to gelation with cations, such as Ca^{++} (Kim and Lee, 1992). However, the cation crosslinked alginate network can degrade through removal of calcium ions with chelators such as, citrate, lactate and phosphate, however these ions

generally do not appear in human intestinal fluid (Bhagat *et al*., 1994). Alginates form strong complexes with polycations such as chitosan (Gombotz and Wee, 1998) and glycol chitosan (Sakai *et al*., 2000) and these complexes do not dissolve in the presence of Ca^{++} chelators and can be used to stabilize the gel and reduce its porosity.

Previously, spray drying was investigated to produce alginate based particles. Takeuchi *et al*., (1998, 2000) investigated the properties of lactose-chitosan-alginate composite particles produced by rotary atomizer for direct tabletting purposes. Coppi *et al*., (2001,2002,2004) studied production of alginate microparticles for oral drug delivery purposes, where BSA, L-lactade hydrogenase and a peptide antibiotic, polymixcin, were used as model systems. In addition, several researchers studied spray dried alginate particle systems focusing on production of particles with specific applications, such as encapsulation of volatile materials (Rosenberg, 1990), and immobilization of cells (Begin *et al.*, 1990).

However, there have been no studies on alginate as spray dried encapsulation matrix, involving the effects of operational parameters on particle properties, such as size, morphology, residual activity and particle recovery, particularly for production of nano-particles where bioactive biologicals, such as enzymes are encapsulated. These operating parameters include formulation, concentration and feed rate of the feed solution, and inlet temperature of the drying air. Moreover, the proposed methodology of Coppi *et al*. (2001,2002,2004) to produce alginate particles for oral protein delivery purposes, involved spray drying of an alginate/protein solution forming particles which were subsequently subjected to several additional steps to enhance the properties, such as physical crosslinking of the particles in $CaCl_2$ aqueous solution and surface treatment of

the particles with chitosan to reinforce the alginate network and to improve adsorption across the intestinal epithelia. Moreover, particles were subjected to a second and final drying step involving freeze drying to remove water and to recover the particles. These multiple steps can alter some of the properties of the particles. For example, the entrapped drug can be released to the aqueous medium during the gellation step, or pH sensitive proteins can be affected during surface treatment of the particles due to the solubility requirements of chitosan (pH<5.5). Moreover, lypholization causes additional stress on particles and proteins, such as changing the particle morphology and denaturation of the proteins due to dehydration (Wang, 2000).

In the present study, alginate and alginate-sugar (trehalose) formulations were investigated using the protease subtilisin as a model protein, in terms of how they can affect the residual activity, since it is important in spray drying to determine the residual activity, when heat sensitive materials, such as proteins, are needed to be encapsulated. Subtilisin was selected due to it's simple activity assay. The effect of inlet temperature, feed rate, and protein:polymer and protein:polymer:disaccharide ratio, on the properties of the resulting particles, including mean diameter, residual activity, storage stability, moisture content, and product recovery was studied. Moreover, an alternative single encapsulation step procedure was proposed for oral administration of proteins. The present study, involved introduction of dilute Ca^{++} ions to a dilute alginate feed solution, along with the protein and glycol-chitosan, where physical crosslinking of the alginate and polyion complex formation takes place forming sprayable low viscosity gel. The resulting particles were investigated, for size, protein content and protein release kinetics. Two other model protein systems (lysozyme, and bovine serum albumin). were also used

along with subtilisin, since it's been known that the properties of the proteins, such as molecular weight and pI, can play an important role during their release from alginate matrices (Gombotz and Wee, 1998) . The distribution of the model protein throughout the polymer matrix was examined by using confocal laser scanning microscopy with florescent labeled protein.

1.3 Spray Drying

The first detailed description of drying of a liquid system through a spray and hot gas system appears in an 1872 patent. However spray drying started to be widely used in the dairy and detergent industries in the 1920's. Currently, it is used in many industries such as pharmaceutical, food, electronics, chemical and cosmetics. Antibiotics (such as penicillin), vitamins (such as ascorbic acid and vitamin B12) and enzymes (such as amylase, protease, lipase and trypsin) are some of the materials spray dried in the pharmaceutical industry (Çelik and Wendel, 2006). In spray drying, a liquid slurry or low viscosity paste is converted into a free flowing powder in one unit operation. Figure 1.1 shows a general schematic of the spray-drying process. The liquid feed is pumped through a nozzle, where it is dispersed into fine droplets. In the drying chamber, hot air promotes simultaneous mass and heat transfer forming dry particles. The resulting particles are separated into a collection vessel. A bag filter system is used to prevent the fine particles from escaping to the atmosphere.

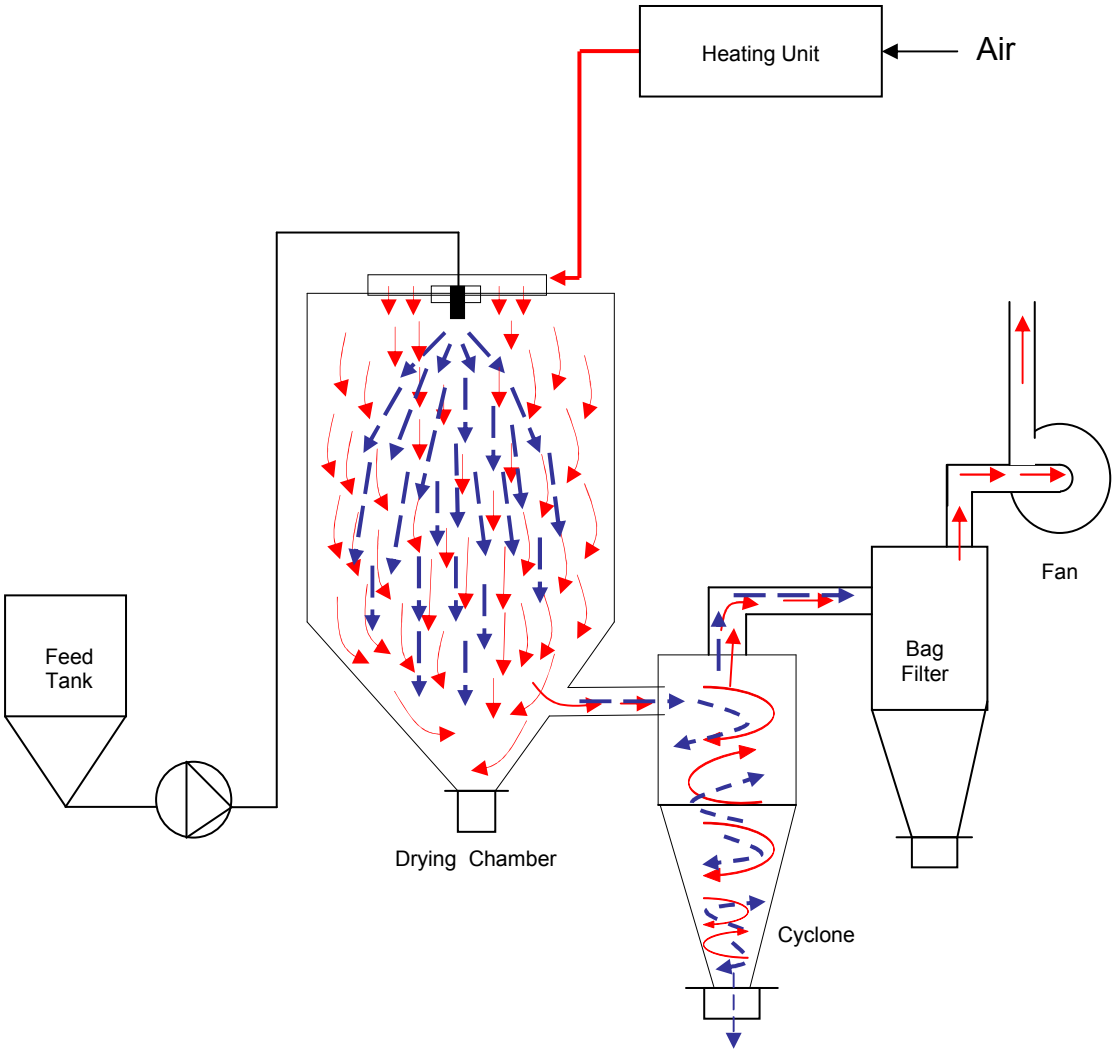

Figure 1.1 . Schematic illustration of a co-current spray dryer, where the gas and the feed are flowing in the same direction. Red arrows represent heated air, whereas blue arrows represent liquid droplets forming particles.

Spray drying is now used in many industries for several reasons. It can be operated as a batch or continuous process and may be operated for months without interruption (Masters,1991). In addition, the physical properties of the resulting product, such as particle size and shape and moisture content can be controlled through equipment configuration and manipulation of the process variables. Also most evaporation takes place in milliseconds to a few seconds, well suited for heat-sensitive products, such as proteins and enzymes.

1.4 Spray Drying Stages

The spray drying process consists of three fundamental stages. The first stage is atomization of liquid feed into fine droplets. In the second stage, spray droplets encounter the heated gas stream and evaporation of the liquid from the droplet occurs, resulting in the final dried particles. The final stage involves separation of the dried powder from the gas stream and recovery of the particles in the collection vessel.

1.4.1 Atomization

The atomization stage produces a spray of droplets having a high surface to mass ratio. There are several atomization systems available which may be classified according to the nozzle design. Examples include rotary atomization, pressure atomization or two-fluid (pneumatic) atomization. The selection of the nozzle type will affect droplet size and subsequent particle size distribution as illustrated in Figure 1.2 (Masters, 1991). In rotary atomization, the feed fluid is centrifugally accelerated to high velocity before being discharged into the drying air atmosphere, which creates a spray of droplets. Spinning wheels are used in larger-scale spray drying equipment (Masters, 1991). In pressure atomization the fluid is fed to the nozzle under pressure which causes the fluid to be dispersed into droplets as it leaves the nozzle. The formation of droplets occurs by conversion of pressure energy within the liquid feed into kinetic energy of thin moving liquid sheets. The sheets break up under the influence of the physical properties and by the frictional effects with the air (Masters, 1991).

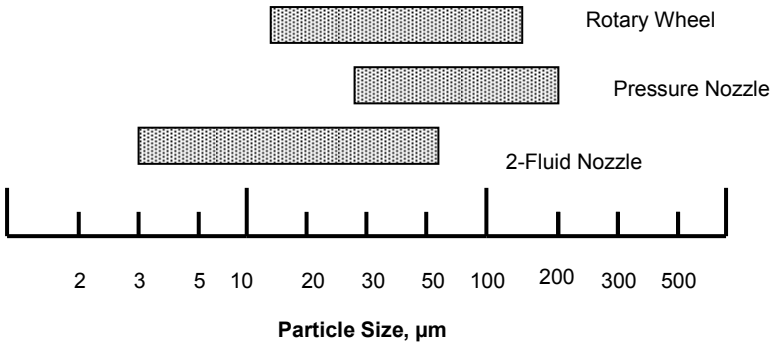

Figure 1.2 The particle size ranges produced by different nozzle systems. Figure is adapted from Çelik and Wendel (2006).

The formation of droplets is influenced by the physical properties of the liquid and by frictional effects with the air. In the two fluid nozzle, the liquid feed is transported to the nozzle at a low flow rate where it encounters a high velocity gas stream (Masters, 1991). The mixing of the feed and the gas stream causes the feed to break up into fine droplets. Two fluid nozzles are generally used in laboratory-scale and small to medium sized pilot plant spray-dryers. The droplet size is also influenced by the surface tension and the viscosity of the liquid feed, and most importantly by the fluid velocity at the nozzle orifice and by the air/liquid mass flow ratio (Masters, 1991).

1.4.2 Spray-Air Contact

Immediately after atomization, droplets encounter the heated gas stream, which is usually air. The evaporation of water from the spray involves simultaneous heat and mass transfer. On contact between the atomized droplets and the drying air, heat is transferred by convection from the air to the droplets and consumed as latent heat during moisture evaporation. The variables that affect the drying of the droplets in the drying chamber of a laboratory-scale dryer are inlet temperature of the drying air (T_{inlet}) and the outlet temperature (T_{outlet}), together with the relative humidity and the flow rate of the drying air. The drying of a droplet to form a particle is considered as a three-step process as illustrated in Figure 1.3. In the initial rate period, the drying rate increases until the surface temperature of the droplet reaches the wet bulb temperature of the drying air, $T_{wb,}$ which corresponds to 100% relative humidity. In the constant rate period, the drying rate is constant, where the surface temperature of the droplet remains constant due to the continuous evaporation of the solvent. In the falling rate period, the drying rate decreases and crust formation occurs. During this third step, a high surface evaporation rate can lead to formation of a dry crust surrounding the droplet (Masters, 1991). The crust may collapse during further drying, resulting in particles with a deformed shape.

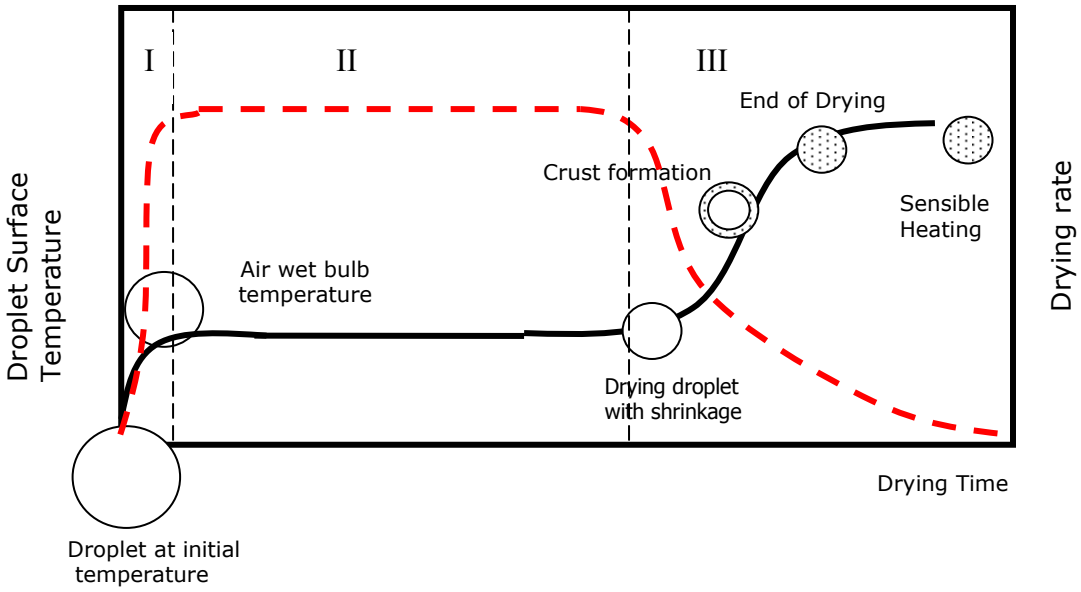

Figure 1.3 Schematic illustration of droplet surface temperature and of the crust formation of the particles. I, II and III represents initial, constant and falling rate periods. The figure is adapted from Çelik and Wendel (2006). Black lines represent the surface temperature of the droplets vs time, whereas red dot lines represent the drying rate of the particles vs time.

The drying rate of a droplet during the constant rate period is dependent on the difference (ΔT) between the air temperature and the temperature at the droplet surface, $T_S = T_{wb}$. A decrease in inlet temperature and hence also in outlet temperature, T_{out}, results in a lower ΔT, and therefore in a lower drying rate (Maa and Hsu, 1997). T_{inlet} is considered as an independent variable, whereas the corresponding T_{out} is determined by T_{inlet} and also the drying air flow rate and the liquid flow rate, Q_{lf}. T_{out} is the dominating temperature within the drying chamber since the high drying rate during the constant rate period consumes much heat energy and causes the air temperature to fall rapidly on contacting the droplets. In the second drying period, the drying rate continuously decreases as illustrated in Figure 1.3 III, since the formation of an outer shell occurs at the surface (Masters, 1991). The evaporation rate and the properties of the shell material

particles can influence the morphology of the particles, such as fast drying can deform the particles and slow drying can leave the material wet and sticky (Çelik and Wendel ,2006).

1.4.3 Separation of the Dried Product

Particles are separated from the air stream by cyclonic air flow in a conical chamber base, or by the ability of the particles to fall out of the air flow to a flat chamber base. Irregardless of the type of separation used, some form of collection equipment is required after the drying chamber. Collection equipment can be dry or wet cyclone bags, bag filters, scrubbers or electrostatic precipitators. Cyclone separators have tangential air entry, where the gas-particle stream enters a cyclindrical and conical chamber. The downward spiraling motion results in powder collection in the bottom vessel, although some powders tend to persist on the cyclone wall. The air leaves through a central opening at the top (Masters, 1991).

1.5 Alginate

Alginate is one of the most widely used and studied polymers for encapsulation, since it is biochemically inert and gels under mild conditions. Alginate is a naturally occurring polysaccharide, produced commercially from algae or bacteria. It forms hydrogels in the presence of multivalent cations. Alginate consists of linear polysaccharide copolymer chains of 1-4 linked β-D mannuronic acid (M) and α-L guluronic acid (G) of widely varying composition and sequence (Gombotz and Wee, 1998). The monomers are arranged mainly into three types of blocks: G, M and MG blocks as shown in figure 1.4.

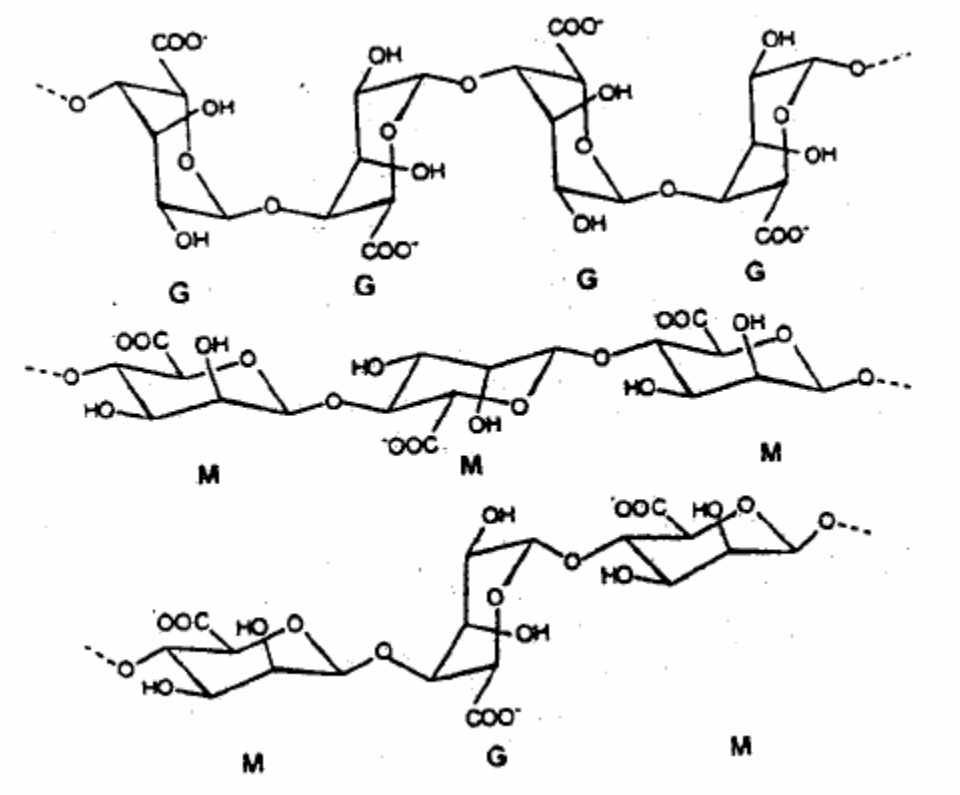

Figure 1.4 Alginate block types G = guluronic acid
M = mannuronic acid. Obtained from Tønnesen and Karlsen (2002).

Alginate-salt of monovalent ion such as sodium, is water-soluble. In the presence of multivalent cations such as Ca^{++}, strong binding occurs between the two neighboring G blocks, resulting in the formation of extended alginate networks where the G blocks form stiff junctions as illustrated in Figure 1.5. The G blocks form cavities that function as binding sites for ions (Skjak-Break, 1990).

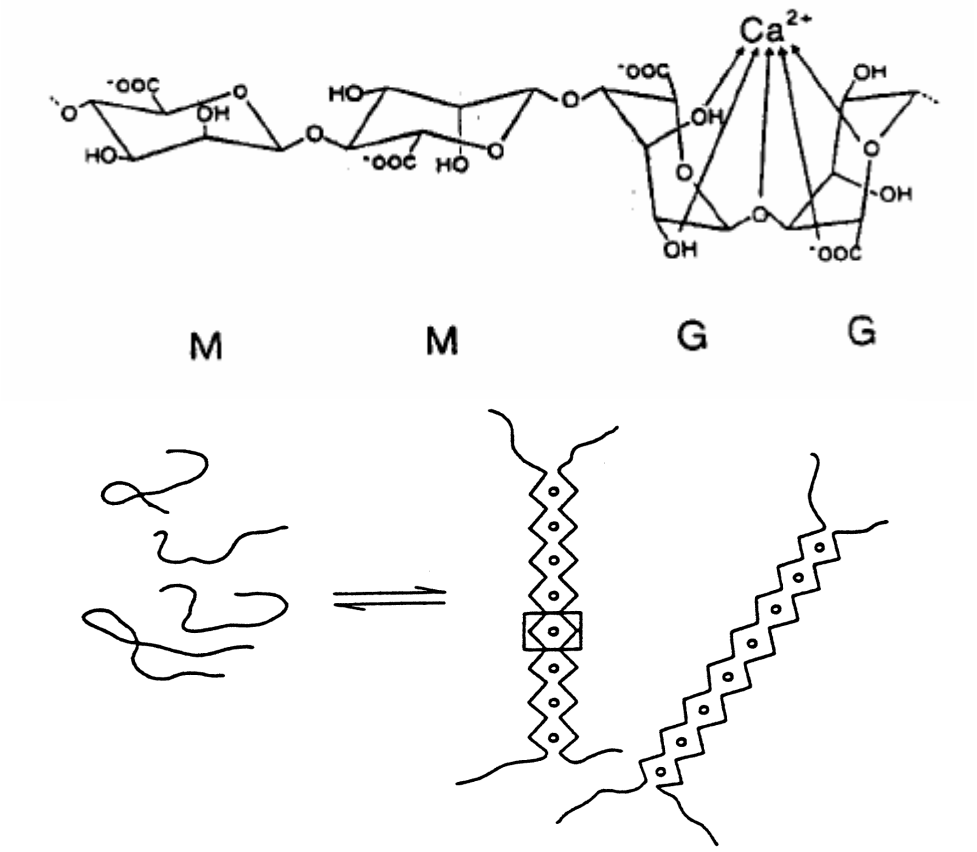

Figure 1.5 **I.** Probable binding mode between the calcium ion and two G residues of alginate. Obtained from Tønnesen and Karlsen 2002. **II** The conversion of alginate chains to buckled ribbonlike structures which contain arrays of Ca^{++} ions. (Gombotz and Wee, 1998)

The selective binding of cations to the alginate accounts for its capacity to form gels. The regions of M and MG blocks are not involved in network formation. Alginate-Ca^{++} gels consisting of alginates rich in G blocks (high G content) are generally hard and relatively brittle, whereas gels containing alginate with a relatively high content of M blocks (low G content) are softer and can undergo larger deformations (Gombotz and Wee, 1998). Due to the viscosity limitation of the spray drying system, low viscosity alginate was used in this research.

1.6 Glycol-Chitosan

Chitosan is a linear polysacharride composed of randomly distributed ß-(1-4)-linked D-glucosamine and N-acetly-D-glucosamine as shown in Figure 1.6. Chitosan is derived from the shells of shrimp and other sea crustaceans (Illum,1998). It is biodegradable and biocompatible and able to form a polyion complex with anionic polymers, such as alginate. These complexes do not dissolve in the presence of calcium chelators and are used to both stabilize and reduce porosity of the alginate matrix (Wee and Gombotz, 1998).

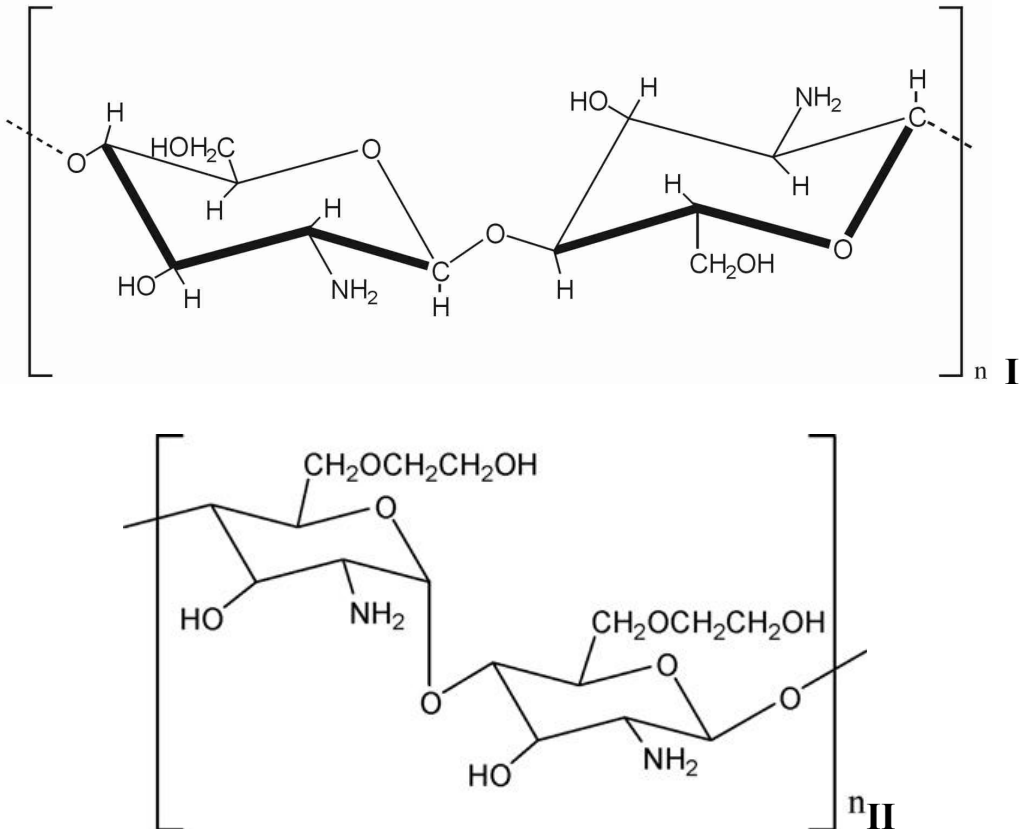

Figure 1.6 Chemical Structure of chitosan (I) (Hu *et al*, 2005)
and glycol-chitosan (II) (Sakai *et al*., 2000)

Chitosan is normally insoluble in water above pH 6 due to its rigid crystalline structure and requires acids to be protonated (Lee *et al.*, 2007). This creates a limitation when pH sensitive proteins are encapsulated, thus chemical modifications of chitosan have been studied to increase its water solubility. A poly(ethlylene-glycol)–chitosan hybrid system was produced, which is water-soluble over the entire pH range (Hu *et al*., 2005). In present study glycol-chitosan was used mainly due to polymer solubility at neutral pH, and ability to form polyion complexes with alginate. The structure of glycol-chitosan is illustrated in Figure 1.6. Glycol-chitosan alginate complexes were also studied previously. Sakai *et al.* (2000) made glycol-chitosan/alginate polyion complex microcapsules to protect implanted encapsulated islets from the host immune response.

1.7 Subtilisin, Lysozyme and Bovine Serum Albumin (BSA)

Molecular weight and size of the proteins and their pI values play an important role in the release of proteins from alginate matrices (Gombotz and Wee, 1998). In this study, three model proteins having different molecular weights were used; bovine serum albumin (BSA), subtilisin and lysozyme. However, during the investigation of the effect of spray drying operational parameters on alginate nanoparticle properties, subtilisin with a well studied spray drying history and simple assay was selected, since it is important to determine the residual activity of the particles in the spray drying process where heat sensitive materials are affected by several stresses. Subtilisin is a protease and one of the most important groups of industrial enzymes as it accounts for 60% of industrial microbial enzyme sales (Banarjee, 1999). It is used in detergent formulations to aid in removing proteinaceous stains, has a molecular weight range of 25-35 kDa and an isoelectic point of 9.4 (Ottessen, 1970). Subtilisins are produced by various *Bacillus*

species such as *Bacillus subtilus* and *Bacillus licheniformis*. Previously, polysaccharide (dextran)-sugar (trehalose,sucrose) mixtures were used in spray drying to encapsulate subtilisin into dry formulations to improve its process and storage stability. Several researchers studied the effect of operational parameters on enzyme particle properties. Samborska *et al*. (2005) investigated the effects of operational parameters on spray-dried activity of α-amlyase. In their study increasing feed solution rate and maintaining low outlet air temperatures, provided better protection of enzyme activity. Depaz *et al.* (2002) focused on the effects of a number of additives including dissacharides (sucrose and trehalose), polymers (dextran and maltodextrin) and dissacharide-polymer mixtures on the stability of the subtilisin, both during drying and storage. It was reported that the additives capable of hydrogen bonding inhibit the unfolding of subtilisin during drying, hence improved shelf life. Namaldi *et al*. (2006) studied the effect of temperature and additives (glucose and maltodextrin) on residual activity of serine alkaline protease. It was found that the residual activity of the protein continuously decreased by increasing drying temperature and presence of additives, and increased the residual activity at drying temperatures of 110 ^{o}C. The properties of the proteins used in this study are represented in Table 1.1.

Table 1.1. Properties of model proteins used in this study: BSA , Lysozyme, Subtilisin

	BSA	**Subtilisin**	**Lysozyme**
Number of Residues	583	275	129
Molecular weight	66.4 kDA	25-35 kDA	14.7 kDa
Theoretical pI	5.82	9.4	11.35

1.8 Trehalose

One of the challenges to preserve the stability of the enzymes and proteins during drying processes is the dehydration stress. Thus water replacing excipients are used as additives, such as sucrose, lactose, maltose and trehalose, which form hydrogen bonds with proteins, during water removal (Allison *et al*, 1999). Trehalose is a disaccharide, having high glass transition temperature (T_g = 84 ^oC, for particles produced at T_{inlet}=150 ^oC), making it the most suitable sugar for protecting proteins from denaturation during drying (Adler and Lee, 1998). The molecular structure of trehalose is presented in Figure 1.9. Trehalose has been used previously as an protective agent in protein spray drying applications in order to improve stability of the proteins. Adler and Lee, (1998) spray dried lactate-dehydrogenase in the presence of trehalose. More that 90% of the activity was retained and when the particles were stored at room temperature for 25 weeks, no activity loss was observed. Furthermore spray drying of recombinant human growth hormone was studied (Maa *et al.*, 1997). Broadhead *et al*., (1994) spray dried *β*-galactosidase in the presence of trehalose and the activity of the enzyme was completely recovered.

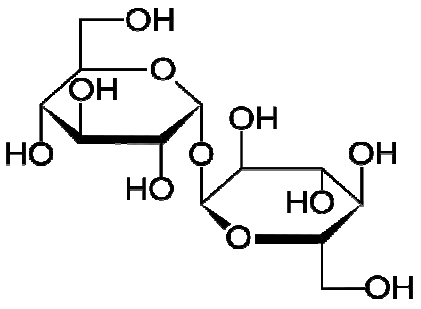

Figure 1.9 Molecular structure of trehalose

CHAPTER 2.0 OBJECTIVES

A number of microencapsulation strategies have been described in the literature, which result in wet suspensions of microparticles, and often involve several processing steps including the use of toxic solvents. The goal of the present study was to examine spray drying as a microencapsulation alternative, since it offers single step operation, producing dry particles, with the potential for extending the microparticle size into the nano-range. Nanoparticles are becoming increasingly important in the pharmaceutical field, such as toward the oral dosage of peptide or protein based therapeutics. A variety of model proteins were nano/microencapsulated in the present investigation, using alginate polysaccharide as a biodegradable matrix material, since it is water soluble, biocompatible, highly inert toward protein drugs, and is bioadhesive increasing drug residence time at the site of intestinal absorption.

Specific objectives are as follows:

1. Spray drying process parameters will be studied including protein to alginate ratio, alginate concentration, feed rate, and inlet temperature, in terms of how they may affect the properties of spray-dried micro- and nano particles such as mean size and distribution, residual activity of the encapsulated subtilisin, water content, product yield and particle morphology.
2. The residual activity of a model protein, subtilisin, will be evaluated in terms of how activity is affected by spray drying, with the addition of protectants, such as trehalose.

3. The long term stability of the spray dried micro- and nano particles will be determined.
4. *In vitro* release of low molecular weight model proteins (bovine serum albumin, subtilisin and lysozyme) will be investigated in simulated gastrointestinal environments.
5. The effect of additives such as glycol-chitosan, on release profiles, particle size and morphology will be evaluated.
6. The potential of spray drying for the preparation of nanoparticulate proteins for oral administration will be compared with other formulation methodologies.

CHAPTER 3.0 MATERIALS AND METHODS

3.1 Materials

Low viscosity sodium alginate (Na-A) (Sigma-Aldrich, Oakville, Canada) with specifications; 250 cP for 2% solution at 25^{o}C; molecular weight about 147 000; 61% mannuronic acid and 39% guluronic acid; batch number 112K0931. Subtilisin enzyme concentrate (Purafect UF concentrate, Lot L 20031) was supplied by Genencor International Inc. (Palo Alto, USA). Bovine serum albumin (BSA), lysozyme, glycol chitosan, maltose, sucrose, trehalose, peptide substrate (N-succinyl-L-Ala-L-Ala-Pro-L-Phe-p-nitroanaline) and other excipients were purchased from Sigma-Aldrich (Oakville, Canada).

3.2 Methods

3.2.1 Preparation of Feed Solution

Sodium alginate at noted concentrations was dissolved in deionized water using a magnetic stir plate, then dearated for 30 min. Model proteins at noted concentrations were dissolved in distilled water and added to the alginate solution. If the alginate solution was needed to be mixed with $CaCl_2$ and glycol-chitosan solutions, the alginate solution at various concentrations containing a model protein (subtilisin, lysozyme and BSA) at desired formulation rate was slowly mixed with $CaCl_2$ solution in a beaker aided by mechanical mixer for 5 minutes. The three-blade upward directing marine-type impeller was placed close to one third of the liquid depth to eliminate air entrainment, and rotated at 250 rpm. For the formulations with glycol chitosan, glycol-chitosan at

desired amount was dissolved in distilled water and added to the alginate solution containing the protein and dilute Ca^{++}. The solution was mixed in a beaker aided by mechanical mixer for 5 minutes by a marine-type impeller at 250rpm. During spray drying, all the feed solution formulations were continuously mixed with a magnetic stirrer. The dryer nozzle and glass walls of the particle collection vessel were cooled with tap water during the drying operation. Activity, yield, particle size and distribution and residual moisture content were determined on formulated particles.

3.2.2 Determination of Subtilisin Concentration and Activity

Subtilisin concentration was determined spectrophotomerically using ultraviolet absorbance at 280 nm. A protein standard curve was prepared with BSA in tris-HCl buffer solution. The catalytic activity of subtilisin was determined spectrophotometrically at 410 nm with 1 mg/ml N-succinyl-L-Ala-L-Ala-Pro-L-Phe-p-nitroanaline as substrate in 100 mM Tris, 0.005 % Tween 80 pH 8.6 (DelMar EG), as described in Chan, (2003). The particles dissolved in tris buffer system, and solution was sampled to run activity assay. The residual activity of subtilisin within spray dryed particles was determined by dividing the activity after rehydration of the particles, by the activity obtained by the stock solution prior to spray drying.

3.2.3 Protein Release from Micro- and Nano Particles in GI Simulated Environment

Protein release was carried out under simulated gastrointestinal (GI) conditions by suspending 10 mg particles into 20 mL 0.1M hydrochloric acid solution at pH 1.2, 37^{o}C for 2h, followed by transfer to 0.05M phosphate buffer at pH 6.8 for 3h. Experiments were performed in triplicate with mixing. At appropriate time intervals, 1.2 mL aliquots were removed, centrifuged and supernatant removed to be assayed for protein content spectrophotometrically (Cary 1, Varian, Australia) at 595 nm using the Bradford modified method (Coomassie plus kit, Pierce, Fisher, Canada). In order to keep the volume constant, 1.2 mL of the buffer solution was replaced. The percentage of released protein and encapsulation efficiency were calculated assuming that all the protein was released after the first 4 hours from the point when the particles were initially added to pH 1.2. The protein encapsulation efficiency of the particles was determined by the ratio of the initial protein load to the particle formulation to the spectrometrically determined amount of the proteins after rehydration. Particle yield was determined by dividing the amount of recovered spray dried particles by the initial mass of solids introduced to the spray dryer.

3.2.4 Characterization of Micro- and Nano Alginate Particles

3.2.4.1 Determination of residual moisture content

The residual moisture content of the spray dryed particles was determined by ratio of the weight difference before and after lyophilization. The particles were assumed to be moisture free after lyophilizing for 48hr.

3.2.4.2 Determination of the size distribution of the particles

Spray dryed particles were sized using a laser diffraction particle sizer (Malvern Mastersizer 2000 with dry particle sizing accessory, Sirocco 2000). Around 100 mg of particles were analyzed for each batch. The operating pressure was 3 bars and vibration speed of the microplate tray was 30% of maximum. For each batch of particles, the mean diameters were calculated in triplicate. The size distribution was estimated by a SPAN factor, which is defined by the ratio;

$$\mathrm{SPAN} = \frac{(D_{90} - D_{10})}{D_{50}}$$

where $D_{90\%}$, $D_{50\%}$ and $D_{10\%}$, are the mean diameters at which cumulative volume percent of 90, 50 and 10% of the particles are determined. A high SPAN indicates a wide size distribution, whereas a low value indicates a narrow size distribution.

3.2.4.3 Particle morphology and protein distribution within the particles

Morphology was examined by scanning electron microscopy (JEOL, JSM-840) with gold coated particles. Protein distribution within the particle matrix was determined by confocal laser scanning microscope (Leica TCS SP2, Germany). A 3 mL amount of BSA-FITC solution (10 mg/mL) was added to a sodium alginate, Ca^{++}, chitosan solution (alginate/protein ratio of 9:1, for 0.3% Na-A solution) and the particles were analyzed by "pro plus basic" Leica operating software. The imaging was performed with dry particles in order to prevent swelling and release of the protein.

CHAPTER 4.0 RESULTS AND DISCUSSION

Spray drying was used to produce micro- and nano particles carrying model proteins embedded within an alginate polymer matrix. One of the applications under consideration for protein loaded nanoparticles is oral drug delivery. The effect of operational parameters on the resulting properties of particles carrying an active biological was investigated (chapter 4.1) and a single step method proposed to enhance the properties of the micro- and nanoparticles for oral drug delivery applications (chapter 4.2).

4.1 Spray-dried Alginate Microparticles Carrying Active Biologicals

4.1.1 Stability of Subtilisin in the Feed Solution

The stability of subtilisin enzyme in the feed solution during the process period is important to ensure highest levels of dry product activity. The activity of the subtilisin feed solution was monitored for 1h, which is the total process time. Acitivity was assayed spectrophotometrically at 410 nm with 1 mg/mL N-succinyl-L-Ala-L-Ala-Pro-L-Phe-p-nitroanaline as substrate in Tris–HCl buffer (100 mM Tris-HCl, 0.005% Tween, pH 8.6) and resultant activities presented in Figure 4.1.1.

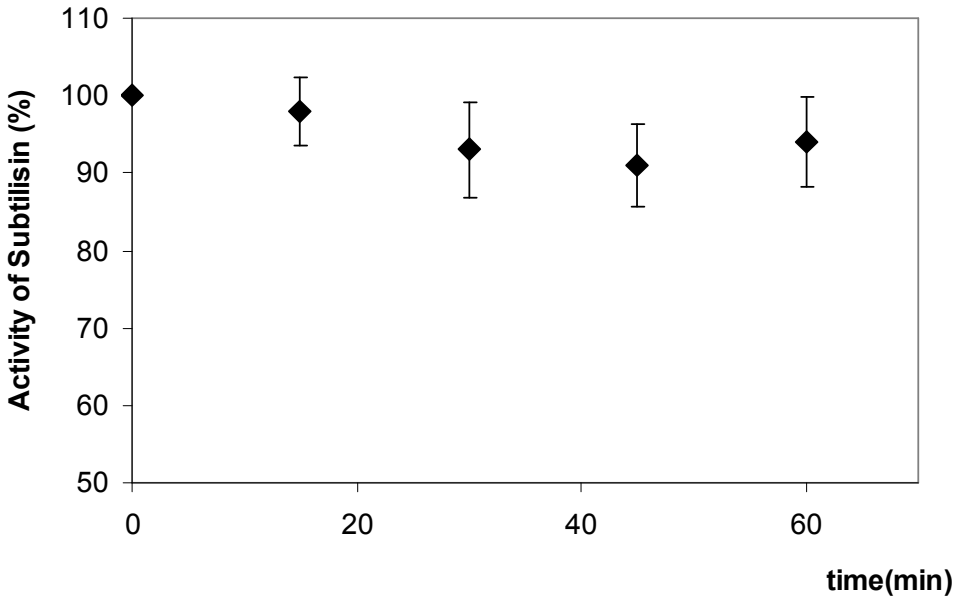

Figure 4.1.1 Activity retention of subtilisin in 2% alginate feed solution Alginate:subtilisin = 9, pH = 7.1 at 20 ^{O}C. Data shown represent mean values and error bars represent one standard deviation around the mean based on a minimum of 3 replicates

The optimum activity of subtilisin is known to be within the pH range of 7 and 8.5 (Chan, 2003), and since the pH of the feed solution was within this optimal range, the average residual activity of the subtilisin was around 95 %, during the 1 h period. It may be concluded that the subtilisin remains highly active during the spray drying process period.

4.1.2 Residual Activity of Subtilisin within Particles

Inlet temperature of the drying air (T_{inlet}) is an important parameter for spray drying processes and can affect the properties, such as residual activity of the protein in the resulting particles, especially with thermo-sensitive biologicals. To study the effect of inlet air temperature on residual activity of subtilisin, alginate-subtilisin solution (2% alginate, subtilisin:alginate ratio 1:9) was spray dried at three different inlet temperatures. For each batch, liquid feed rate (Q_{lf}), aspirator rate (Q_{da}), atomization pressure (P) and subtilisin loading were kept constant. In addition, free soluble subtilisin solution (0.2%) was spray dried at T_{inlet} =150^{o}C to determine the activity loss of subtilisin without the presence of alginate. The resulting particles were assayed for residual activity and results presented in Table 4.1.1.

Table 4.1.1Residual Activity of subtilisin within alginate microparticles produced by spray drying at different T_{in}. Operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi, Protein loading = 0.1 g subtilisin/g particle

T_{inlet} ºC	T_{outlet} ºC	**Residual Activity[%]**
125	63	76±2
150	69	81±2
175	81	77±3
150*	75	34±4

* Free soluble subtilisin C = 0.2%,w/v

Increasing T_{inlet} from 125 ^{o}C to 175 ^{o}C, consequently T_{outlet} from 63 ^{o}C – 81 ^{o}C, did not significantly affect the final residual activity yield of the subtilisin within particles, as for all three conditions, the activity yield ranged from 76 to 81%. In sharp contrast,

when subtilisin was spray-dried alone, more than 65% of the initial activity was lost. This shows that alginate is playing a stabilizing role for the enzyme during spray drying. As mentioned earlier, stability of the proteins can be affected by several stresses during spray drying, including thermal stress due to the drying air, shear stress due to the shear forces in the nozzle during atomization, adsorption due to the generation of new air/water interfaces and dehydration stress caused by the rapid evaporation of the water (Lee *et al*., 2002). However, globular proteins such as subtilisin are considered to be rigid and tend to resist changes in conformation upon adsorption at interfaces (Tripp *et al*., 1995) and show pressure stability up to 200 MPa (Webb *et al*., 2000). Therefore, activity loss due to conformational changes as a result of adsorption and shear stresses might be minor compared to dehydration and thermal stresses.

Immediately after atomization, the droplet surface temperature approximates the wet-bulb temperature of the inlet air. For the free soluble subtilisin solution which has a low solids concentration (0.2%,w/v), the enthalpy-humidity chart of the pure water system can be used. For inlet temperature of 150^{o}C, the surface temperature (T_{sur}) value can be estimated to be 41^{o}C, thus subtilisin solution droplets experience much lower temperature than the hottest region of the dryer. However, the maximum temperature of the microparticles can be assumed to attain the outlet temperature of the drying air where T_{outlet} = 75 ^{o}C, and the time period of exposure of the drying droplets to the elevated temperature is approximately 5-30 sec. (Broadhead, 1992). However, it has been known that polysaccharides such as dextran, have the ability to form an amorphous phase with proteins which limits the conformational changes due to the stresses involved in spray drying (DePaz *et al.,*2002). As mentioned earlier, alginate which is a polysaccharide, may

also form an amorphous phase with subtilisin molecules creating a matrix structure around the subtilisin, through hydrogen bonds which limits conformational changes, caused by dehydration and thermal stresses.

Recovery of the particles is also an important parameter in terms of investigation into the scaling up of the spray drying process. The water content and product yield of the final microparticles were also studied and the results presented in Table 4.1.2.

Table 4.1.2 Water content and recovery of the microparticles produced at different inlet temperatures by spray drying. Operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi, C = 2% w/v

T_{inlet} [ºC]	T_{outlet} [ºC]	Product recovery [%]	Moisture Content [%]
125	63	21± 2	7.1±2
150	69	33± 2	5.6±2
175	81	37± 3	5.5±2

As can be seen, the final water content of the particles change from 7.1 to 5.5% when the inlet temperature of the drying air increased. The product yield increased as the inlet temperature increased from 125 ºC to 175 ºC. At 125ºC, deposition of particles on the cyclone wall was observed, which might be due to insufficient droplet/particle drying within the drying chamber and higher moisture content particles, adhering to the cyclone wall, leading to a lower product yield. At inlet temperatures 150ºC and 175ºC, higher moisture removal and product yield was observed. In both systems, the moisture content of the particles was less around 6% and around 35% of the particles are recovered in the collection vessel. In terms of industrial scale up, higher inlet temperatures could be

selected, due to the better water moisture removal and higher product recovery, especially as higher temperatures to 175 ºC do not significantly affect enzyme activity.

4.1.3 Retention of Subtilisin Activity with Different Formulations of Alginate

In spray drying of proteins, the ratio of amorphous phase to protein is important as it affects the final residual activity within the microparticles. The subtilisin in the formulation was varied from 0.1 to 0.33 g subtilisin/g particle keeping the total solid concentration constant. The formulation was spray dried at two different inlet temperatures, 150 ºC and 175 ºC, and the results are presented in Figure 4.1.2.

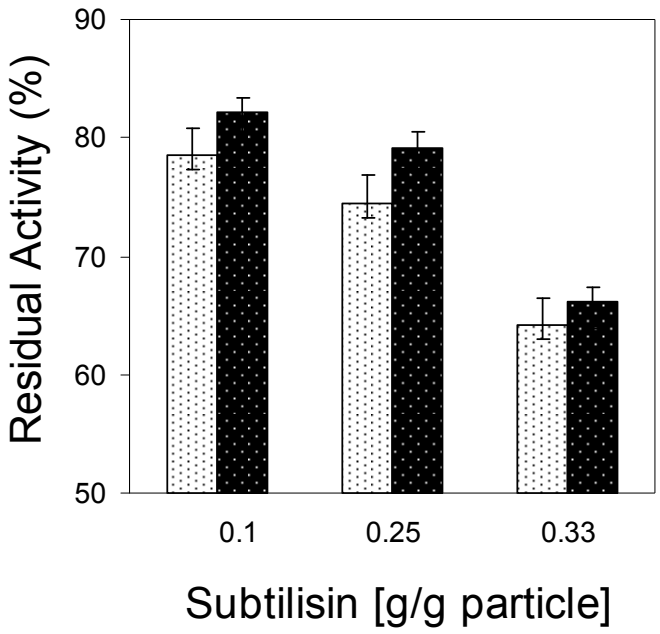

Figure 4.1.2 Effect of protein loading and inlet temperature on residual activity of subtilisin. 175 ºC (light columns), 150 ºC (dark columns). Operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi.

The formulations containing 0.1 g subtilisin/g particle showed around 80 % residual activity, and as the subtilisin concentration increased to 0.33 g subtilisin/ g particle, a decrease in the residual activity was observed. The residual activities for 0.25 and 0.33 g subtilisin/g particle were around 75 % and 65 %. Figure 4.1.2 also showed that the effect of inlet temperature on the residual activity of subtilisin is minor compared to the ratio of alginate:subtilisin. As the subtilisin in the formulation increases, the alginate concentration decreases, as does the residual activity.

4.1.4 Effect of Feed Rate on Particle Size

Liquid feed rate is the major influencing factor on T_{out} and together with atomization air pressure and liquid properties (viscosity and surface tension), it also determines the spray droplet size (Maa *et al.*, 1997). The effect of feed rate at constant atomization pressure on moisture content, residual activity and product recovery is presented in Table 4.1.3. For each batch, other spray drying parameters such as liquid feed rate, aspirator rate, atomization pressure and other process parameters such as total solute concentration in the feed solution and subtilisin loading were kept constant.

Table 4.1.3 Effect of liquid feed rate on outlet temperature, particle size and residual activity of subtilisin. Operating conditions; Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi, Protein loading = 0.1 g subtilisin/ g particle

Q_{lf} mL/min	Toutlet [ºC]	D[0.1]	D[0.5]	D[0.9]	SPAN	Water Content	Residual Activity[%]	Product Recovery [%]
10	58	3.12	6.69	30.1	4.09	8.1±1	71±2	21± 2
7	61	2.32	6.31	22.2	3.19	7.2±2	72±2	21± 2
5	69	1.91	4.91	13.5	2.34	5.6±2	81±2	33± 2

Liquid feed rate affected the mean particle size (D[0.5]), the particles size decreased from 6.69 to 4.91 μm by decreasing the feed rate, whereas the outlet temperature of the drying air increased from 58 to 69^oC . The effect of liquid feed rate and the atomization air pressure on spray droplet size is defined by the air/fluid mass ratio, which represents the energy available for atomization (Masters, 1991). When atomization pressure was kept constant and liquid feed rate decreased, the energy available for atomization increased, therefore spray droplet size decreased and consequently the size of the dried particles. As a result of higher liquid feed rate, the

amount of water evaporated was increased, leading to a lower outlet temperature as observed in Table 4.1.3. The water content of the particles also increased with increasing liquid feed rate. Although, higher feed rates result in lower outlet temperatures, the residual activity in the final particles decrease about 10%. This difference can be explained due to particle deposits present inside the walls of the cyclone. As mentioned earlier, when the particles leave the drying chamber with high moisture content, they adhere to the walls of the cyclone. However, the temperature of the cyclone wall is close to the outlet temperature of the drying air. As the process continues, the moisture content of the particles might further decrease, and with the help of drying air they might further travel to the collection vessel. This will affect residual activity, due to the high surface temperature of the cyclone wall.

In terms of particle size, the Buchi-290 lab scale spray dryer is thought to be able to produce particles with diameter range of 1-10 μm (Lee, 2002). From Table 4.1.3, it may be concluded that in order to produce alginate nanoparticles having mean diameter less than 5 μm, a low feed rate of 5 mL/min should be selected. Size and dimensional distribution of the particles are presented in Table 4.1.4.

Table 4.1.4 Size and dimensional distribution of alginate microparticles. Operating conditions; Q_{lf}= 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi, C = 2% w/v Protein Loading = 0.1 g protein/ g particle

Diameter Range (µm)	Distribution
<1	3 %
1-2	25%
2-3	26%
3-4	18%
4-5	24 %
5-10	10%

Around 90 % of the particles were within the particle size range desired for oral absorption though the intestinal mucosa.

4.1.5 Effect of Alginate Concentration on Particle Size Distribution

In addition to solution feed rate, liquid properties such as viscosity and surface tension can also affect the particle size in spray-drying. The alginate concentration was thus varied from 0.2 to 2%, and the particle properties are presented in Table 4.1.5.

Table 4.1.5 Effect of alginate concentration on particle size and residual activity of subtilisin. Operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P_{aa} = 80 psi, Protein Loading = 0.1 g subtilisin/ g particle

C [%]	Viscosity [cps]	T_{out}	D[0.1]	D[0.5]	D[0.9]	Span	Residual Activity [%]	Product Recovery	Moisture Content
0.2	43	85	1.31	2.15	3.58	1.05	68±3.6	21±2.2	5.2±2.4
0.5	93	78	1.57	3.67	7.3	1.56	73±3.4	19±2.5	5.3±2.1
1	127	73	1.83	4.12	11.8	2.41	74±2.2	37±2.4	5.3±2.2
2	189	69	1.91	4.91	13.5	2.34	81±2.4	33± 2.1	5.6±2.2

The total solid content in the spray drying feed solution may affect the particle size since higher solids content in droplets shortens the constant rate period because less water must evaporate to build a crust, consequently forming larger particles. This case will occur, where the solutes do not have large impact on solution viscosity. The viscosity values of the feed solutions with corresponding alginate concentrations are presented in Table 4.1.5. As seen, higher alginate concentration, increases solution viscosity, consequently producing larger particles. The change in total solids concentration from 0.2 to 2% increases the mean particle size (D[0.5]) from 2.15 to 4.91 μm. The span value also increases with higher alginate concentration, and thus viscosity of the feed solution. This indicates that the microparticles produced with lower alginate concentration resulted in a narrower size distribution. However, the residual activity and particle recovery decreased around 15%, as the alginate concentration decreased from 2% to 0.2% in the feed. As mentioned earlier, the particles are collected through a cyclone system under centrifugal force. However, when the particle size is less than 2 μm, the gravitational force may not be sufficient for recovery, allowing particles of diameter < 2 μm to pass

into the outlet air (Prinn *et al.*, 2002). Therefore for the particles prepared with low alginate concentration, there is a higher chance that the particles can escape, leading to lower particle recovery. In addition, volumetric distribution of the particles can be also affected, due to insufficient trapping of the particles having lower particle size. Recovery of the nano-size range particles can be increased with electrostatic filters. The outlet temperature of the spray dryer was increased to 85 ^{o}C as the alginate concentration in the feed solution decreased to 0.2% leading to a lower residual activity of subtilisin encapsulated within particles.

The volumetric size distribution of the particles is presented in Table 4.1.6. It can be seen that for all the conditions tested, less than 5% of the particles are within the submicron range. For all conditions, around 90% of the particles are within the range of 1-5 µm, which is an appropriate size for oral absorption within the intestinal mucosa.

Table 4.1.6 Size and dimensional distribution of alginate microparticles. Operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi, Protein loading =0.1 g protein/ g particle.

Total Solid Concentration [C]	**2%**	**1%**	**0.5%**	**0.2%**
Mean Diameter [µm]	4.91 µm	4.12 µm	3.67 µm	2.15 µm
<1	3 %	3%	4%	5 %
1-2	25%	31%	35%	43%
2-3	26%	24%	29%	31%
3-4	18%	17%	16%	15%
4-5	24 %	17%	10%	4%
5-10	10%	8%	5%	2%

However, the volumetric size distribution of the particles may extend more into the nanorange, where lower alginate concentration was used. At lower alginate concentration, there is a higher probability that the particles will have a smaller size making separation more difficult affecting the particle population collected. These results show that the mean particle size and the volumetric population of the particles depend on the alginate feed solution concentration, and also particles prepared with low alginate concentration have narrower size distribution.

SEM images of the particles are presented in Figure 4.1.3. The particles were dimpled and spherical morphology.

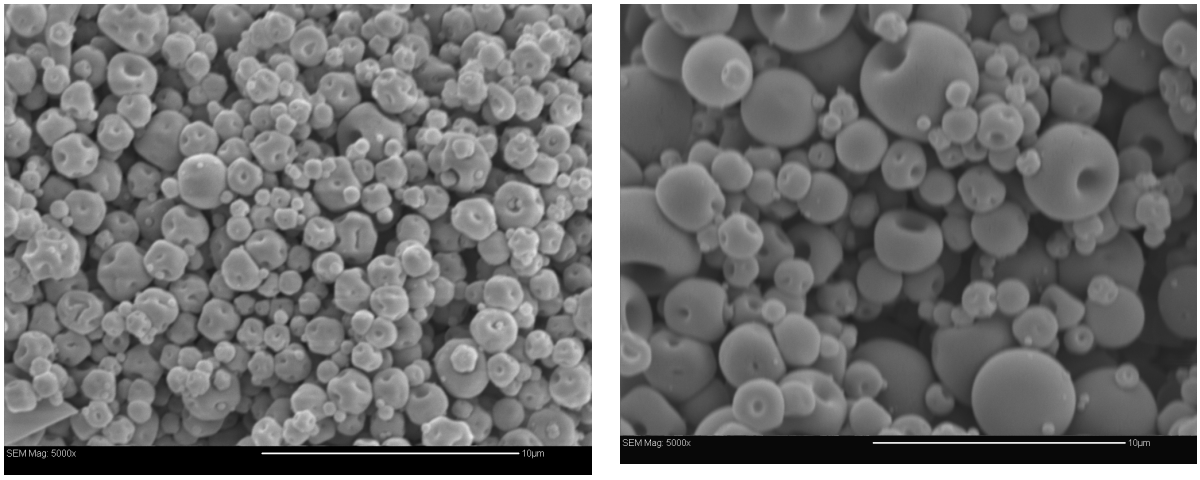

Figure 4.1.3. SEM images of spray dried alginate particles carrying subtilisin, Left image: Alginate particles prepared with 0.2% alginate in the feed solution. Scale bar represents 10 µm. The right image show alginate particles prepared with 2% alginate. Scale bar represents 10 µm.

4.1.6 Residual Activity of Subtilisin with Different Amounts of Trehalose

As mentioned earlier, water removal can lead to conformational changes in the protein/peptide, leading to loss of activity. Many proteins/peptides have been successfully dried with minimal activity loss by use of stabilizing additives. Additives can protect during the dehydration process by forming hydrogen bonds with the protein. Several mono-and disaccharides, and several polyols and amino acids are known stabilizers against water removal stress. Examples include lactose, trehalose, sucrose, mannitol, sorbitol, lysine, histidine or arginine (Arakawa *et al*., 1993). Amongst these examples, trehalose is known to be the best sugar for stabilizing proteins during spray drying processes (Adler and Lee, 1998).

The total solids concentration of the feed was kept constant and the trehalose amount changed from 0 to 0.25 then 0.33 g/g particle, with the subtilisin loading kept constant at 0.1g/g particle. The effect of trehalose loading on residual activity of subtilisin is presented in Figure 4.1.4.

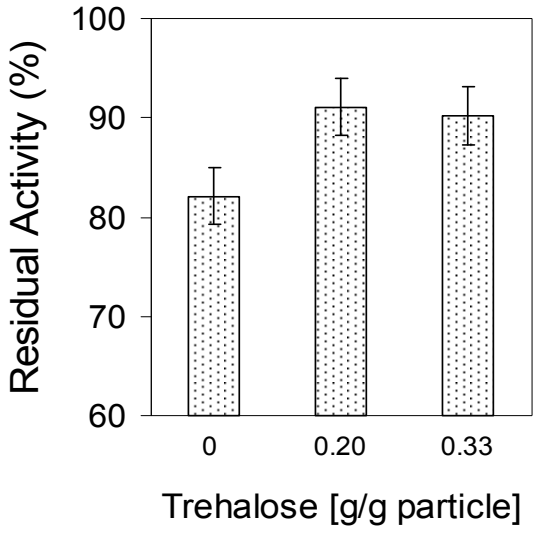

Figure- 4.1.4. Effect of trehalose loading on residual activity of subtilisin within microparticles. Spray drying operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 667 L/h, P_{aa} = 80 psi, C = 2% w/v, Protein loading = 0.1 g subtilisin/ g particle.

The presence of 20% trehalose in the formulation increases the residual activity of subtilisin up to 90%. Further increase in trehalose did not affect the residual activity. It might be concluded that the mixture of alginate and trehalose appear to promote the stabilization of subtilisin during spray drying. This might be due to the ability of trehalose to hydrogen bond with the proteins in the place of water during dehydration. A second study was done with a higher protein loading (0.33 g subtilisin/g particle), and the results are presented in Figure 4.1.5.

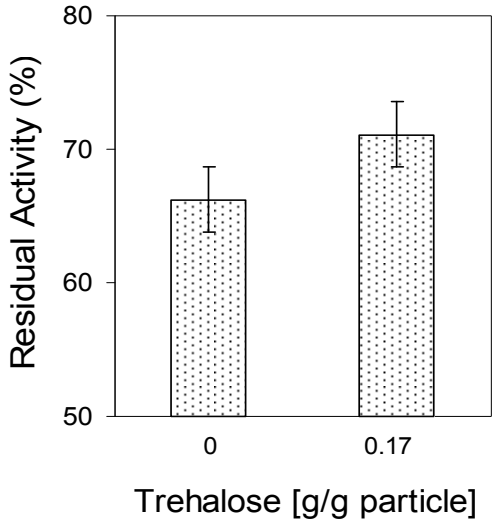

Figure 4.1.5 Effect of trehalose loading on residual activity of subtilisin within microparticles. Spray drying operating conditions: Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 667 L/h, P_{aa} = 80 psi, C = 2 % w/v, Protein Loading=0.33 g protein/ g particle.

When the trehalose in the formulation was increased to 0.17 g/g particle, the residual activity of the particles increased by 7 % compared to the activity of the particles without the presence of trehalose. It can be concluded that the presence of trehalose in alginate increases the stability of subtilisin within the particles, which results in higher residual activity. It was also shown that more then 60% of the residual acitivity can be recovered for particles having high protein concentration (0.33g/g particle). Similar trends were also observed using sugar excipients with polysachharides. Bare *et al.* (1999) spray dried cold adapted subtilisin in the presence of gum arabic and lactose mixture, observing about 55% absolute increase in residual activity of subtilisin. DePaz *et al.* (2002) showed that dextran-trehalose mixture showed full recovery of subtilisin activity during freeze drying.

SEM image of the trehalose-alginate loaded with subtilisin are presented in Figure 4.1.6. The particles are spherical and have a smooth surface morphology and the smaller particles were dimpled.

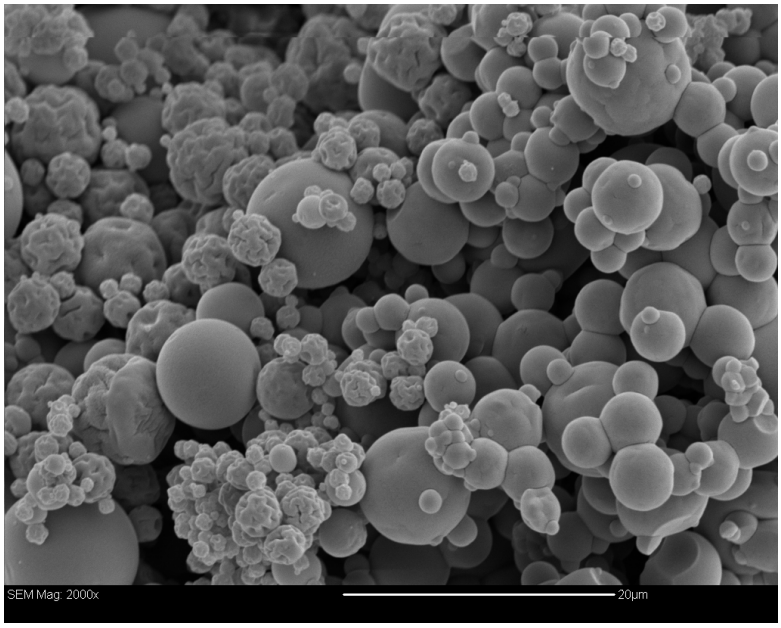

Figure 4.1.6. SEM image of the spray dried alginate-trehalose particles carrying subtilisin. Alginate:trehalose ratio was 9:1. Scale bar represents 20 µm.

4.1.7 Effect of Storage Time on Residual Activity of Subtilisin

Along with the stability of the particles during spray drying, the storage stability was also tested. Spray-dried powders were stored at 25^{o}C and 25% relative humidity for over 4 months. Figure 4.1.7 shows the residual activity of the particles with storage time.

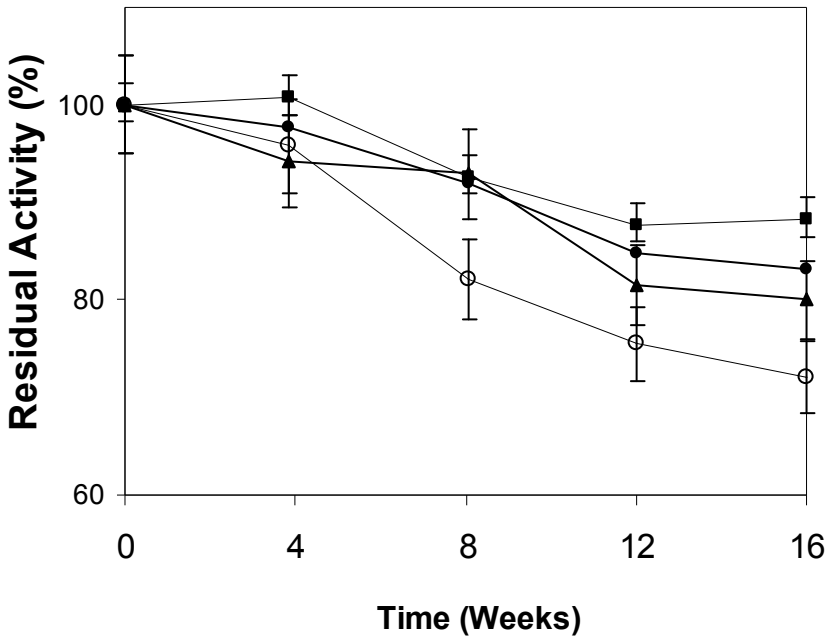

Figure 4.1.7 The residual activity of subtilisin loaded particles stored at 25 ^{o}C and 25% relative humidity relative to the activity of freshly prepared particles. Formulation consists of trehalose:subtilisin (g/g particle) of 0.2:0.1(■), 0:0.1(●), 0:.0.25(▲), and 0:0.33(O)

As may be seen in Figure 4.1.7 the alginate-trehalose loaded particles retained more then 90% of their initial activity during 4 months of storage. As the subtilisin in the formulation was increased (0.1-0.33 subtilisin g/g particle) a decrease in the final residual activity was observed. Also presence of trehalose in the formulation had a positive effect

on storage stability compared to particles produced without addition of any trehalose. Within the first month of the storage period, all particles had more than 90 % residual activity, however trehalose loaded particles showed nearly no activity loss within the first month, but activity gradually decreased during the following 3 months. A similar trend was observed for particles prepared without addition of trehalose, showing a slower residual activity decrease rate within the first month. These trends show that within the four months of storage, trehalose loaded particles showed the best storage stability.

4.2 Alginate Micro- and Nano Particles Produced by Spray Drying

In the first section of the results and discussion chapter some of the operating parameters effecting the process parameter and particle properties were studied. In this section an alternative single step procedure was proposed in order to enhance the properties the alginate particles carrying proteins for oral drug delivery purposes.

Spray drying has been used previously by Coppi *et al.* (2001, 2002, 2004) to produce alginate micro- and nano particles carrying proteins, such as BSA and a peptide antibiotic, polymyxin. Although spray drying is a single step process, these earlier published methodologies involved several additional steps to produce the final particulate product. In this chapter, an alternative spray drying method is proposed and discussed to produce alginate micro- and nano particles in a single step operation.

The method proposed by Coppi *et al.* (2001) as outlined in Figure 4.2.1, involved spray drying of an alginate/protein solution forming particles which were subsequently

subjected to several additional steps to enhance the properties, such as ionic crosslinking of the particles in $CaCl_2$ aqueous solution and treatment of the particles in chitosan solution to reinforce the alginate network and to improve adsorption across the intestinal epithelia. In the final step, freeze drying was used as a second drying step, to remove water and to recover the particles. However, these steps can alter some of the properties of the particles. For example, the entrapped drug can be released to the aqueous medium during the gellation step, and during surface treatment with chitosan. Moreover, pH sensitive proteins can be affected due to the solubility requirements of chitosan (pH<5.5). In addition, lyophylization (freeze drying) causes additional stress on particles and proteins, such as changing the particle morphology and denaturation of the proteins due to dehydration (Wang, 2000).

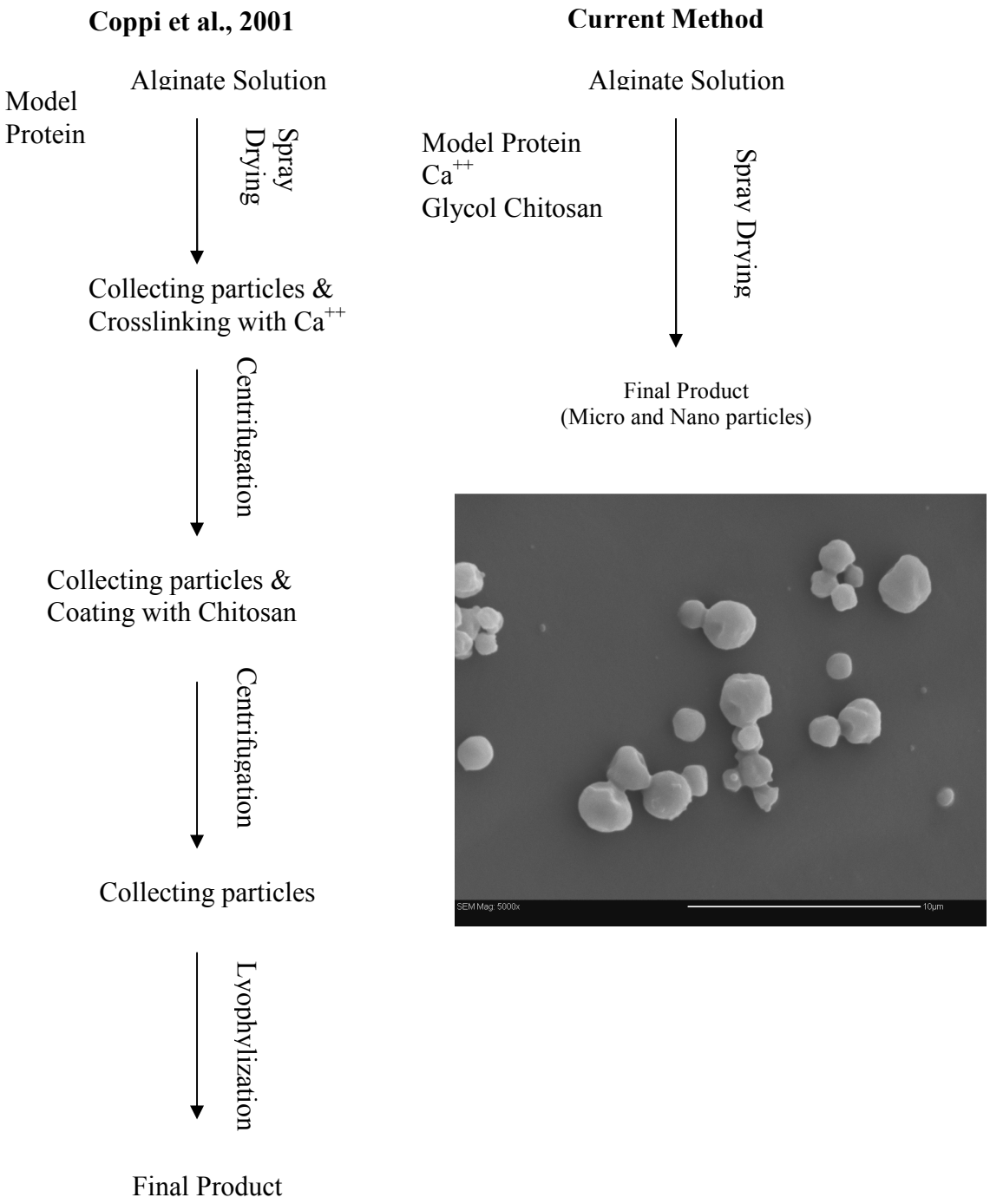

Figure 4.2.1**:** Schematic description of Coppi *et al.* (2001) and current method. The scale bar represents10 microns.

As an alternative, the single step procedure which forms the basis of the present study, involved introduction of dilute Ca^{++} ions to a dilute alginate feed solution, along with the protein and glycol-chitosan, where physical crosslinking of the alginate by dilute calcium solution takes place forming sprayable low viscosity gel. In addition, glycol-chitosan was used as an additive, to further reinforce the alginate network and improve absorption of the particles. The SEM image of the particles produced is presented in Figure 4.2.1. The final particles had smooth surface and spherical morphology, with particle size ranging from a couple hundred nanometers to several microns. In the following chapters, the stability, particle size distribution, morphology, formulation aspects and protein release from the particles will be presented and discussed.

4.2.1 Stability and Size of Alginate Micro- and Nano Particles

One application for nanoparticles is in the oral delivery of therapeutic proteins. Nanoparticles can appear in the lymph or blood circulation, following 10 min to 3 h after dosing depending on the particle size and nutritional conditions (Hussein *et al.*, 2001), thus it is important to test the stability and the properties of the particles, as affected by formulation parameters. The effect of Ca^{++} present in the alginate feed solution, on stability and properties of micro and nano particles, such as particle size and protein release, was examined. The amount of Ca^{++} (3-10 mM) and alginate solution concentration (0.2-0.5%) was varied. Alginate and Ca^{++} concentrations were low due to the viscosity limitation of the spray nozzle. At Ca^{++} ion concentrations greater than 10 mM, immediate viscous gel formation occurred, which was not suitable for atomization.The stability of the resulting particles was tested by suspending in saline

solution (0.9%) at pH 6.8, and particles were observed under light microscope. Results are presented in Table 4.2.1.

Table 4.2.1. Stability of micro- and nano particles produced by spray drying. Spray dryer operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi. The particles were suspended in saline solution (0.9%) for 3 h. The "-" sign represents complete dissolution of the particles and the "+" sign represents presence of particles.

Ca^{++} Concentration in the Feed Solution	**Alginate Concentration in the Feed Solution (%,w/v)**		
	0.2 %	**0.3 %**	**0.5 %**
3 mM	-	-	-
5 mM	+	+	-
10 mM	+	+	+

Table 4.2.1 shows that the stability of the micro and nano particles was affected by the concentration of Ca^{++} ions. The particles prepared with 3 mM Ca^{++} completely dissolved at pH 6.8 in 3 h. However, the particles prepared with higher Ca^{++} showed stability during 3 h of the test. Crosslinking of the alginate chains with calcium ions takes place in the feed solution. With increasing calcium ion concentration , there is a higher degree of crosslinking, increased the stability of the resulting particles. When there is insufficient crosslinking and entanglements of the polymer chains, the particles swell and disolve when dispersed into a saline solution, particularly as the non-gelling sodium in the saline displaces the calcium in the particle matrix. It was observed that the particles produced with higher calcium concentrations were more stable in saline solution (0.9%), therefore preparation of particles with higher calcium concentration in the feed solution is more suitable for oral delivery purposes.

The objective was to produce particles within an appropriate size range to be taken up by the intestinal mucosa. Thus, the effect of alginate concentration on particle size was examined. The particles were sized by laser diffraction and the size distribution is presented in Table 4.2.2. The mean size of particles increased from 3.17 μm to 4.18 μm as the alginate concentration in the feed solution increased from 0.2 % to 0.5 %, while keeping the calcium concentration constant. As the alginate concentration increased, higher SPAN values were obtained, which indicates broader size distribution. This trend might be due to the increase in the viscosity of the solution, since the higher the alginate concentration in the feed solution, the higher is the viscosity of the feed, consequently leading to larger droplets and larger particles. All the resulting particles prepared with different amounts of alginate had appropriate size for oral absorption.

Table 4.2.2 Size distribution of the alginate micro- and nano particles with different amount of alginate in the feed solution. The particles were produced with different amounts of alginate , 0.2, 0.3 and 0.5%. The Ca^{++} ion concentration was constant at 10 mM. Spray drying operating conditions; Q_{lf} = 5 mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi.

Alginate concentration in the feed solution	D[0.1]	D[0.5]	D[0.9]	SPAN
0.2	1.61	3.17	11.5	3.12
0.3	2.12	3.41	13.2	3.25
0.5	2.72	4.18	19.1	3.92

In order to further investigate and improve the particle properties, 0.3% alginate and 10 mM Ca^{++} in the feed solution formulation were selected for further study, due to the lower recovery of the particles with 0.5% alginate, which might be due to the insufficient drying.

4.2.2 Protein Release from Micro- and Nano Alginate Particles

For oral delivery, it is important to examine the release profile of proteins from the encapsulated matrix. In order to study the effect of Ca^{++} on protein release, Ca^{++} concentration was varied (5 mM, 10 mM) and a control batch prepared without calcium. BSA was added along with 0.3% alginate, where the protein:alginate ratio was held constant at 1:9. The effect of Ca^{++} on protein release is presented in Figure 4.2.2, where the released percentage is plotted with time. The release studies were carried out in saline solution (0.9 %) at pH 6.8.

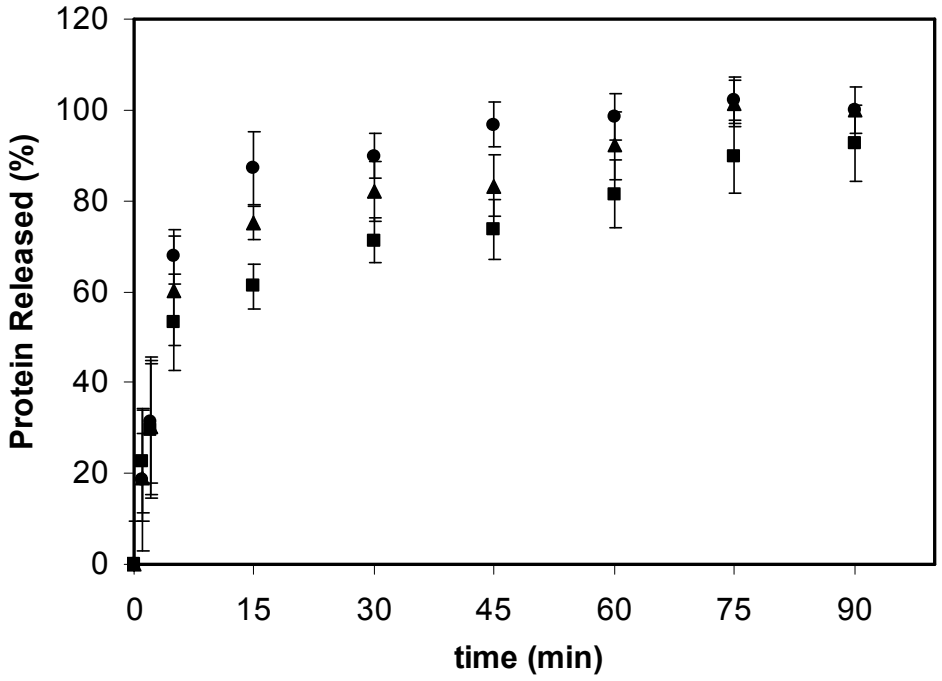

Figure 4.2.2: Release profile of BSA from alginate particles produced with 0 (●), 5 (▲) and 10 mM (■) Ca^{++}. Release was carried out in saline solution at pH 6.8. Particles were formed from 0.3% alginate at protein to alginate ratio 1:9. Data shown are the standard deviation of the mean values of a minimum of 3 repeated experiments.

All of the profiles showed a burst release profile, with about 60% of the BSA released in the first 5 min. Particles without Ca^{++} dissolved in 15 min and more than 90% of the BSA was released. Particles prepared with 10 mM Ca^{++} showed a nearly constant release rate after 15 min and more than 90% of the protein was released at the end of 75 min. Initially, the burst release profile might be due to the swelling and disintegration of the particle structure, however particles prepared with calcium, showed a sustained release profile after 15 min. The effect of calcium on the release profile, is likely due to the change in permeability of the particles due to the higher degree of physical crosslinking taking place in the feed and polymer entanglements during the formation of the particles.

4.2.3 Protein Release from Particles Formulated with Glycol-chitosan and Calcium Alginate

As mentioned earlier, the objective was to investigate an encapsulation method that would form nanoparticles in single step operation. Glycol chitosan, a positively charged polysaccharide, was used as an additive to enhance the properties of the particles, since it is known to reinforce the negatively charged alginate network and to improve the intestinal uptake of the particles, while decreasing the particle permeability. Glycol-chitosan was selected as it is water-soluble at pH 7.4 and forms polyion complexes with negatively charged polymers, such as alginate (Sakai *et al.,* 2000).

The aim of this experiment was to understand the effect that the amount of glycol-chitosan had on the release profile of BSA. The ratio of glycol-chitosan:alginate:protein was varied and protein release plotted with time in Figure 4.2.3. Release was carried out in different simulated pH environment of the gastro intestinal track.

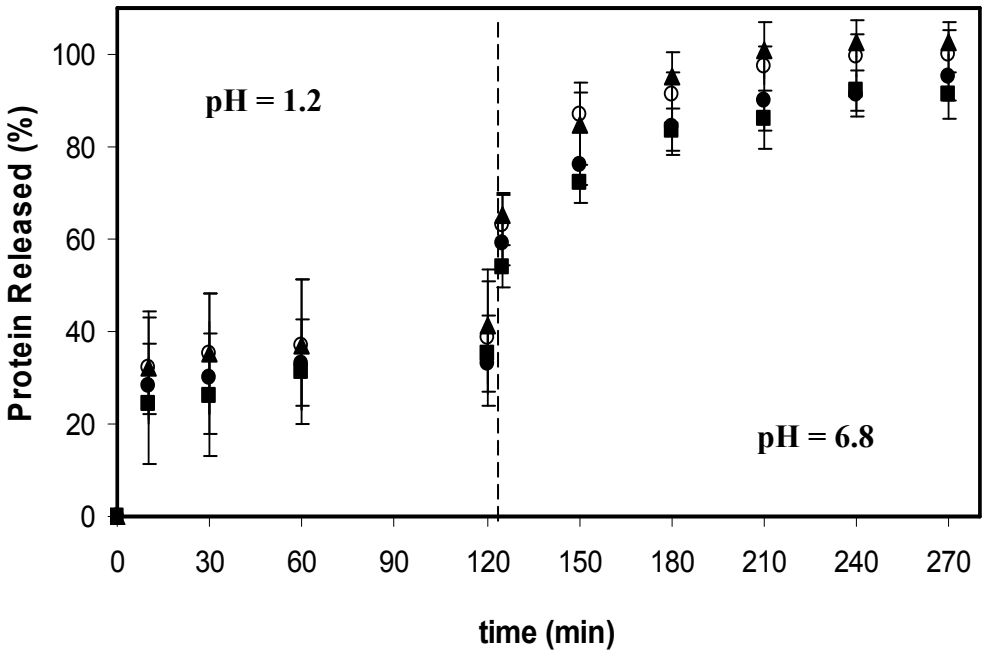

Figure 4.2.3: Release profile of BSA from glycol chitosan-alginate particles produced by spray drying prepared at formulation ratios of glycol chitosan:protein:alginate, 0:2:18 (O); 2:2:16 (▲); 3:2:15 (●); and 4:2:14 (■) in hydrochloric acid at pH 1.2 and phosphate buffer at pH 6.8. The feed solution consisted of 0.3% alginate and 10 mM Ca^{++}. Data shown are the standard deviation of the mean values of a minimum of 3 repeated experiments.

Particles formulated with glycol chitosan showed around 40% release after 2 h in the low pH, stomach simulation medium. When the particles were transferred into a simulated intestinal media, a sharp release was observed within the first 5 min, which might be due to particle swelling. The particles formulated without glycol chitosan or low glycol chitosan content, reached equilibrium within the first 30 min of the simulated intestinal environment, whereas the formulation having higher glycol chitosan (4:2:14) reached the equilibrium after 120 min. Figure 4.2.5 indicates that protein release was affected by increased glycol-chitosan in the formulation. This result was expected, since glycol chitosan can form a polyion complex with alginate decreasing solute diffusivity.

However, the buffer system used can also affect protein release, since phosphate ions can remove calcium ions from the crosslinked alginate network, consequently the permeability of the particles is affected. In addition, Ca^{++} concentration was not changed as the chitosan to alginate amount was varied, effectively increasing the ratio of Ca^{++} to alginate, consequently the crosslinking of the alginate chains might also increase, which would affect solute diffusivity.

Alginate particles were also formulated with subtilisin and lysozyme, which are smaller proteins than BSA. In these experiments the ratio of chitosan:protein:alginate was 4:2:14. Release profile of the particles is presented in Figure 4.2.4.

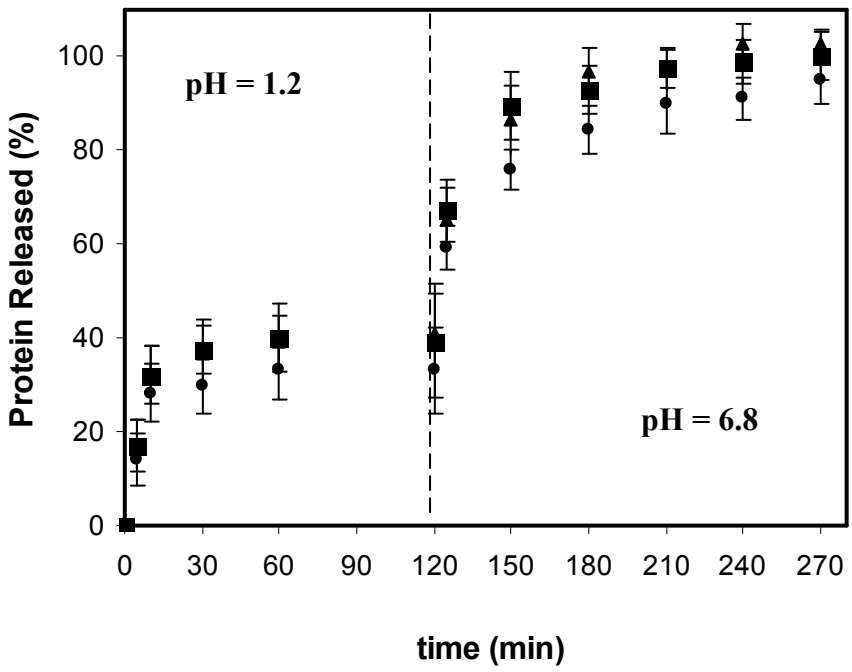

Figure 4.2.4 Release profile of BSA (●), subtilisin (▲) and lysozyme (■) from chitosan-alginate particles in hydrochloric acid buffer at pH 1.2 and phosphate buffer at pH 6.8. Particles were formulated from 0.3% alginate and 10 mM Ca^{++}. Data shown are the standard deviation of the mean values of a minimum of 3 repeated experiments.

All particles released more than 35% of the protein load within 30 min in the simulated stomach environment. After 2 h, the particles were transferred to a simulated intestinal environment at pH 6.8. Within the first 5 min, a second burst release profile was observed, possibly due to the swelling of the particles. Although the positively charged lysozyme (pI = 11.4) and subtilisin (pI = 9.4) had different molecular weights, they showed similar release profiles, possibly due to the large pore size of the resulting particles compared to the molecular size of the proteins. However, BSA showed a slightly slower release profile, which might be due to the larger molecular size of the protein compared to the pore size of the particles.

4.2.4 Physical Properties of the Chitosan-Alginate Particles.

Chitosan-alginate particles were tested for particle size, protein loading and morphology. The final protein loading within the particles and the particle size distribution is presented in Table 4.2.3.

Table 4.2.3 Comparison of particle size distribution of chitosan-alginate particles carrying different proteins. The chitosan:protein:alginate ratio was 4:2:14. The feed solution contained 0.3% alginate and 10 mM Ca^{++}. Spray drying operating conditions; Q_{lf} = 5mL/min, Q_{da} = 38 m^3/h , Q_{aa} = 600 L/h, P = 80 psi.

Protein	D[0.1]	D[0.5]	D[0.9]	SPAN	Encapsulation [%]
BSA	2.16	3.17	12.8	3.36	96±2
Subtilisin	2.11	3.53	12.1	2.83	94±1
Lysozyme	2.01	3.16	13.1	3.51	95±2

The mean particle size D[0.5], was found to be around 3.5 μm for subtilisin loaded particles, while those loaded with BSA and lysozyme had mean size around 3.16 μm. SPAN values showed a relatively broad size distribution. For all of the particles, encapsulation yield was more than 94%.

The protein loaded particles showed spherical morphology with smooth surface as illustrated in Figure 4.2.5.

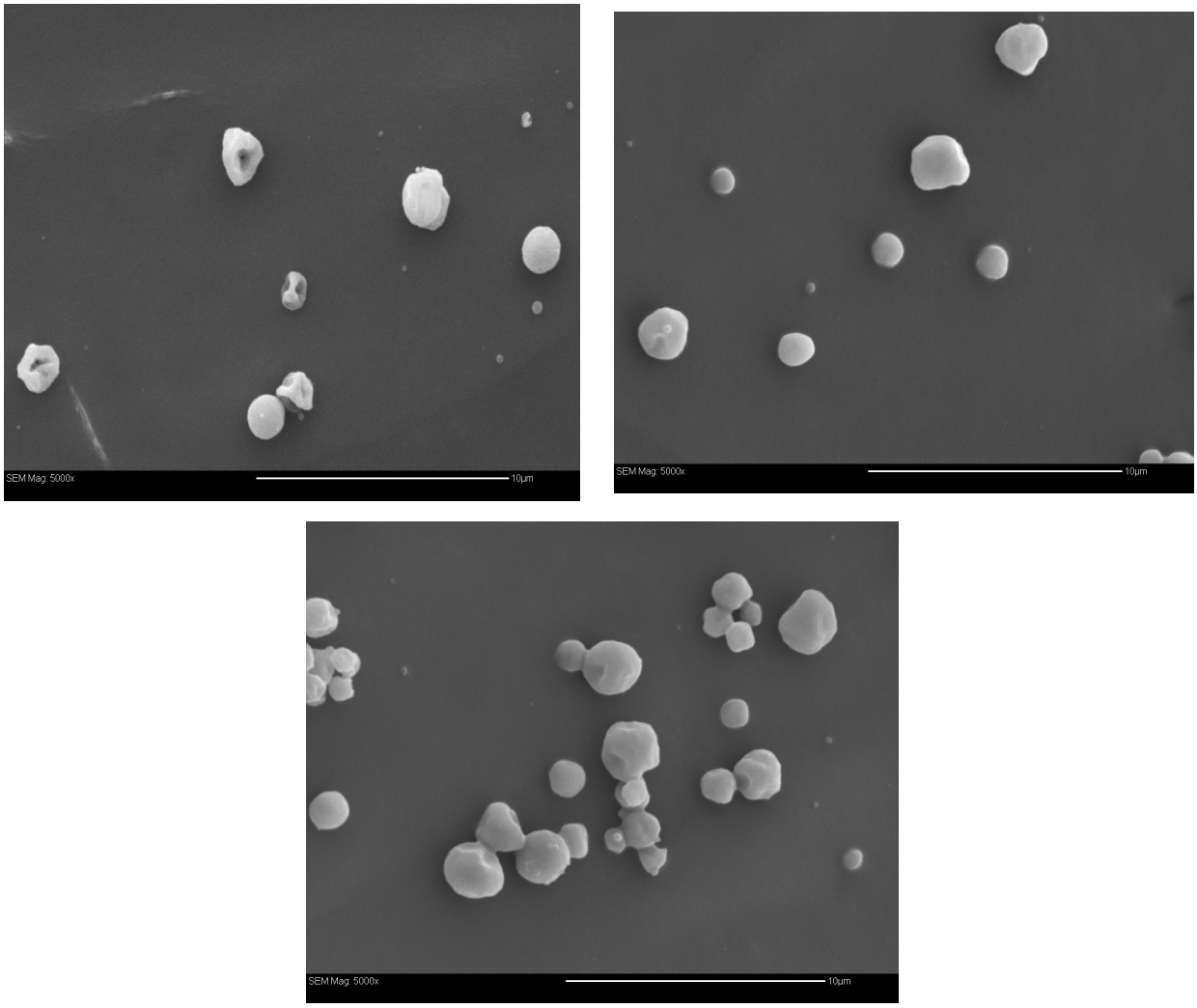

Figure 4.2.5. SEM images of protein loaded particles. The scale bar represents 10 microns. The particles are loaded with lysozyme (a), subtilisin (b) and BSA (c).

4.2.5 Protein Distribution Within the Particles

The distribution of the protein throughout the polymer matrix was examined by confocal laser scanning microscopy with florescent labeled BSA, as shown in Figure 4.2.6. The particles show a donut-like structure with hollow center, and protein deposition toward the particle surface. This might be due to evaporation of the water

from the droplet surface, carrying solutes from the particle core to the surface during formation.

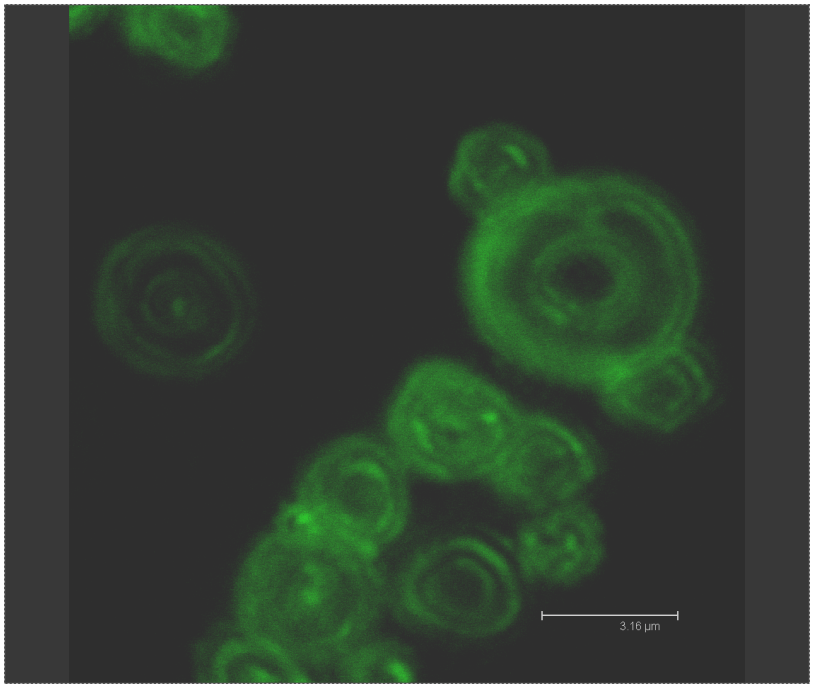

Figure 4.2.6 Confocal laser scanning microscope image of glycol-chitosan alginate micro- and nano particles carrying BSA. The green sections represent the presence of FITC labeled BSA. Scale bar represents 3 µm.

As mentioned earlier, in lab scale spray dryers, the time required to dry a single atomized droplet is expressed in milliseconds. During that extremely short period of time, solutes such as proteins can be assumed to be stationary during the formation of the particles, however this may not be the case for formulations where total solid concentration is low and the selected encapsulation matrix material can form porous networks, such as the present alginate/protein (BSA) system.

During the formation of particles in spray drying, two characteristic times are important, the first being the time required for a solute to diffuse from the edge of the

droplet to it's center, defined by R^2/D, where R is the radius of the droplet and D is the solute diffusion coefficient, and second being the total drying time, t_d, for a droplet. The ratio of these two characteristic times defines the peclet number, $Pe = R^2/ t_d D$ which characterizes the relative importance of diffusion to convection. If the drying of a droplet is sufficiently slow, Pe<1, and solutes have time to diffuse throughout the droplet, yielding relatively dense particles. When Pe>1 the particles have insufficient time to diffuse from the surface to the center of the droplet (Tsapis *et al.,*2002). Also when Pe>1, the water molecules must diffuse from the center to the surface, since evaporation only occurs from the surface, carrying along solutes from within the droplet towards the surface. Therefore when Pe>1, the diffusion of the solutes from the center to the surface of the particle might be more favorable than diffusion of solutes from the surface to the center, due to the mobile phase, water, which is diffusing from the particle core to the surface. This would be a stronger possibility, if the solid concentration is low. In addition, the encapsulation matrix material, which tends to form porous polymeric network structure, can also play an important role in the final distribution of the solutes within the particle matrix. For example, if the proteins have a smaller size than the pores of the encapsulated polymeric network, as the water migrates to the surface for evaporation, the protein can migrate as well without interruption.

For the present system, the total drying time of the droplet was calculated as 8.5 ms, whereas the diffusion coefficient of the BSA molecule was estimated to be 990 $\mu m^2/s$. Pe was calculated as 240, (calculations appear in sections to follow) which indicates that for proteins, the driving force is dominated by convention, instead of diffusion. It might be concluded that the proteins which were at the surface of the droplet during formation of the particle, did not have sufficient time to diffuse from the surface to

the center. Moreover, the mobile phase, water, continuously diffused from the center to the surface of the droplet, while it carried the other solutes, such as polymer chains and proteins as illustrated in Figure 4.2.7. The hollow region at the center of the particles also confirms this theory that the other solutes, such as polymer chains, were being drawn outwards during evaporation, forming a hollow core.

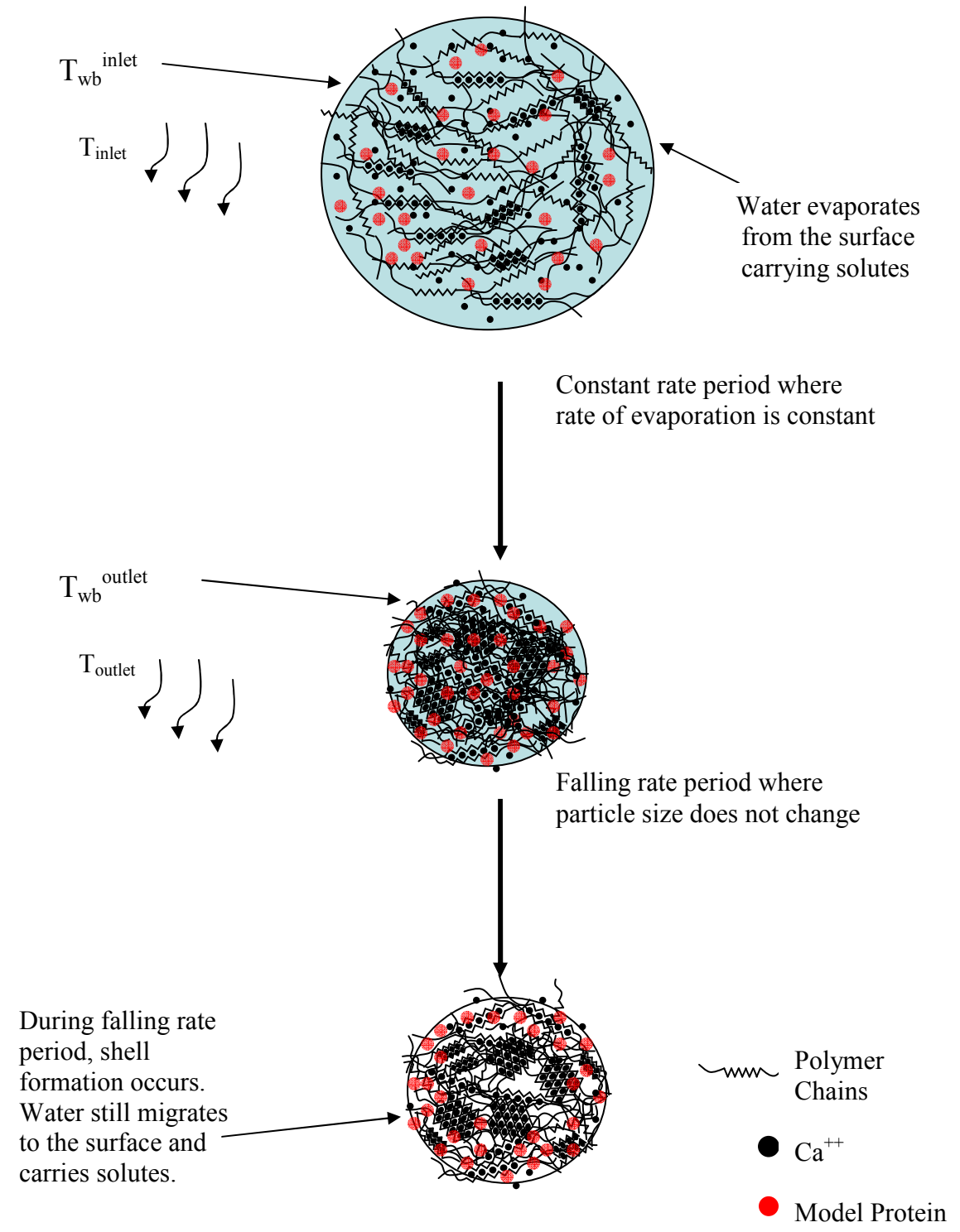

Figure 4.2.8 Schematic illustration of particle formation in spray drying and the nature of protein deposition at the surface of the particles.

In order to discuss protein distribution within the particle, the Peclet number was calculated. The total drying time of a single droplet was calculated through a series of heat and mass balance equations, and diffusion coefficient of the protein solute, was estimated through the Stokes-Einstein equation.

4.2.5.1 Estimation of Total Drying Time of Single Alginate Droplet.

The drying of a spray droplet containing low concentration of alginate (0.3%) can be divided into two phases, initial constant rate period and falling rate period. Previously total drying time of a droplet was calculated by Adler and Lee (1999), during these calculations heat and mass transfer equations presented in Master's (1991) was used. In order to estimate the Pe number of the alginate droplets during spray drying process the regarding equations was used. In the initial constant rate period, the rate of evaporation was assumed to be constant, whereas in the falling rate period, the particle size was assumed to be constant due to the crust present at the droplet surface (Masters,1991). In the initial constant rate period, water concentration at the droplet surface and hence the rate of evaporation, dW/dt was calculated with the regarding equation;

$$\frac{dW}{dt}\Big|_{cr} = \frac{2\pi k_d D_{av} \Delta T}{\lambda}$$

where, ΔT is the log mean temperature difference;

$$\Delta T = \frac{\left(T_{inlet} - T_{wb}^{inlet}\right) - \left(T_{outlet} - T_{wb}^{outlet}\right)}{\ln\left[\left(T_{inlet} - T_{wb}^{inlet}\right)/\left(T_{outlet} - T_{wb}^{outlet}\right)\right]}$$

T_{wb}^{inlet} is the wet bulb temperature of the spray droplet surface and T_{wb}^{outlet} is the web-bulb temperature of the dried particle surface. For the low solids alginate system T_{inlet} = 150 C, and the enthalpy humidity chart of water can be used. T_{inlet} = 150 ^{o}C and thus T_{wb}^{inlet} = 41 ^{o}C, from the enthalpy-humidity chart. For T_{outlet} = 85 ^{o}C, T_{wb}^{outlet} = 55 ^{o}C. The ΔT is determined to be 61 ^{o}C. The thermal conductivity of the water vapor, k_d in the stagnant layer around the droplet is 0.565 kcal/m h ^{o}C at ΔT =61 ^{o}C.

D_{av} is the average droplet/particle diameter (D_d/ D_p). The droplet diameter can be estimated through a back calculation, since the final mean diameter of the particle is known, 3.5 μm. It can be assumed that the total solid concentration within the droplet is the same as the solid concentration in the feed solution, 0.3%,w/v. The droplet diameter could be calculated, if the weight of the particle is known. By using the confocal laser scanning microscope image of a single particle, the weight of the single alginate nano particle, W_p, can be estimated through the following equation;

$$W_p = 4/3\ \pi\ (D_p^3 - D_h^3)/8\ \rho_a$$

where D_p is the particle diameter, 3.5 μm and D_h is the diameter of the hollow region, 1 μm , and ρ_a is the bulk density of the alginate, 754 kg/m^3 (Sriamornsak and Sungthongjeen, 2007). W_p was found to be 3.63 10^{-14} kg. The diameter of the droplet, D_d

can be calculated as follows. If the initial feed solution total concentration is 0.3%,w/v, what is the volume required to produce a particle weighing 3.63 10^{-14} kg.

$$V_d = \frac{4}{3}\pi(r_d)^3 = \frac{3.63*10^{-14}\,kg}{0.3g}*100ml = 12.1*10^{-9}\,cm^3$$

$$r_d = 14.2\mu m$$

$$D_d = 28.4\mu m$$

By averaging the D_d and D_p which are assumed to be 28.4 μm and 3.5 μm respectively, D_{av} = 16 μm. λ is the latent heat of vaporization of water = 540 kcal/kg. The evaporation rate can be calculated to be;

$$\frac{dW}{dt}\Big|cr = \frac{2\pi k_d D_{av}\Delta T}{\lambda}$$

$$= \frac{2\pi * 0.565\ \text{kcal/m h}\ ^oC * 16\mu m * 61^oC}{540kcal/kg}$$

= 6.4 kg 10^{-6} /h

The total mass of water removed, ΔW_{cr} , in constant-rate period can be calculated through;

$$\Delta W_{cr} = \frac{4}{3}\pi(r_w^3 - r_d^3)\rho W_1$$

where r_d is the droplet radius, r_p is the particle radius, ρ is the density of the alginate/water solution equal to 996 kg/m^3 . r_w is the diameter of the droplet and assumed to be 10 μm and r_d = 3.5 μm. W_1 is the water content of the spray solution (0.997kg/kg). W_{cr} can be calculated;

$$\Delta W_{cr} = \frac{4}{3}\pi(r_d^3 - r_p^3)\rho W_1$$

$$= \frac{4}{3}\pi(14.2^3\,\mu m^3 - 1.875^3\,\mu m^3) * 996\text{kg/m3} * 0.997\text{kg/kg}$$

$$= 1.185\ 10^{-11}\ \text{kg}$$

The drying time of the droplet in the constant rate period, t_{cr}, can be calculated by (Masters,1991) ;

$$t_{cr} = \frac{\Delta W_{cr}}{dW/dt|cr} = 1.185\ 10^{-11}\ \text{kg} / 6.4\ 10^{-6}\ \text{kg/hr} = 6.7\ \text{ms}$$

During the calculation of the falling rate, the crust or solid shell formation occurs and it is assumed that the particle size does not change further, and the rate of evaporation is given by $dW/dt|fr$;

$$\left.\frac{dW}{dt}\right|_{fr} = \frac{dW}{dt} * \text{wt of dry particle}$$

where,

$$\frac{dW}{dt} = \frac{12k_d\Delta T}{\lambda D_c^2 \rho_s}$$

and k_d a is the thermal conductivity of the drying air around the particle, 0.0236 kcal/h m ^oC at $\Delta T = 61^oC$. D_c is the critical particle diameter which is equal to the final diameter of the particle, 3.5 μm. ρ_s is the average particle density during the falling rate period, and the particle density is assumed to be 773 kg/m^3, by volumetric average of the water and alginate content of the particles at the critical point. dW/dt can be calculated as follows;

$$\frac{dW}{dt} = \frac{12k_d\Delta T}{\lambda D_c^2 \rho_s} = \frac{12 * 0.0236 kcal/h.m^oC * 61^oC}{540 kcal/kg * (3.5\mu m)^2 * 774 kg/m^3} = 937/s$$

$$\frac{dW}{dt}\Big|fr = 937/s * 3.63 * 10^{-14} kg = 3.4 * 10^{-11} kg/s$$

The total mass of water removed during the falling rate period can be calculated by difference in initial weight of the droplet (W_{in}) and the water removed during constant period (ΔW_{cr}) and the final weight of the particle (W_p).

$$\Delta W_{fr} = W_{in} - (\Delta W_{cr} + W_p)$$

For a droplet having diameter of 28.4 μm, where ρ is the density of alginate/water solution, 996kg/m^3.

$$W_{in} = 4/3\ \pi\ (D_d/2)^3\ \rho$$
$$= 1.195\ 10^{-11}\ kg$$

$$\Delta W_f = 1.195\ 10^{-11}\ kg - (1.185\ 10^{-11}\ kg + 3.63\ 10^{-14}\ kg)$$

$= 6.37\ 10^{-14}$ kg

$$t_{fr} = \frac{\Delta W_{fr}}{dW/dt\big|fr} = 6.37\ 10^{-14}\ \text{kg}\ /\ 3.4\ 10^{-11}\ \text{kg/s} = 1.87\ \text{ms}$$

The total time required to dry a nano alginate particle is

$t_d = 6.7\text{ms} + 1.87\text{ms} \sim 8.5\ \text{ms}$

4.2.5.2 Estimation of Time Required for a BSA Molecule to Diffuse from the Surface to the Center of the Droplet

The diffusion of BSA within the droplet can be calculated by Stokes-Einstein Equation:

$$D_s = \frac{k_B T}{6\pi\eta R_H}$$

where k_B is Boltzman's constant, η the viscosity of the solvent (water), T is the temperature and R_H is the hydrodynamic radius of the solute.

For BSA system

$k_B = 1.3806503 \times 10^{-23}\ \text{m}^2\ \text{kg}\ \text{s}^{-2}\ \text{K}^{-1}$

T = 41+85 / 2 = 63 ^{o}C = 331.3 K (the average of wet bulb temperature and outlet temperature)

η = 0.544 cp at 63 ^{o}C

R_H = 4.5nm acquired from Böhme and Scheler, 2007

$$D_s = \frac{1.3806503\times10^{-23}\ \frac{\text{m}^2\ \text{kg}}{\text{s}^2 * K} * 331.13\ \text{K}}{6\pi * 0.544\ \frac{\text{g}}{\text{m}\cdot\text{s}} \cdot * 4.5x10^{-9}\ m} = 99\ \mu\text{m}^2/\text{s}$$

4.2.5.3 Calculation of Peclet Number

$Pe = R^2 / t_d D$

Where R is the radius of the droplet and td is the total drying time of the droplet and D is the diffusitivty constant of a BSA molecule.

$Pe = (14.2\mu m)^2 / (99\ \mu m^2 / s * 8.5ms) = 240$

4.2.6 Comparison of Present Study with a Previous Method

As mentioned, several methods have been proposed to produce micro- and nano particles by spray drying. But the proposed methods involved use of organic solvents and several process steps. Previously, Coppi *et al*.,(2001) proposed production of alginate micro- and nano particles by spray drying for oral drug delivery purposes. However, the proposed method included several additional steps to enhance properties of the alginate particles, including crosslinking the particles in $CaCl_2$ aqueous solution and treatment of the particles with chitosan to reinforce alginate network and a final lyophilization step was used for recovery. (schematic description of the process illustrated in Figure 4.2.1). Several attempts were made in the present investigation to reproduce the method proposed by Coppi *et al.,* 2001. During aqueous treatment of the particles with calcium and chitosan, particle aggregation and disintegration were observed. Furthermore, the lyophilization step altered the morphology of the particles and fabric like structures were formed. The Coppi method was difficult to reproduce due to additional steps involved. Moreover, the published data showed around 30 % protein release during these additional steps.

The current proposed method and the previously published method are compared in Table 4.2.4. The present study and the previously proposed methodology are compared based on the model protein, BSA, which was used in both protocols.

Table 4.2.4. The comparison of current proposed method with Coppi *et al.,* 2002.

	Coppi *et al.*, (2002)	**Present Study**
Total Process Time	14 hours	100 minutes
Final Protein Loading	63 %	94 %
Total Protein Released in Simulated Stomach Environment	30 %	35 %
Mean Particle Size	2.5 μm	3.5μm

The total time required to produce 2 grams of micro- and nano alginate particles carrying proteins. Assuming 12 hour lyophilization process.

The present proposed method was much faster being a single step protocol, thus it is relatively easier to reproduce. The two methods showed similar release profiles in simulated stomach environment, around 30% of the encapsulated BSA was released in the previous work done by Coppi *et al.,* 2002, whereas in the present study around 35 % protein release was observed. However, it should be noted that in the present study the protein association of the particles is around 30 % higher, thus around 30% more protein is likely to be carried to the intestinal environment. For both systems, the mean particle size (<5μm) was within the desired range for oral absorption across the intestinal epithelia. The comparison of the two system shows that the present single step protocol is more efficient, in terms of process time and protein encapsulation.

CHAPTER 5.0 CONCLUSIONS

Stable calcium alginate/chitosan protein-loaded micro- and nanoparticles were produced using a proposed single step methodology involving the spray drying of a polymer/protein solution. This method eliminates the need for toxic solvents and multiple processing steps involved in alternative methods, and leads directly to a stable dry product within a short processing time. The following findings and conclusions represent contributions to our understanding of the production of alginate micro- and nanoparticulate proteins by spray drying.

1 Increase in inlet air temperature up to 175 ^{o}C of the spray dryer did not significantly affect subtilisin residual activity, but improved the product yield. The highest particle yield of 40% was achieved at 175 ^{o}C and highest activity yield of 80% achieved at 150 ^{o}C. Particle yield was also found to be dependant on the feed rate, with highest yield at 5 mL/min.

2 The moisture content of the spray dried particles was found to be dependent on the inlet temperature of the drying air. The moisture content was 7 % for T_{inlet} = 125 ^{o}C, whereas it was decreased to 5.5 % for T_{inlet} = 150 ^{o}C and 175 ^{o}C.

3 Subtilisin residual activity increased with increase in alginate:subtilisin ratio. Presence of alginate in the feed solution increased the residual activity of the subtilisin by more than 50% compared to spray drying of free subtilisin feed solution. The particles containing 0.33 g subtilisin/ g particle, exibited more than 65 % residual activity. The encapsulation yield of subtilisin was more than 95%.

4. The formulations containing 0.2g trehalose /g showed residual activity of 90%. Increase in the amount of trehalose in the formulation (0.33g trehalose /g particle) did not significantly affect the residual activity.

5. Particles formulated with 0.2 g trehalose / g particle exhibited greater protein stability than the formulations without presence of trehalose, with over 4 months of storage, maintaining more than 80% of their initial activity. The formulations prepared with higher subtilisin:alginate ratio showed less protein stability during the shelf life experiments.

6. Spray dried particles showed spherical morphology. The larger particles showed more spherical morphology, whereas smaller particles were dimpled. Trehalose loaded particles showed a smoother surface.

7. The alginate concentration in the feed solution affected the particle size. Particles prepared with less alginate had smaller mean diameter and narrower size distribution. The size ranged from 2.51 μm to 4.91μm, which was within the desirable range for absorption across the intestinal mucosa.

8. Chitosan-Ca-alginate particles were produced in a single step process by spray drying. The mean diameter was around 3.5 μm and particles had spherical morphology and smooth surface. The particles showed 35 % protein release in a simulated stomach environment.

9. In *vitro* release profiles of the resulting particles did not show significant variation for different molecular weight proteins.

10. The confocal laser scanning microscopy image of the particles showed the presence of fluroscent-labeled protein towards the outer surface of the particles. The particles had a donut shaped structure with a hollow central region.

11. Peclet number and drying time was calculated for the particles and found to be 340 and 8.5ms. These results show that during particle formation, convection was more dominant than diffusion of the solutes, leading to the formation of a hollow central region and protein distribution toward the surface of the particle.

12. The smallest mean particle size of 2.5 μm was achieved with 0.2% alginate feed concentration with the spray drying operation conditions; Q_{lf} = 5mL/min, Q_{da}= 38, m^3/h, Q_{aa} = 600 L/h, P = 80 psi. The formulation consisting of an alginate:trehalose:subtilisin ratio of 7:2:1 gave the highest subtilisin residual activity.

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7.0 APPENDIX

7.1 Calculation of Residual Activity of Subtilisin with Alginate micro particles after spray drying.

The catalytic activity of subtilisin was determined spectrophotometrically at 410 nm with 1 mg/ml N-succinyl-L-Ala-L-Ala-Pro-L-Phe-p-nitroanaline as substrate in 100 mM Tris, 0.005 % Tween 80 pH 8.6. The activity was also expressed in international units (IU), which is defined as μmoles of substrate hydrolyzed per minute at 25 ^{o}C and pH 8.6. The activity of the protease was determined through Beer-Lambert law.

$$A = \varepsilon c l$$

$$\Delta C/\Delta t = \Delta A/ \varepsilon l$$

$$\Delta C/\Delta t = \text{slope} / \varepsilon l$$

A is the absorbance reading, ε is the molar extinction coefficient (8480 M^{-1} cm^{-1} for subtilisin), c is the concentration of product and l is the optical path length of the cuvette. (1cm)

7.2 Determination of Activity of Subtilisin within Microparticles

Initially 1mL of tris buffer solution was added to cuvette. The cuvette was placed into a 25 ^{o}C waterbath (set point 25.2 ^{o}C) and incubated for 10minutes. Then 10 μL of N-succinyl-L-Ala-L-Ala-Pro-L-Phe-p-nitroanaline stock solution was added. Around 20mg

of particles, having 0.1g subtilisin/g particle was dissolved in tris buffer system (at 25 ^{o}C). 10 μL of enzyme solution was sampled and added to the cuvette for sphectrometrically determination of the activity. Also a standard cuvette was placed in the blank photometers cell. Absorbance readings were taken at 0.33 sec time intervals and overall data acquisition was 1minutes.. Slope of the absorbance vs time curve was determined for the linear abs range of 0.2-0.8. The residual activity of the particle can be determined by dividing the acitiviy of the stock solution by the experimental activity of the subtilisin after spray drying.

7.3 Sample Calculation for Activity of the Subtilisin Stock Solution

$\Delta C/\Delta t = \Delta A/ \varepsilon l$

$= slope / \varepsilon l$

$= 0.81 / (8480 * 1)$

$= 0.0000963$ M/min

$= 0.0963$ mM/min

Total Volume in cuvette is 1020 μL

Reaction Rate = 0.0963 mM/min *1020 μL = 0.0982 μmole/min

10 μL sample can convert the syntetick protein subtrate into 0.0982 μmole/min product per min, 1 L protease can catalyse:

$= 0.0982$ μmole/min (1L/10 μL)

$= 9820$ μmole/min/L

The activity can be also expressed as in international units (IU), which can be defined as the amount of enzyme that catalyzed the production of 1μmoles of p-nitroaniline per minute at 25 ^{o}C in 100Mm Tris/HCl buffer at pH 8.6.

= 9820 μmole/min/L protease = 9820 IU/L

= 9.82 IU/mL

The dilution fold applied to the enzyme was 1981

The protease activity of the stock solution = 9.82 IU/mL * 1891= 18569 IU/ml

7.4 Sample Calculation for T_{inlet} = 150 ^{o}C, with 0.1 g subtilisin/g particle.

The same calculation procedure can be followed to determine the experimental Activity of the subtilisin encapsulated within the microparticles. For T_{inlet} = 150 ^{o}C, with 0.1 g subtilisin/g particle. The slope was determined to be 0.89 26 mg of particles dissolved in 10mL tris/HCl buffer solution.

$\Delta C/\Delta t = \Delta A/ \varepsilon l$

= slope / ε l

= 0.89(8480 *1)

= 0.0001049 M/min

= 0.1049 mM/min

Total Volume in cuvette is 1020 μL

Reaction Rate = 0.1049 mM/min *1020 μL = 0.1070 μmole/min

10 μL sample can convert the syntetick protein subtrate into 0.1070 μmole/min product per min, 1 L protease can catalyse:

= 0.1070 μmole/min (1L/10 μL)

= 10700 μmole/min/L

The activity can be also expressed as in international units (IU), which can be defined as the amount of enzyme that catalyzed the production of 1μmoles of p-nitroaniline per minute at 25 ^{o}C in 100Mm Tris/HCl buffer at pH 8.6.

= 10700 μmole/min/L protease = 10700 IU/L

= 10.70 IU/mL

The dilution of the sample can be calculated through the protein loading and the dissolution volume of the medium. 26mg of particles was dissolved in 10 mL of tris/HCl buffer system, where the particles had a protein loading of 0.1g subtilisin/g particle. Protein concentration of the sampled solution can be calculated as;

= 0.1 g subtilisin/g particle * 26mg particle / 10mL

= 0.26mg/mL

The initial stock solution of the enzyme was 359mg /mL.

The dilution fold is found to be; = 359mg/mL / 0.26 mg/mL = 1380 times

The protease activity of the original solution = 10.70 IU/mL * 1380 = 14774 IU/mL

Residual activity % = (experimental activity/ stock solution activity)*100

= (14774 IU/mL / 18569 IU/ml)*100

= 79.5 %