Microencapsulation of Protein with EUDRAGIT S 100 Polymer

by

Dan Li

School of Chemical Engineering
The University of Adelaide

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SUMMARY

Lactose intolerance is a common and inconvenient medical condition and can cause severe discomfort. People who experience lactose intolerance often take lactase enzyme supplements when they wish to consume dairy products. As a consequence, they normally consume dairy products that are rendered lactose free or else a lactase enzyme supplement is taken concurrently. Normally, these are pills or tablets that dissolve and release the enzyme in the stomach. However, the enzyme may be denatured in the low pH conditions of stomach. Hence, a higher dose is required to ensure that an effective concentration can survive and pass into the small intestine – the site of the enzyme's physiological action. This problem is being addressed by microencapsulation methods: surrounding the enzyme with protective materials in the form of small particles. These protect the enzyme in the stomach and allow release in the small intestine.

The goal of this research was to investigate an appropriate microencapsulation method for this purpose. An oil-in-oil solvent evaporation method was used to produce microparticles containing BSA protein with a EUDRAGIT S 100 – methacrylic acid and methyl methacrylate copolymer. BSA was used as a cost-effective surrogate for lactase during the research. Sonification was employed during the emulsification step. The microparticles produced at different sonication amplitudes or power outputs were uniform with similar morphologies, typically spheres. Microparticle size decreased with sonicator energy output from 120 μm to 12 μm as the amplitude changed from 40% to 70%. The encapsulation efficiency at amplitude levels of 50%, 60% and 70% was between 70% and 80%. However, the encapsulation efficiency recorded at the 40% setting was much lower, around 40%. The release profiles of those microparticles were studied at different pH. There was a slight leakage from the microparticles at low pH. Above pH 7, total release was achieved within 2 hours. The results of this research confirm that the microparticles could encapsulate lactase as part of a treatment of lactose intolerance.

ACKNOWLEDGEMENTS

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1 INTRODUCTION

Many populations and ethnic groups, particularly in Asian countries are lactose intolerant: they possess insufficient lactase enzyme necessary to digest lactose, the principal sugar in dairy products. Lactose intolerance is a difficult medical condition to manage and may cause severe discomfort. As a consequence, dairy consumption must be limited unless the products are rendered lactose free or lactase enzyme supplements are ingested simultaneously.

Presently, the sole commercially available lactose-free dairy product is milk. This product has only recently become available in several European countries. Additional processing to remove the lactose incurs significant expense. This limits commercial acceptance. Lactase enzyme supplements have been available for several decades and are widely used. They are normally in pill or tablet form, and must be consumed concurrently with the dairy product. Unfortunately, the enzyme may be denatured in the low pH conditions of the stomach. This necessitates a higher dose to ensure an effective concentration survives and passes into the small intestine – the site of the enzyme's physiological action. This problem is being addressed by microencapsulation methods: encapsulating the enzyme with protective materials normally as a small particle. The protective material protects the enzyme in the stomach, then allows release from the particle in the small intestine.

The goal of this research is to apply a suitable coating material, and to investigate a microencapsulation method that could be used to achieve this purpose for the lactase enzyme. Chapter 2 of this thesis previews the relevant literature, including the prior art in the microencapsulation of lactase. Chapter 3 describes the experimental methods used. A suitable encapsulating material and microencapsulation method were selected from the literature. Bovine serum albumin (BSA) was used as a protein surrogate for the lactase enzyme to minimize the cost of the research. The microencapsulation method involved an emulsification step. Unlike other relevant studies, sonication was employed for this step. Chapter 4 presents and discusses the results that were obtained. Part of the investigation involved understanding under what condition and at what rate the

microparticles released the BSA. Conclusions of this study and recommendations for further work are presented in Chapter 5 and 6.

2 LITERATURE REVIEW

The goal of this project is to develop and to gain experience with an existing microencapsulation technique that may be suitable for the oral delivery of lactase supplements. This chapter reviews the literature relevant to this project. The problem of lactose intolerance and the existing treatment strategy involving lactase supplements are briefly described. The potential for microencapsulation to resolve the current disadvantages of lactase supplements is addressed. Previous work investigating the microencapsulation of lactase is reviewed. The suitability of utilising an alternative and cheaper protein (BSA) as a surrogate for lactase during development of a microencapsulation technique is considered.

2.1 Lactose Intolerance

Lactose intolerance (LI) is the inability to digest lactose. Lactose, or β-galactosidase, is a disaccharide with one glucose molecule bound to one galactose molecule (Rutgers University, 2002) (Figure 2.1). This type of sugar is present in milk and dairy products. An individual's inability to digest lactose results from a shortage of the enzyme lactase. It is normally produced by the cells that line the small intestine (Marks & Lee, 2003). Lactase is a protein with a molecular weight of 116 kDa and an isoelectric point of approximately 4.6 (Roche Diagnostics GmbH, 2000; Sigma-Aldrich, 2004). It plays a vital role in metabolism, because it breaks down milk sugar into simpler forms that can then be absorbed into the bloodstream to provide an energy source. When insufficient lactase is available to digest the lactose consumed, the results, though never serious, may be very annoying and troublesome. When undigested lactose reaches the colon, it is degraded by bacteria into lactic and other acids, which create the symptoms of LI. These symptoms include nausea, abdominal cramps, rumbling, bloating, rectal gas (flatus) and watery diarrhea. These symptoms occur about 30 minutes to 2 hours after eating or drinking foods containing lactose (National Digestive Diseases Information Clearinghouse, 2004). The severity of symptoms varies depending on the amount of lactose each individual can tolerate. Not all individuals who are deficient in lactase acquire such symptoms; those who do are considered to be LI.

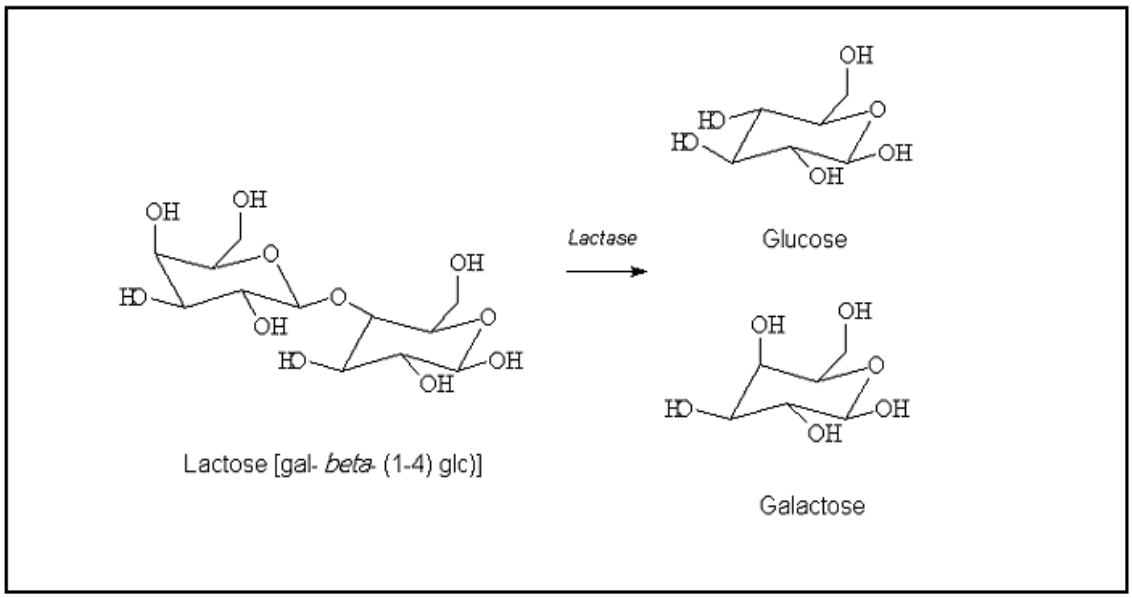
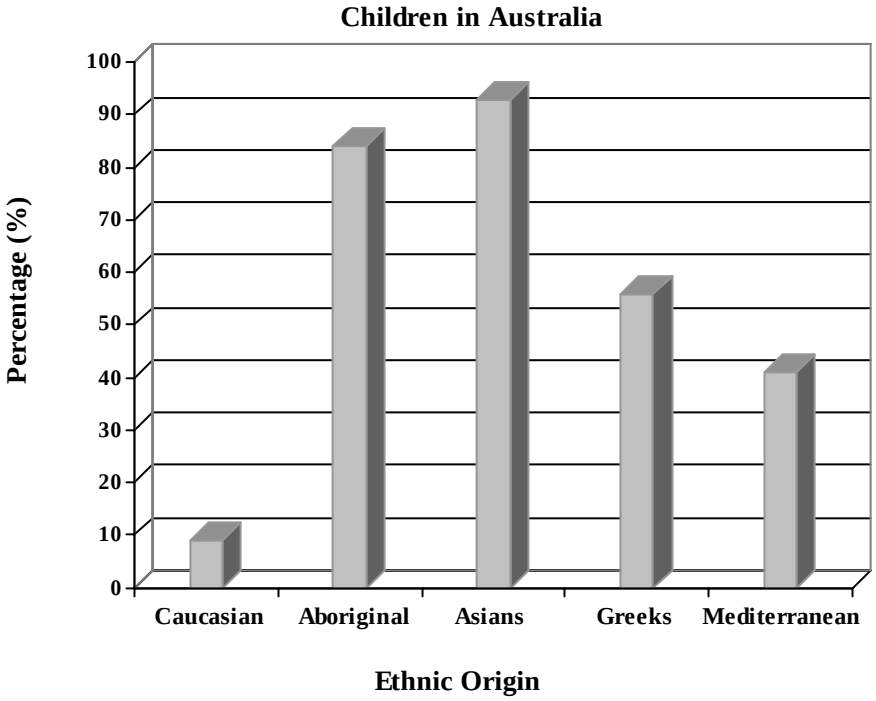


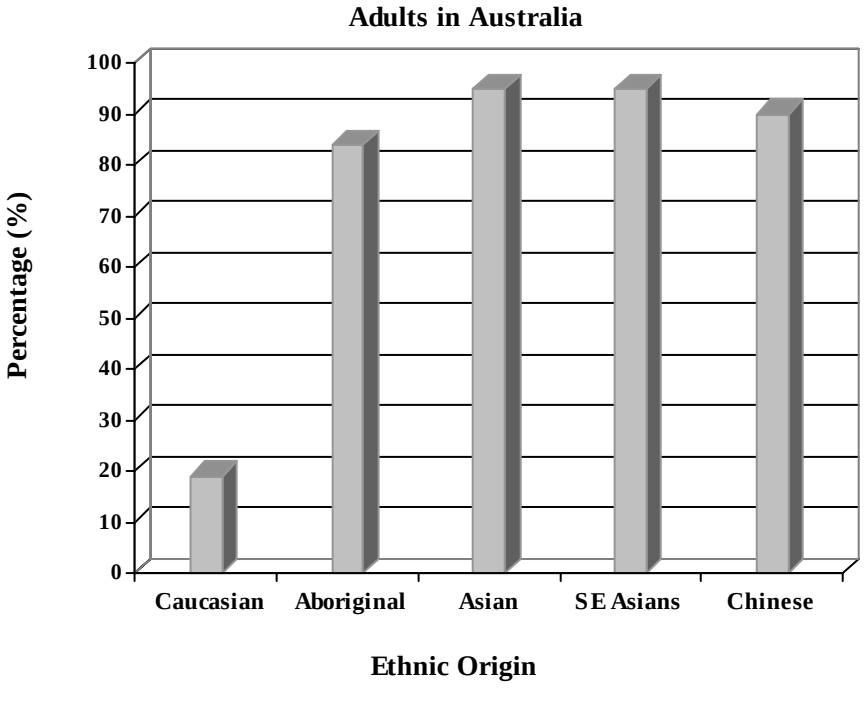
Figure 2.1: Overall chemistry of lactose digestion.
(Reproduced from University of Illinois (2002))

Some causes of LI are well known. For example, certain digestive diseases and injuries to the small intestine may reduce the amount of enzymes produced (National Digestive Diseases Information Clearinghouse, 2004). Generally speaking, infants and children have lactase to digest mother's milk. As they grow, the level of enzyme begins to decrease in many individuals. Normally, an individual does not experience LI until they are quite old.

Certain ethnic and racial populations are more prone to develop LI (Stafne, 2004). More than ninety percent of Asians, both adults and children, face this problem (Figure 2.2). Eighty-five percent of Australian Aboriginals are LI. One half of the Greek and Mediterranean children in Australia possess insufficient lactase to digest lactose. In addition, roughly twenty percent of Caucasians fail to produce sufficient lactase as adults.



(a)



(b)

Figure 2.2: Lactose intolerance among different ethnic origins in Australia: (a) children in Australia; (b) adults in Australia.

(Reproduced from Stafne (2004))

2.2 Treatments for LI

Currently, a diverse range of treatments is suggested to deal with LI, depending on the patients. The most common method for very sensitive individuals is an elimination diet. Patients avoid lactose-containing foods which include dairy products as well as bread, baked goods, cereals, instant potatoes, lunchmeat, salad dressings, etc. (Rutgers University, 2002). However, dairy products are a major source of calcium and are necessary for good heath and strong bones. Calcium deficiency may lead to osteoporosis and bone fractures (Marks & Lee, 2003). Hence, dairy products are important for human's daily needs.

The second option is to consume lactose-reduced dairy products (National Digestive Diseases Information Clearinghouse, 2004). The only widely available commercial example of this is lactose-reduced milk. Presently, this food is available at many supermarkets. However, the current treatment to remove lactose from milk is by addition of lactase enzyme. The commercial products are expensive, and thus cost limits public acceptance. Following treatment, the taste of the milk is significantly sweeter, because the lactose is hydrolysed to glucose and galactose. Glucose and galactose are roughly four times sweeter than lactose (Onwulata *et al.*, 1989; Solomon *et al.*, 1985). Unfortunately, this increase in sweetness tends to be undesirable for many consumers.

Another means for reducing the symptoms of LI is ingestion of chewable lactase tablets, capsules, or softgels prior to ingestion of milk-containing foods (Alavi *et al.*, 2002). Such preparations are available without a prescription to help people digest foods containing lactose. Unfortunately, the efficacy of chewable tablets containing lactase is often poor. The stomach's strong acid pH may denature lactase. Furthermore, other enzymes in the gastrointestinal (GI) tract may destroy lactase before it reaches the small intestine (Alavi *et al.*, 2002) – the natural site of lactase's physiological action. This problem is presently being addressed by "microencapsulation" strategies. This involves encapsulation of the enzyme with protective materials to form microparticles. The protective material protects the enzyme from denaturation in the stomach, and releases the enzyme from the microparticles in the small intestine. The property of GI tract, especially pH, is an important factor to consider in the design of the microparticles produced in this study.

2.3 The GI Tract

The GI tract includes the oesophagus, stomach, small intestine, large intestine or colon, rectum, and anus, as depicted in Figure 2.3. The stomach and the small intestine are the two key sections of interest in this work.

The pH varies widely from the stomach to the small intestine. The pH of gastric juice in the stomach may range from 1.0 to 2.5. Evans *et al.* (1988) demonstrated that pH rose as material passed from the stomach to the end of the small intestine. The mean pH in the proximal part was measured to be 6.6 (±0.5) during the first hour of intestinal recording and that in the terminal ileum was 7.5 (± 0.4).

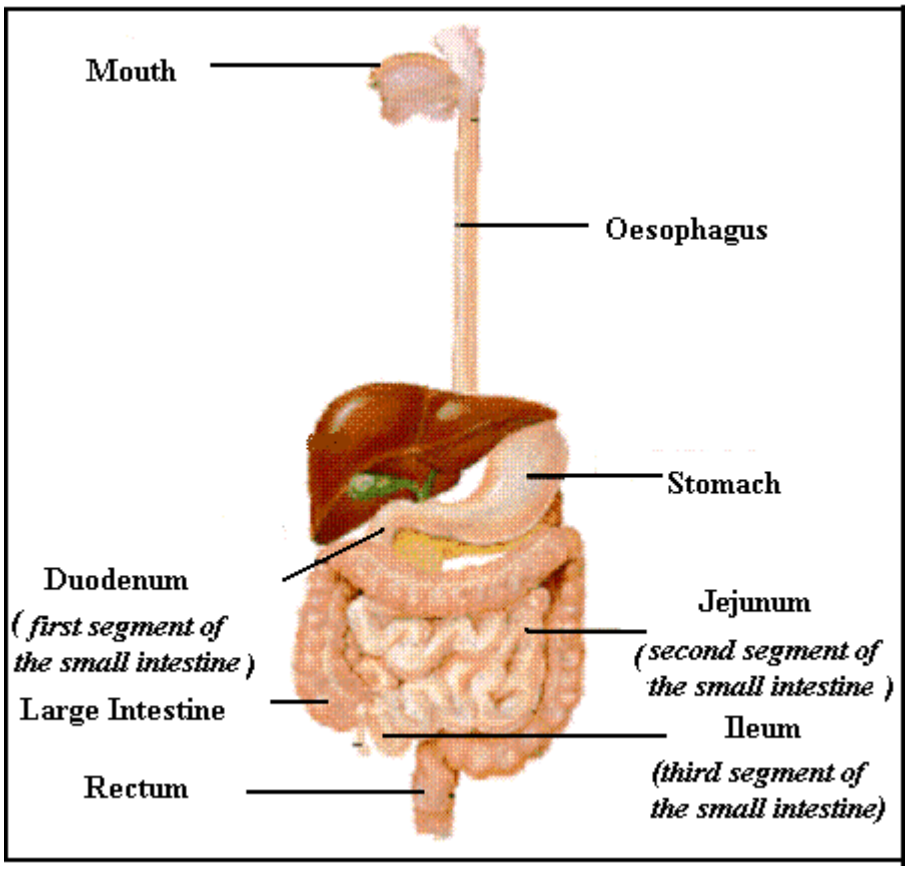


Figure 2.3: The gastrointestinal tract (GI tract).
(Reproduced from University of Texas (1996))

The residence time for food along the tract is influenced by numerous physiological and other factors; nevertheless, there are some generally accepted GI residence values for

various sections of the tract. The residence time in the stomach is constant and equal to 120 minutes. In the small intestine, there is a difference in the mean transit times, but it is roughly 3 hours (ranging from 1 to 6 hours) (Versantvoort *et al.*, 2004). Normally, a mean transit time of 3 hours for food digestion is assumed (Coupe *et al.*, 1991; Davis *et al.*, 1986; Davis *et al.*, 1984). Figure 2.4 summarizes these details. The transit time is approximately 10 minutes for the duodenum, 2 hours in the jejunum, and 1 hour for the ileum.

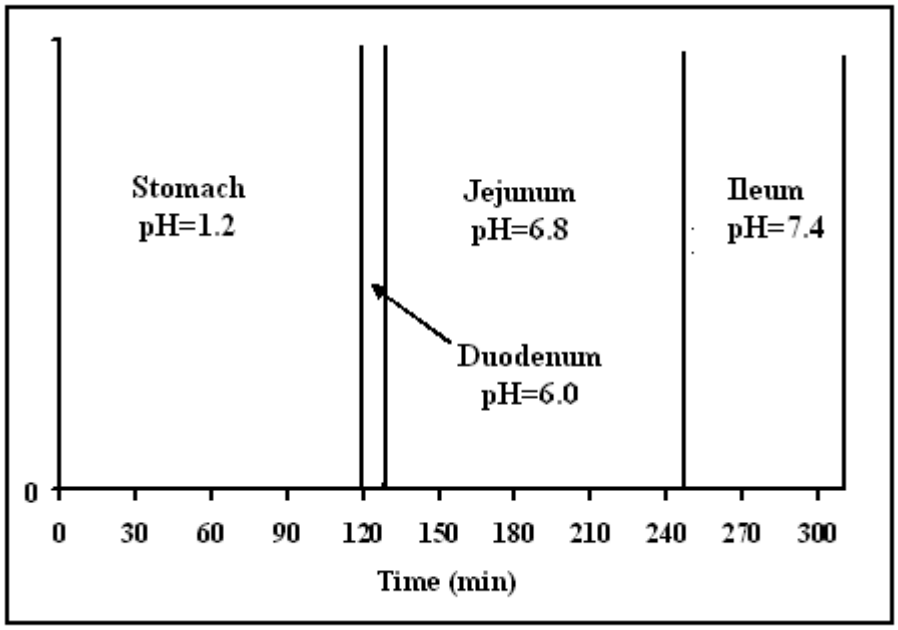


Figure 2.4: Dissolution pH and residence time for food digestion.
(Reproduced from Klein *et al.* (2005))

Clearly, the lactase enzyme should be protected against the low pH in the stomach for 2 hours and then released at a required site, namely the small intestine, after approximately 3 hours.

2.4 Present Research about Microencapsulation of Lactase

Microencapsulation is not a new method and it is widely used in other industries for similar purposes, especially in food, pharmaceuticals and biotechnology. There are

various types of microencapsulation strategies and some examples are illustrated in Figure 2.5. The resulting microparticle may be homogenous where constituents to be encapsulated and encapsulating agents are evenly mixed and dispersed. Alternatively, the microparticle may be heterogenous with distinct material phases containing the constituents surrounded by the encapsulating agent.

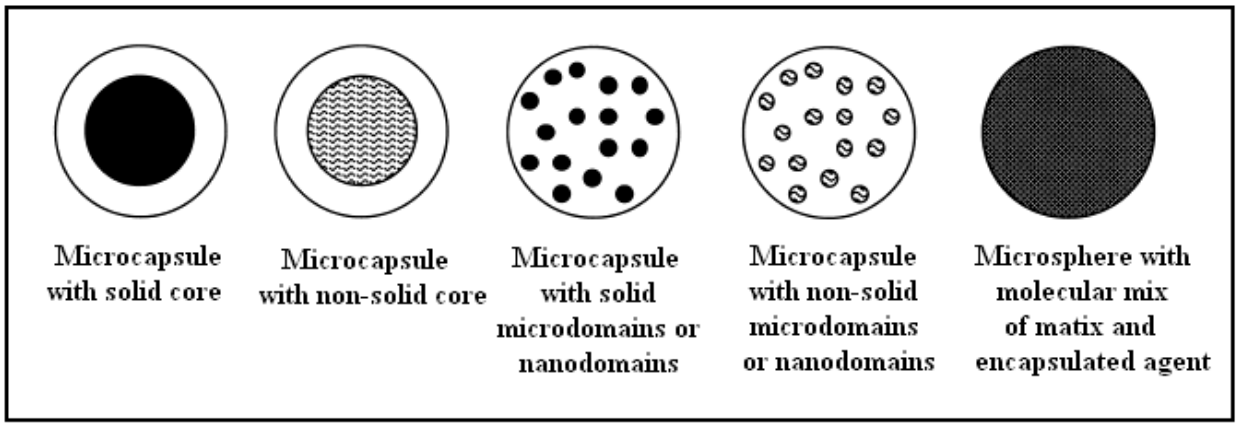


Figure 2.5: Microcapsules and microspheres.
(Reproduced from Birnbaum & Brannon-Peppas (2003))

A number of previous studies have been conducted to investigate microencapsulation of lactase, employing a variety of materials. Natural materials were the initial and common choice. The research conducted by Wang & Shao (1993) is the earliest report describing microencapsulation technology applied to lactase. Complex microcapsules were prepared to deliver lactase. Briefly, semipermeable microcapsules were first formed by enveloping lactase within spherical, ultrathin semipermeable membranes. The lyophilised powders of those semipermeable microparticles containing lactase were mixed with a starch solution to form an emulsion in paraffin. The emulsion was mixed with enteric-soluble materials (lac and cellulose acetate phthalate) and chloroform was added to solidify the particles. The final microcapsules were then treated with fats to increase their hydrophobicity. The particles possessed a mean diameter of approximately 800 μm and the yield following encapsulation was 85.2%. All of the encapsulated lactase was released following incubation into synthetic intestinal juice in 2 hours. However, there was a significant leakage of lactase enzyme from the capsules and approximately 65% of protein was preserved after 2 hours in the synthetic stomach fluid. Furthermore, the production process was complex and time-consuming.

Pommersheim *et al.* (2003) and Dashevsky (1998) used alginate beads to microencapsulate lactase. Alginate beads were also employed by Taqieddin & Amiji (2004) and an external chitosan shell was added to form a "core-shell" structure. The microcapsule core was produced by crosslinking sodium alginate with either calcium or barium ions in the presence of lactase. Then the crosslinked alginate core was uniformly coated with a chitosan layer and crosslinked with aqueous sodium tripolyphosphate (Na-TPP) solution. A loading efficiency of sixty percent was obtained with the Ca^{2+} alginate and one hundred percent loading efficiency was obtained with the Ba^{2+} alginate. However, the size of particles determined using SEM images exceeded 1mm. Furthermore, release from the microparticles for pH conditions in the stomach and small intestine was not confirmed. As well, chemical modification of the natural materials was required. This would render the manufacturing process complex and expensive.

Lipids have also provided a protective coating for delivering lactase enzyme (Kim *et al.*, 1999). Briefly, a lipid mixture, composed of egg phosphatidyl choline (EPC) and cholesterol, was dissolved in anhydrous chloroform. The chloroform was evaporated and the dried lipid film was suspended in phosphate buffer. The small unilamellar vesicles (SUV), obtained during suspension, were mixed with a phosphate buffer containing lactase. After freezing, phosphate buffer was added to the freeze-dried preparation and mixed. The resultant dehydration–rehydration vesicles were centrifuged and the formed a pellet of multilamellar vesicles (MLV) which was resuspended in phosphate buffer. The MLV were then mixed with phosphate buffer containing trehalose and lyophilized to obtain the dried liposomes containing lactase. Release studies confirmed that the enzyme could be preserved in the gastric juice and then released in the small intestine. Unfortunately, values for encapsulation efficacy and particle size were not reported.

Clearly, natural materials possess advantages such as non-toxicity, biocompatibility and biodegradability, but their properties appear sub-optimal for this application. Natural materials are often difficult to source in a sufficiently pure form. Subsequent chemical modification is commonly required in order to ensure adequate encapsulation and retention (Otani *et al.*, 1998). This means that manufacturing is complex and costly for commercial application. Furthermore, the studies suggest that some natural materials

may be unstable in the stomach and release significant lactase prematurely, due to their susceptibility to the low pH environment of the gastric juices (Wang & Shao, 1993). Consequently, recent studies have focussed primarily on synthetic materials.

Kwak *et al*. (2001) were amongst early researchers to study synthetic materials. They used commercially available materials, including polyglycerol monostearate (PGMS), medium chain triacylglycerol (MCT), sorbitan monolaurate (SPAN-20), sorbitan monooleate (SPAN-80), sorbitan trioleate (SPAN-85) and polyglycerin polyricinoleate (ESPR). The mixture of lactase and the emulsifier materials was nebulized through a nozzle to form a microcapsule suspension. The encapsulation efficiency varied from 50% to 75%. In this study, no release profiles or particle size data were reported. The process is much simpler than those exploiting natural materials, but the materials were costly and encapsulation efficiency was not sufficiently high.

EUDRAGIT polymers were applied by Squillante *et al.* (2003) and Alavi *et al.* (2002) for the microencapsulation of lactase. EUDRAGIT polymers are a common and popular encapsulation material employed by the pharmaceutical industry. Both studies used an oil-in-oil solvent evaporation method to produce EUDRAGIT microparticles containing lactase. Briefly, the polymeric organic solution containing the lactase was added to external paraffin oil and microparticles were formed by homogenization. The encapsulation efficacy was roughly 80% whilst the particle size ranged from 50 to 200 µm. Preliminary work confirmed that these products were stable in the low pH buffer and soluble at the high pH – a characteristic of the small intestine.

Compared to natural materials, synthetic polymers are normally more expensive. However, they are available at high purity and the efficiency of encapsulation is higher. As well, the production process for microparticles is simpler. In this study, it was decided to focus on the use of EUDRAGIT. This decision was based on the easy availability of EUDRAGIT polymers in forms that were stable at acid pH but dissolved at near neutral pH. These are critical properties for a microparticle to protect lactase in the stomach and subsequently release it in the GI tract. This is discussed in detail in the next section where the selection of most appropriate EUDRAGIT polymer is considered. Furthermore, EUDRAGIT has an established track record of safety in pharmaceutical applications. This would ensure easy commercial acceptance for this application.

2.5 Selection of the EUDRAGIT Polymer Type

EUDRAGIT® is a trademark of Röhm GmbH & Co. KG, Darmstadt in Germany, first marketed in 1950s (Röhm Pharma Polymers, 2003). EUDRAGIT polymers L100 and S100 varieties are co-polymers of methacrylic acid and methyl methacrylate (Table 2.1). The choice of constituents and their relative ratios determine the pH at which the polymer becomes soluble in aqueous solution. In particular, EL 100 dissolves at a pH of approximately 6, whilst ES 100 will dissolve at a pH of 7. As a consequence, both polymers have been widely applied by the pharmaceutical industry where specific release of a drug in the GI tract is desired (Röhm Pharma Polymers, 2003).

EUDRAGIT polymer RS PO is also a copolymer, and contains acrylate and methacrylates with quaternary ammonium groups as functional groups (Röhm Pharma Polymers, 1991). It does not dissolve at a specific pH and remains insoluble in aqueous solution. However, the polymer is permeable, and a drug may be released slowly and continuously.

Clearly, among the EUDRAGIT polymers previously studied, ES 100 appears to possess the most desirable attributes: no release of enzyme in the stomach at low pH followed by release in the small intestine where the pH is neutral. It was selected for further study.

Table 2.1: Properties of ES 100 and EL 100.

(Reproduced from Röhm Pharma Polymers (2003))

Polymer	Chemical Structure	Dissolution pH
EL 100	Methacrylic acid Methyl methacrylate 50:50	6.0
ES 100	Methacrylic acid Methyl Methacrylate 30:70	7.0

2.6 Description of ES 100

As mentioned earlier, ES 100 is a methacrylic acid and methyl methacrylate copolymer (Figure 2.6) (Röhm Pharma Polymers, 2003). It is insoluble in acid medium and dissolves above neutral pH. Dissolution occurs as a result of structural change of the polymer associated with ionisation of the carboxylic functional group (Figure 2.7).

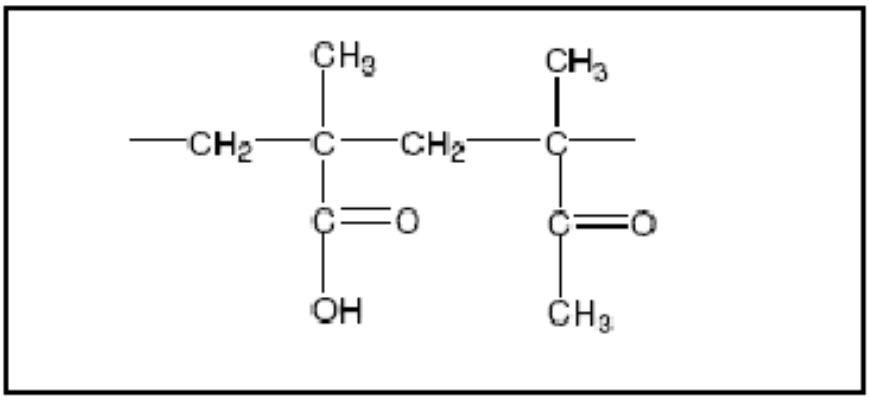


Figure 2.6: Structure of EUDRAGIT S 100.

(Reproduced from Röhm Pharma Polymers (2003))

(The ratio of the free carboxyl groups to the ester groups is approximately 1:2 and the average molecular weight is approximately 135,000.)

At acid pH, ES 100 microparticles possess low permeability due to hydrogen bonding between the hydroxyl groups of the carboxylic moiety and the carbonyl oxygen of ester groups in the polymer molecules. This bonding increases the degree of compactness of

the polymer and decreases its porosity and permeability (El-Kamel *et al.*, 2001), minimising release of an encapsulated agent.

When the pH of the aqueous medium is increased, the ES 100 microparticle starts to dissolve as the carboxylic functional group ionises. The reported theoretical dissolution threshold is pH 7.0 and the pKa of polymer molecules is believed to be approximately 6 (Nguyen & Fogler, 2005). As the microparticle dissolves, the contained drug is released. Moreover, swelling of the ES 100 matrix may accompany the dissolution process contributing to release. It is believed that EUDRAGIT S swells at pH above 6.5 (Bhagwat *et al.*, 2005). Therefore, the release of the active substances may occur due to a combination of swelling and dissolution.

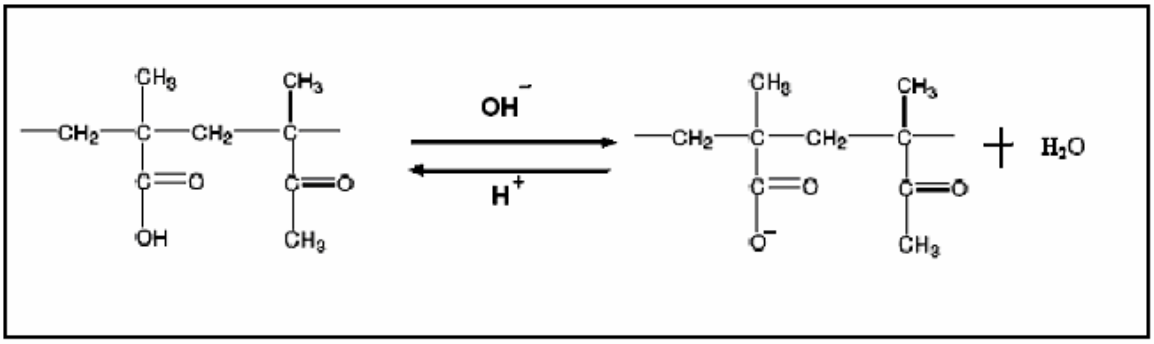


Figure 2.7: ES 100 degradation.
(Reproduced from El-Kamel *et al.* (2001))

2.7 Encapsulation of Lactase with ES 100

Alavi *et al.* (2002) undertook the sole previous study using ES 100 polymer for the microencapsulation of lactase.

In their research, the polymer produced microparticles using the oil-in-oil (O/O) solvent evaporation method. Acetone with 2% water was applied to dissolve the polymer and composed for internal oil phases (Röhm Pharma Polymers, 2003). Sucrose stearate (Figure 2.8) provided droplet stabilization during the process of emulsion formation.

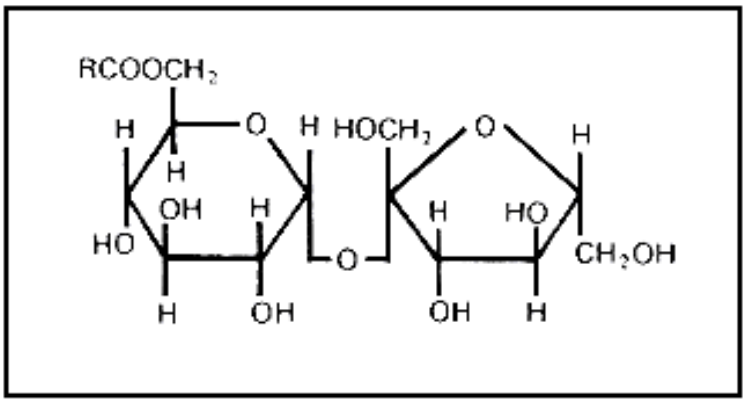


Figure 2.8: Chemical structure of sucrose ester.

(Reproduced from Youan *et al.* (2003))

(Sucrose ester is a nonionic surface active agent manufactured from a pure sugar and vegetable oils. It is an ester compound consisting of sucrose and fatty acids.)

In brief terms, the process was as follows, lactase and emulsifier were dispersed into an organic solution of ES 100 to form a coarse dispersion. Liquid paraffin was added and the solution was agitated using a high shear homogenizer. Subsequent solvent evaporation resulted in the formation of the microparticles.

The produced microparticles were spherical and with a mean diameter of approximately 200 µm. Enzyme release from particles was measured following exposure at pH 1.2 and 7.2. These data permitted calculation of the encapsulation efficiency of the microparticles. 100 ml of chilled pH 4.8 buffer was loaded with microparticles with theoretical equivalent to 10 mg lactase (calculated from the weight of individual ingredients and their proportion). The solution was filtered to obtain an estimate of "surface lactase" or the lactase not incorporated in the microparticles. A second 100 ml volumetric flask of pH 7.2 buffer was prepared. An accurately weighed microcapsule sample equivalent to 10 mg of lactase was added followed by agitation with a magnetic stirrer to allow estimation of the total lactase in the product. The difference between surface lactase and total lactase permitted calculation of the amount of trapped or encapsulated lactase. The maximum encapsulation efficiency was approximately 80%.

Unfortunately, transient release studies were not performed by Alavi *et al.* (2002), hence no estimates of the rate of lactase release were available. This information is required to characterize the temporal performance of the microparticles as they travel

through the GI tract from the stomach into the small intestine. It is important that sufficient time is available for the microparticles to completely dissolve and release the lactase. Based on the previous discussion in Section 2.3, the microparticles must dissolve within 3 hours after entering the small intestine from the stomach.

2.8 Other Published Studies of Microencapsulation with ES 100

Other studies have investigated the use of ES 100 to encapsulate other proteins and simple molecules. These provide valuable information about alternative preparation methods (see Figure 2.9). They also include data on the release characteristics for ES 100 microparticles.

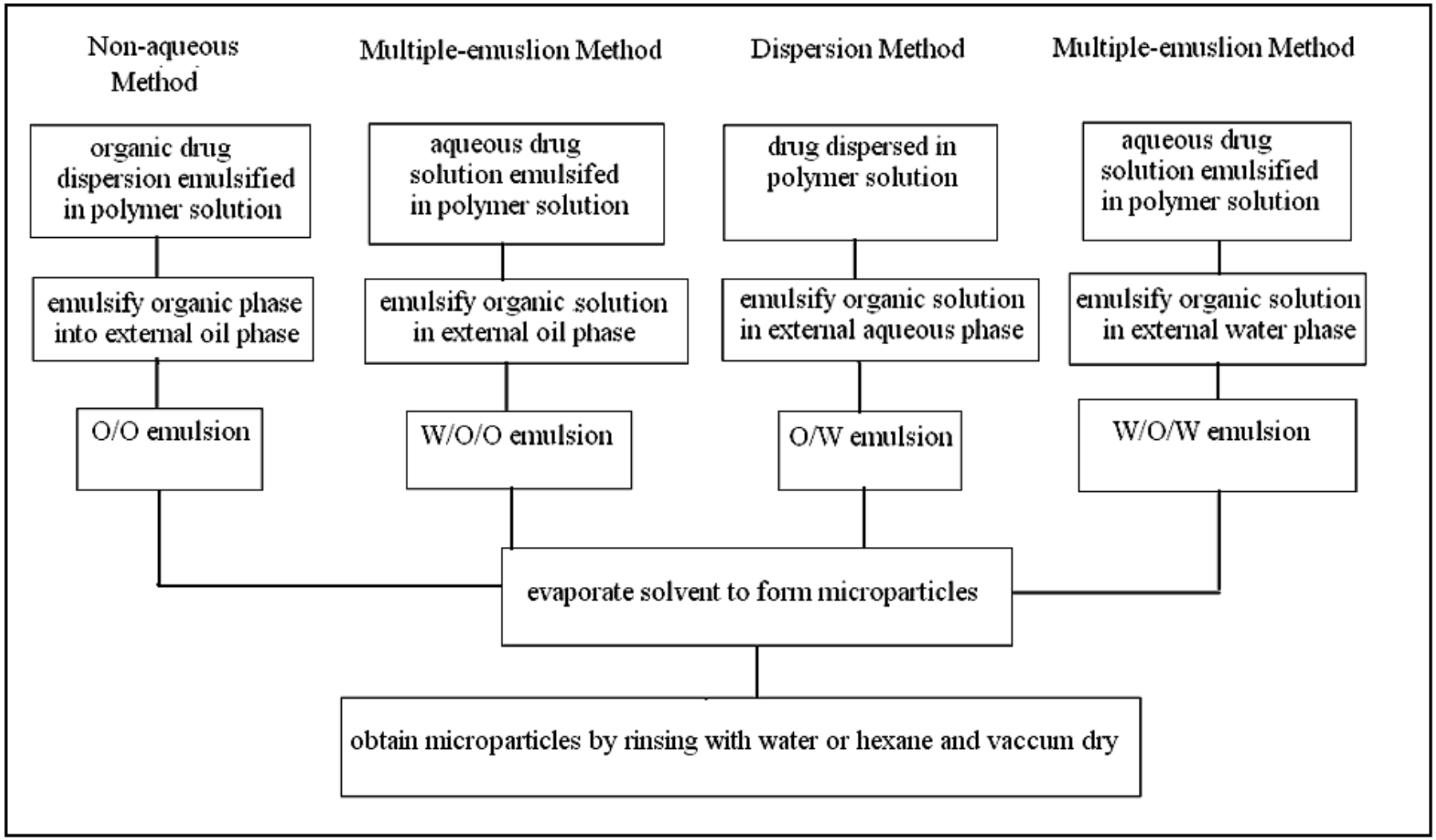


Figure 2.9: Solvent evaporation methods for ES 100 microparticles.

(Reproduced from Alavi *et al.* (2002); Amorim & Ferreira (2001); Jain *et al.* (2005); Lee *et al.* (2000); Rodriguez *et al.* (1998))

Amorim & Ferreira (2001) employed ES 100 to microencapsulate aprotinin (Apro). Apro is the trypsin inhibitor from bovine lung, which has a MW of 6,500. An O/O solvent evaporation technique was used. A bulking agent, casein, was dispersed with the Apro into an ES 100 polymer acetone/ethanol (2:1, V/V) solution, which was then dispersed in paraffin. Solvent evaporation allowed microparticle formation. The encapsulation efficiency exceeded 80%. The particle size ranged from 500 μm to 2 mm and the release rates *in vitro* at pH of 1 and 6.8 were measured. No release could be detected at pH=1, and complete release was achieved within 3 hours at a pH=6.8.

Rodriguez *et al.* (1998) produced a system to microencapsulate and deliver to the colonic region a variety of active molecules including ondansetron (MW=365.9) and budesonide (MW=430.5) respectively. Drug loaded cellulose acetate butyrate (CAB) microspheres were coated with ES 100. The encapsulation of the CAB cores and formation of pH-sensitive microcapsules were achieved using an O/O emulsion solvent evaporation technique. The encapsulation efficiency ranged between 60% and 96%. The ES particle size was 300 μm. Rodriguez *et al.* (1998) conducted release tests at pH of 1.1, 5.5, 6.8 and 7.4. Negligible release was observed when pH fell below 6.8. Approximately 65% of the drug was released from the multi-particles containing CAB cores at pH 7.4 after 5 hours. As well, ES 100 microparticles directly encapsulating the drugs were prepared and tested. Again no release occurred if the pH remained below 6.8. By contrast, total or near complete release of the drugs was achieved at pH 7.4 within 5 hours.

Jain *et al.* (2005) applied another method, water/oil/water (W/O/W) solvent evaporation to entrap insulin (MW ~ 5,800) in ES 100 and recorded encapsulation efficiencies ranging from 5% to 80%. The size of produced microparticles was roughly 32.5 μm. An *in vitro* drug release test was only performed in the phosphate buffer (pH=7.4). Around 60% of the encapsulated core material was released after 6 hours.

Lee *et al.* (2000) encapsulated four active agents (tacrine HCl, acetaminophen, propranolol HCl and theophylline) with MW < 500. The particle size was approximately 200 μm. For the microparticles containing acetaminophen and theophylline, total release of the active

substances occurred at pH=7.4, and roughly 40% to 80% was released at pH=6.8 after 10 hours. A small amount of drug leakage occurred at pH=2. However, entirely different release profiles were measured for the particles delivering tacrine HCl. Only 20% of core material was free at pH=7.4 in 25 hr and almost 100% release occurred at pH=2. In their research, both O/W and W/O/O solvent evaporation methods were exploited. With the O/W method, similar low encapsulation efficiency to those of Jain *et al.* (2005) were recorded, ranging from 10% to 40%. This reduction in encapsulation efficiency was attributed to the different emulsion regimes, influencing the physical state of the drug in the polymer solution (Alavi *et al.,* 2002; Amorim & Ferreira, 2001; Lee *et al.*, 2000). Specifically, the introduction of an external aqueous phase causes partitioning of water-soluble drugs or proteins into the external phase thereby reducing the efficiency of encapsulation (Alavi *et al.*, 2002). Compared to the results obtained from O/W and W/O/W, the W/O/O method (Lee *et al.,* 2000) produced higher encapsulation efficiency for water-soluble encapsulated core materials. The theophylline, which is soluble in oil phase, produced the lowest encapsulation efficiency of below 60%. However, water-soluble drugs could yield 80% efficiency. Clearly, it is believed that an external phase is an important factor for encapsulating core material efficiently. For oil-soluble drugs, the method with external aqueous phase may yield higher encapsulation. Whilst for water-soluble drugs, the method without an external aqueous phase is much more suitable.

These studies confirm O/O solvent evaporation method of Alavi *et al.* (2002) as the preferred preparation method. It achieves the highest encapsulation efficiency, a key parameter for commercial application. As well, the size of microparticles and properties of the core material appear to influence release rates.

2.9 Sonication Application for Emulsification

As mentioned earlier, the emulsification step in the solvent evaporation procedure employed in previous studies have utilized by a mechanical high-shear homogeniser. Unfortunately, in this study such equipment was not available. As a consequence, an alternative emulsification method based on sonication was investigated. A laboratory sonifier capable of producing high-intensity ultrasound waves of 20 kHz frequency at various amplitude settings was used.

Sonication has been widely applied to emulsify immiscible liquids and process low viscosity materials to produce stable emulsions. In particular, several researchers have utilized ultrasound to produce microparticles for O/O solvent evaporation. Normally, the organic solution containing either solid drugs or proteins is emulsified into the external oil phase by sonication for several minutes prior to evaporation (Bruening *et al.*, 2005; Herrero-Vanrell & Refojo, 2001; Mandal *et al.*, 2001; Thote & Gupta, 2005). During sonication, it is imperative to avoid any rise in the temperature of the mixture (Herrero-Vanrell & Refojo, 2001; Mandal *et al.*, 2001). The optimal temperature for solvent evaporation is strongly determined by particle formation and morphology. Rapid temperature rise causes excessive solvent evaporation and larger and irregular particles form (Lee *et al.*, 2000; Zhou *et al.*, 2003). In most instances, an ice bath is used to remove the heat created during sonication and to control the rate of evaporation of the organic solvent (Mandal *et al.*, 2001).

The ability to vary the amplitude of the ultrasound waves is a significant advantage of the laboratory sonifier employed in this study. This allows the output energy and wave intensity used for emulsification to be adjusted and controlled.

2.10 Protein Surrogates for Lactase

Several sources of lactase are known and prices vary depending on the source, purity and activity. The activity of lactase sourced from Escherichia coli (600-1200 units/mg) is significantly higher than that produced from Aspergillus oryzae (8 units/mg). Clearly, the fungal sourced lactase is cheaper. Lactase powders of higher activity mean that fewer microparticles are required. Unfortunately, the high cost of high purity lactase precluded its use for this study and a surrogate (cheaper) protein was employed instead.

Table 2.2 shows relevant properties of commonly used proteins compared to lactase. BSA has a MW and isoelectric point close to that of lactase and is relatively inexpensive.

Table 2.2: Some commonly used proteins.

(Data and prices from Sigma-aldrich (2004))

Protein Category	Source	Isoelectric Point	MW (kDa)	Structure	Price (AUD/10g)
Lactase	(1) Escherichia Coli (600-1200 units/mg) * (2) Kluyveromyces Fragilis (5 units/mg) * (3) Aspergillus Oryzae (8 units/mg) *	4.6	112	Globular	(1) 17,350.00 (2) 131,120.00 (3) 130.00
Bovine Serum Albumin	Bovine Serum	4.9	66	Globular	22.00
Ovalbumin	Chicken Egg White	4.7	45	Globular	216.30
Immunoglobulin	Calf Liver	5.9	78	Globular	173,000.00
Lysozyme	Chicken Egg White	11.0	14	Globular	294.60
Cytochrome c	Bovine Heart	10.2	12	Globular	3,528.00

**** One unit will hydrolyse 1.0 µmole of o-nitrophenyl ß-D-galactoside to o-nitrophenol and D-galactose per min at the specified pH and temperature.***

2.11 Summary and Research Gaps

It was decided to choose ES 100 as the encapsulating agent for lactase in this study, because its dissolution characteristics as a function of pH will allow selective release in the neutral conditions of small intestine – the natural site of the enzyme's physiological action. Only one previous study investigating microencapsulation of lactase with ES 100 (Alavi *et al.,* 2002) has been undertaken. They employed an O/O emulsification and solvent evaporation technique to produce microparticles. A review of work on ES 100 microencapsulation methods suggests that this technique achieves the highest micro-encapsulation efficiency. Emulsification will be achieved by sonication rather than by mechanical shearing. There is currently no published data for production of ES 100 microparticles by sonication. BSA was employed as a surrogate for lactase for the purpose of the project to reduce the cost of the experimental investigations. The dissolution of ES 100 microparticles, and hence release rates of encapsulated protein, as a function of pH are not precisely known.

2.12 Aims

The overall goal of this project was to investigate a method for microencapsulation of lactase. The following specific aims have been formulated based on a detailed review of relevant literature and prior art.

1. To demonstrate and characterise encapsulation of BSA with ES 100 by O/O solvent evaporation based on the method of Alavi *et al.* (2002) but using sonication for the emulsification step.

2. To measure the transient release behaviour of the microparticle thus formed to consider their suitability for the desired application.

3 MATERIALS AND METHODS

The goal of this research was to apply a suitable coating material and investigate a microencapsulation method for the lactase enzyme. BSA was used as a surrogate for the lactase during the research. An oil-in-oil solvent evaporation method, based on the technique developed by Alavi *et al.* (2002), was used to produce microparticles containing BSA protein and encapsulated with EUDRAGIT S 100 – a methacrylic acid and methyl methacrylate copolymer. Sonication was employed during the emulsification step. The particles produced at different sonication amplitudes were characterized by SEM and size analysis. The release profiles of selected microparticles at different pH were investigated to understand how the microparticles would perform in the GI tract.

3.1 Microparticle Formulation

Microparticles were obtained by oil-in-oil emulsification followed by solvent evaporation mentioned as depicted in Figure 3.1. The key parameters for the particle formation process are summarized in Table 3.1.

Two hundred and twenty five (225) mg of ES 100 (Röhm Pharma, Darmstadt, Germany) was dissolved in a solution containing 3 ml of acetone (Ace Chemical, Melbourne, Australia) and 2% (V/V) water. Bovine serum albumin (22.5 mg) (Boehringer-Ingelheim, North Ryde, Australia), 120 mg of sucrose stearate DK ester® (Swift Australia, Sydney, Australia) and 40 mg triethyl citrate (TEC) (Sigma-Aldrich, St Louis, USA) were mixed with the polymer solution to create a coarse dispersion. This was rapidly poured into 20 ml of liquid paraffin at 10 °C. A dispersion step using magnetic stirring for 30 s was employed to form a suspension which was then placed in a beaker immersed in an ice bath. Sonication was performed using a Branson 450 Digital Sonifier (Branson, Danbury, USA) with ⅛" diameter tapered tip over a range of amplitudes. Residual acetone was evaporated at room temperature whilst agitating the suspension with a magnetic stirrer. Samples were centrifuged (5000 rpm for 5 min) and washed with n-hexane (Sigma-Aldrich, St Louis, USA) five times to remove the

paraffin. Microparticles were then freeze dried and stored in a closed container at room temperature prior to analysis.

Table 3.1: Parameters in microparticle preparation.

Parameters	**Processing Condition**
Polymer	EUDRAGIT S 100
Solvent	Acetone
Surfactant	Sucrose Stearate DK F-100
External Oil	Liquid Paraffin
Oil Volume	20 ml
Process for Suspension	Magnetic Stirring for 30 s
Process for Sonication	450 Digital Sonifier with ⅛" Diameter Tapered Tip
Process for Solvent Evaporation	Magnetic Stirring at Speed 7
Centrifugation	5000 rpm, 5 min
Solvent for Washing	Hexane

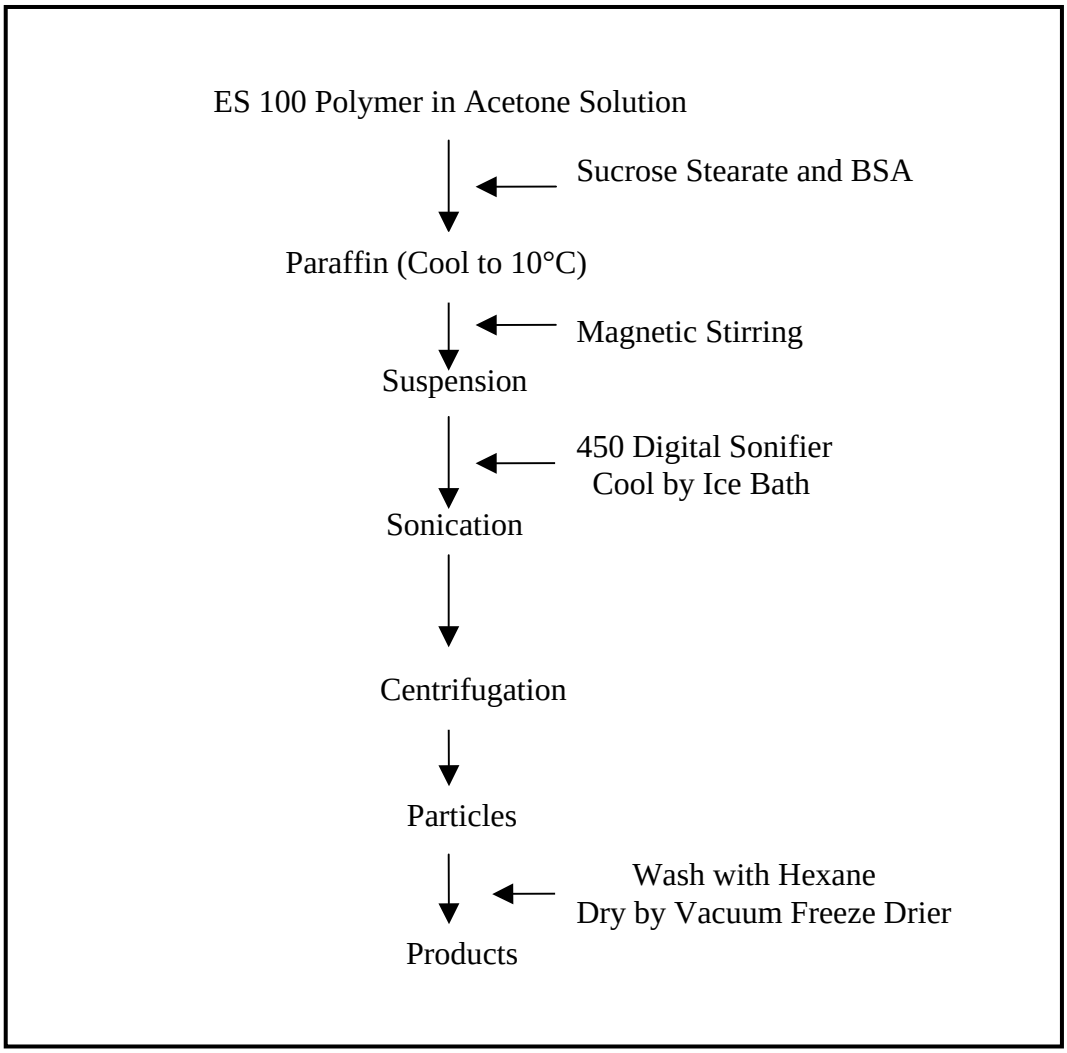


Figure 3.1: Process for producing particles.

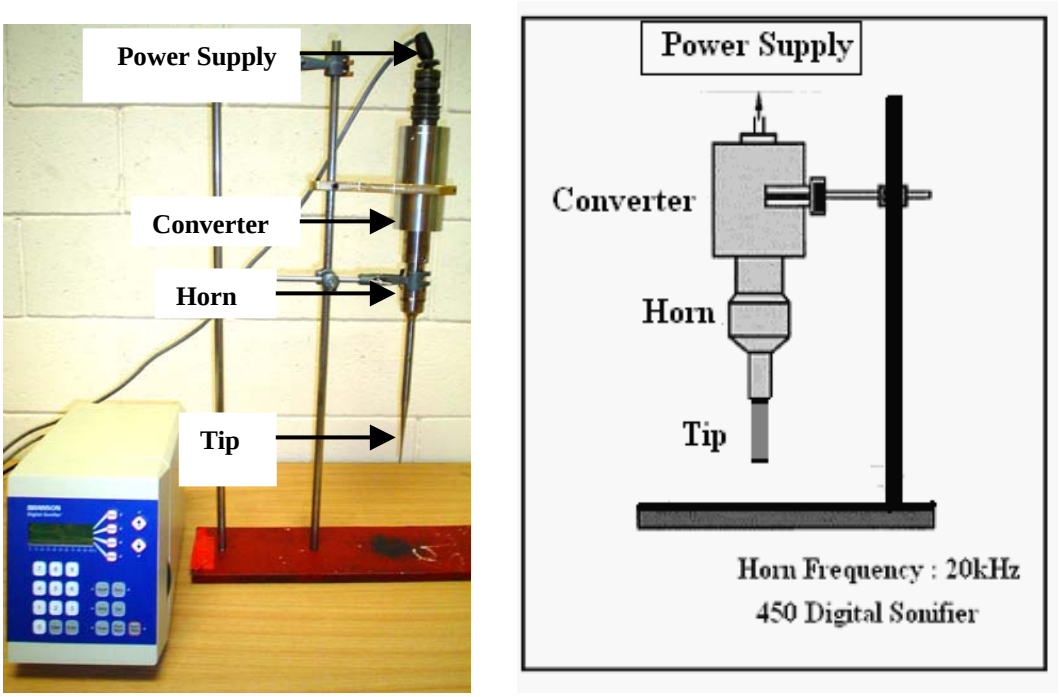
3.2 450 Digital Sonifier

3.2.1 Introduction

Figure 3.2 shows a photo and schematic of the 450 Digital Sonifier used in this study. The Digital Sonifier power supply converts the output voltage to 20 kHz electrical energy (Branson Ultrasonics Corporation, 2004). This high-frequency energy is passed to a converter where it is transduced into ultrasonic mechanical vibrations. The converter vibrates longitudinally and this motion is transmitted to the horn tip immersed in the solution. Shock waves propagate through the liquid, inducing cavitation with subsequent violent particle motion and high velocity particle collisions. The ultrasonic

vibrations may be delivered into a sample using a variety of tips attached to the horn. A ⅛" diameter tapered tip was employed in this research.

In order to estimate the output power of the 450 Digital Sonifier, two methods were employed: (i) direct measurement of acoustic output with a hydrophone and (ii) indirect measurement from heat dissipation into water in an insulated container.

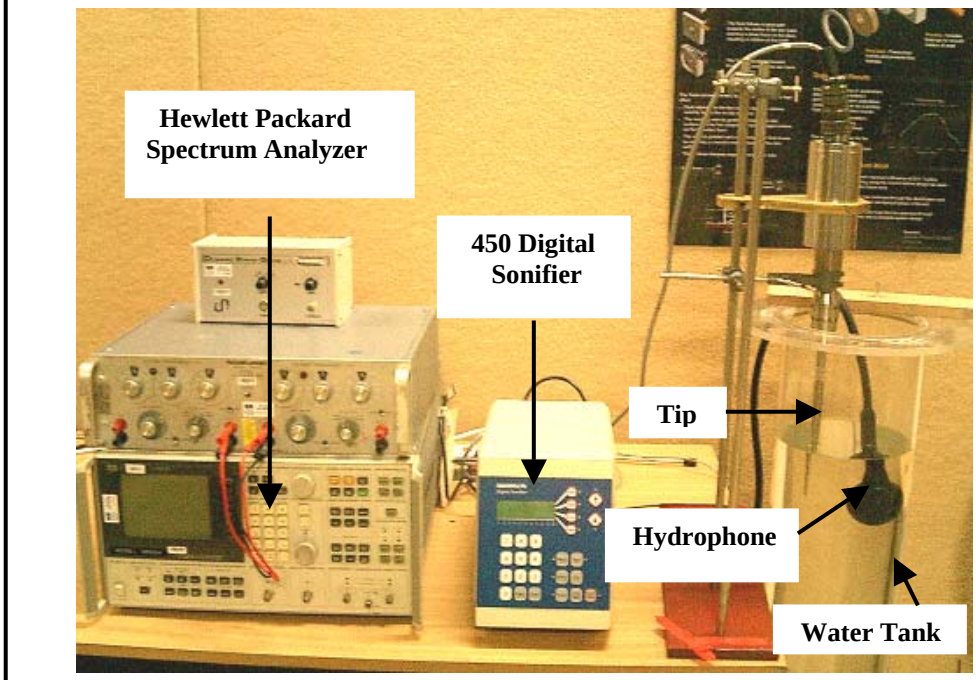


(a) **(b)**

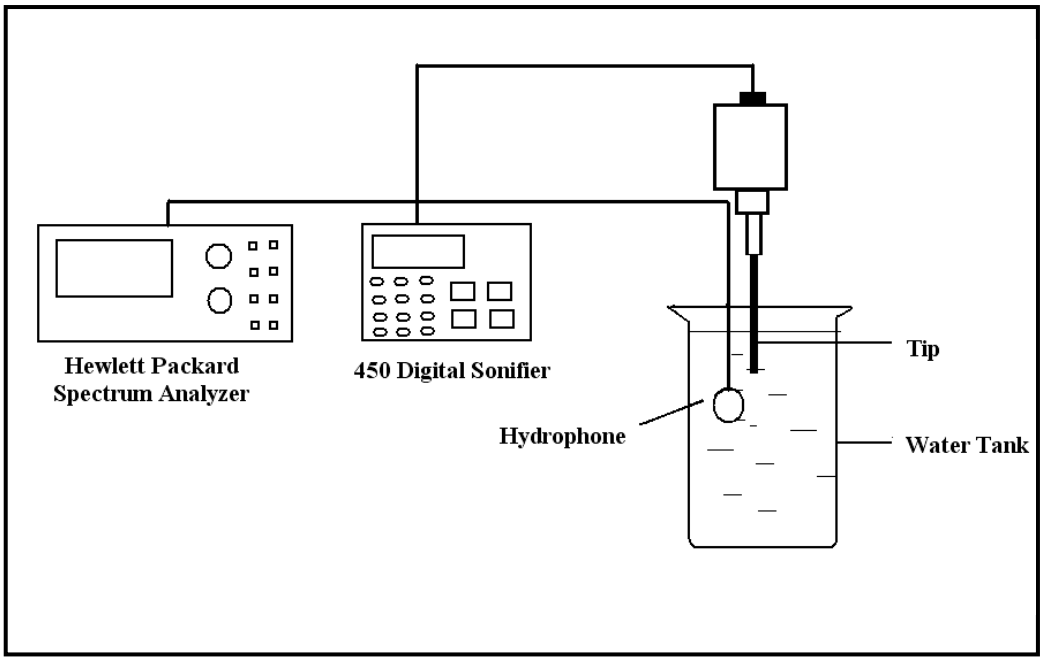
Figure 3.2: 450 Digital Sonifier: (a) photograph; (b) schematic.

3.2.2 Measurement from Acoustic Power

The output of the Sonifier at various amplitudes was measured in water using a hydrophone (Mechanical Engineering School, The University of Adelaide, Australia). The signals from the hydrophone were analysed by a Hewlett Packard Spectrum Analyzer (Mechanical Engineering School, The University of Adelaide, Australia) and converted into a sound pressure. Figure 3.3 depicts the experimental arrangement that was employed.



(a)



(b)

Figure 3.3: Setting for output power: (a) photograph, (b) schematic.

During the tests, the hydrophone and sonifier tip were immersed in a water tank. Four distances between sonifier tip and hydrophone (100 mm, 195 mm, 455 mm and 535 mm) were studied. The water level in the tank was fixed at 835 mm, and the tip was located at 75 mm from water surface in the tank.

The amplitude of the sonifier was set to values between 10% and 70%. The hydrophone output signal was automatically recorded twenty times for a sampling period of 0.5 s at a frequency of 20 kHz. The average value of the hydrophone output was converted to a sound pressure level using a calibration curve (see Appendix A) previously determined for the hydrophone by the technicians from the School of Mechanical Engineering, University of Adelaide.

The power output was estimated from sound pressure level measured using the following equation (Bies & Hansen, 2003).

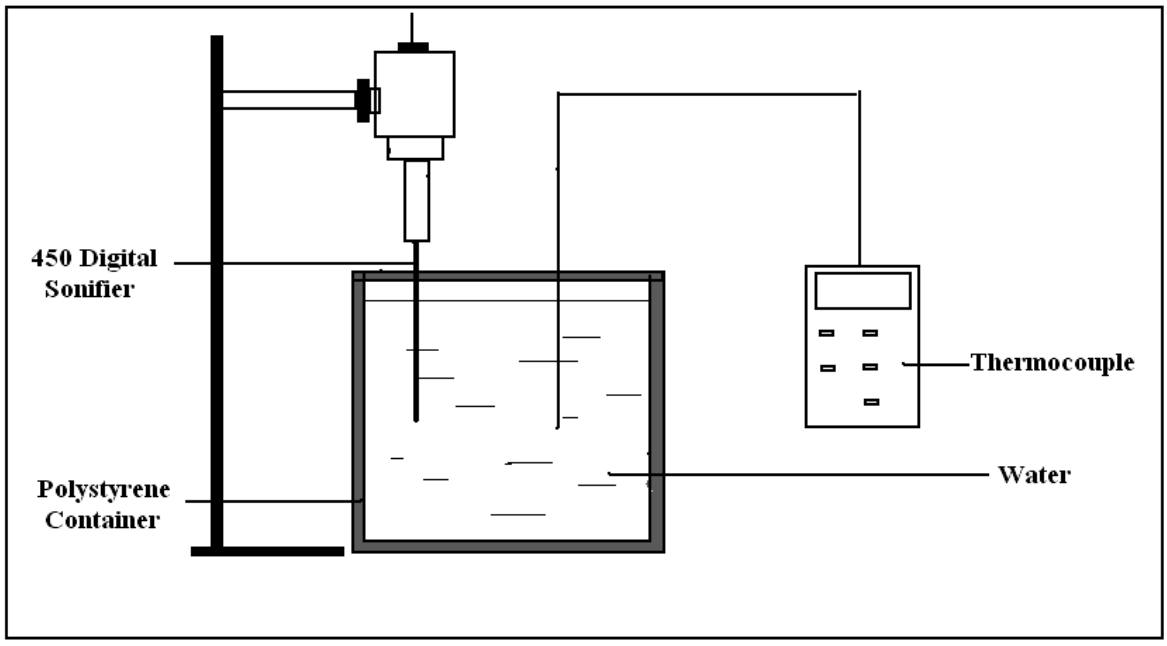
$$W = \frac{P^2 4\pi r^2}{\rho c D_\theta} \qquad \textbf{(3-1)}$$

where P is sound pressure after calibration (Pa), r is the distance between microphone and tip of sonifier (m), D_θ is directivity factor (D_θ = 1), ρ is the density of water (ρ = 1000 kg/m^3), and c is the speed of sound in water (c ~1500 m/s) (Brownstone, 1999).

The above equation neglects the effect of a reverberation field in the tank and assumes that sound pressure emanates from the probe evenly in all directions. This assumption may not be correct.

3.2.3 Output Measurement by Energy Dissipation

(a)



(b)

Figure 3.4: Setting for measurement: (a) photograph, (b) schematic.

Figure 3.4 illustrates the experimental arrangement for measuring power output determined by the energy dissipation from the Sonifier. 200 ml water was added into an enclosed polystyrene container. The time for the temperature to increase by 5 K during sonication was recorded for a range of amplitudes (10% to 70%). Each experiment was replicated thrice.

The energy dissipated by the Sonifier is ultimately degraded to heat and thus may be estimated from the temperature rise observed in the container:

$$\Delta Q = m \times C_p \times \Delta T \quad \textbf{(3-2)}$$

where m is the mass of the water added (m = 200 g), C_p is the water heat capacity (C_p ~ 4.184 J/g K), and ΔT is the rise in temperature (K).

The power output produced by the Digital Sonifier may then be estimated from,

$$W = \frac{\Delta Q}{t} \quad \textbf{(3-3)}$$

where t is the time (s) over which the temperature rise has occurred.

3.3 Morphology and Size Analysis

3.3.1 Visualization by SEM

The microparticles were observed using scanning electron microscopy (SEM). Initially, the microparticles were suspended in n-hexane. The suspension was sonicated at an amplitude setting of 30% for 30 s to disperse individual particles.

Next, a droplet of the resulting microparticle suspension was placed on an aluminium stub (ProSciTech, Queensland, Australia), and then covered with a double-sided carbon tape and the solvent was allowed to evaporate. Specimens were coated with a 3 nm layer of the high-resolution platinum using a Cressington 208 HR high resolution sputter coater (Cressington, England) for 10 min. Specimens were examined with a

Philips XL30 FEGSEM scanning electron microscope (Philips, Eindhoven, Netherlands).

3.3.2 Size Analysis of SEM Images

Particle size measurement was performed using the SEM images and image analysis software analySIS® (Soft Imaging System, Adelaide, Australia). The median particle size for the samples was determined from the cumulative frequency distribution.

3.4 BSA Protein Quantification

3.4.1 Description

Protein concentration was quantified using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, USA). This procedure is based on a colorimetric assay for protein concentration. The assay method exploits the reaction of protein with an alkaline copper tartrate solution and Folin reagent (Lowry *et al*., 1951).

The assay involves a two-step procedure: (1) reaction between protein and copper in an alkaline medium, and (2) subsequent reduction of Folin's reagent ($3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$ & $3H_2O.P_2O_5.14WO_3.4MoO_3.10H_2O$) with the copper-treated protein resulting in a characteristic blue colour maximum at 750 nm within 15 min.

In this work, BSA was selected as the model protein. Hence, BSA solutions of known concentration were used to generate the absorbance vs. concentration calibration. There are two levels for this assay method: high-concentration and low-concentration.

For the high-concentration range, the protein concentration ranges from 0.2 to 1.5 mg/ml. 100 µl of protein solution was mixed with 500 µl of reagent A (Bio-Rad, Hercules, USA) and 4 ml reagent B (Bio-Rad, Hercules, USA) and vortex mixed. After 15 min, the absorbance was read at 750 nm using a Shimadzu UV-1601 Spectrophotometer (Tokyo, Japan).

In the low-concentration range, the concentration of protein lies between 5 and 250 µg/ml. In this case, 200 µl protein solution was mixed with 100 µl of reagent A and 800 µl of reagent B by and immediately mixed. After 15 min, the absorbance was read at 750 nm.

Protein concentration measurements during all of the encapsulation efficiency tests and release profiles for the microparticles were quantified using this protein assay.

3.4.2 Validation Tests

Prior to testing the encapsulation efficiency and time release behaviour of the microparticles, the influence of ES 100 on the Bio-Rad DC protein assay was investigated. This was necessary as absorbance at 750 nm might be affected by presence of the polymer. The test protocol was as follows.

Experiment Design

BSA protein solutions at various concentrations with or without ES 100 were prepared in pH 6.5 buffer for the high and low concentration ranges of the assay (Table 3.2). The concentration of ES 100 used was 5 g/L. The protein solutions were centrifuged at 5000 rpm for 5 min. The assay was performed for each sample. A regression analysis was performed to establish whether the presence of ES 100 had a significant effect on the Bio-Rad DC protein assay.

Table 3.2: Bio-Rad DC protein assay for statistical analysis.

Concentration Range	ES 100	Observation	BSA Concentration (mg/ml)
Low Concentration	With ES 100	1	0.01
		2	0.06
		3	0.12
		4	0.18
		5	0.25
	Without ES 100	6	0.01
		7	0.06
		8	0.12
		9	0.18
		10	0.25
High Concentration	With ES 100	1	0.20
		2	0.50
		3	0.80
		4	1.10
		5	1.40
	Without ES 100	6	0.20
		7	0.50
		8	0.80
		9	1.10
		10	1.40

Regression Analysis

For the low and high concentration ranges, absorbance was linearly regressed against BSA concentration and a binary variable indicating the presence or absence of ES 100, i.e.

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \qquad \textbf{(3-4)}$$

where x_1 is the BSA concentration (mg/ml), x_2 is the binary variable for presence of ES 100, and β_0, β_1, and β_2 are the regression coefficients.

The significance of the regression coefficient β_2 was established by calculating its standard error and test statistic for the hypothesis, where the test statistic was (Montgomery, 2001):

$$t_0 = \frac{\hat{\beta}_r}{\sqrt{\hat{\delta}^2 C_{rr}}} \qquad \textbf{(3-5)}$$

where $\hat{\delta}^2 C_{rr}$ is the diagonal element of the covariance matrix corresponding to $\hat{\beta}_r$.

3.5 Encapsulation Efficiency

The quantification of encapsulation efficiency was performed as follows, using an approach similar to the previous studies (Alavi *et al.* 2002; Squillante *et al.*, 2003). 50 mg of particles were dispersed into tubes containing 3 ml HCl (pH = 1) solution for 5 min. These were centrifuged at 5000 rpm for 5 min and 2 ml of the supernatant solution was removed for the BSA protein concentration quantification (C_1). Thus, an estimate of surface BSA or the BSA not encapsulated in the microparticles was made. Following this process, 3 ml NaOH (pH = 13) was then added to the pellet remaining in the tube. The contents of the tube were sonicated at an amplitude level of 30% for 30 s, followed by vortex mixing for 30 min to ensure complete dissolution of the polymer. 500 µl of sample was taken and the same volume of HCl (pH = 1) was mixed with it to precipitate the ES 100 polymer. The solution was centrifuged at 5000 rpm for 5 min, and the supernatant analysed for BSA protein concentration (C_2). An estimate of total protein in the microparticles was made.

The difference between surface BSA and total BSA indicates the amount of trapped or encapsulated protein. The encapsulation efficiency is therefore,

$$EE = \frac{C_2 V_2 - C_1 V_1}{W} \times 100\% \qquad \textbf{(3-6)}$$

where V_1 is the volume of remaining HCl solution in tube before adding alkaline solution (V_1 = 1 ml), V_2 is the volume of total solution in tube after adding NaOH solution (V_2 = 4 ml), C_1 and C_2 are the concentrations of protein which have been mentioned above (mg/ml), and W is the weight of protein added during the process of microparticle formation (mg).

Each experiment was replicated twice.

3.6 Microparticle Dissolution and Release Studies

3.6.1 Preparation of Buffers and Solutions

Buffer and solution preparation followed methods proposed by Perrin & Dempsey (1974). Acid pH solutions contained 0.05 M KCl, pH corrected with 0.2 M HCl to values of 1, 2, or 4. Near and above neutral pH solutions contained 0.05 M KH_2PO_4, pH corrected with 0.1 M NaOH to values of 6.0, 6.6, 6.8, 7.1, 7.4 or 8.1.

3.6.2 Dissolution Test at Different pH

Conical flasks were filled with 5 ml of aqueous solution at various pH (1.0, 2.0, 4.0, 6.0, 6.6, 6.8, 7.1, 7.4 and 8.1). The conical flasks were placed in an orbital mixer at 37 °C for 10 min. 50 mg of dried microparticles sonicated at an amplitude of 50% were added. The flasks were mixed for another 2 hr.

During this period, 800 µl samples were removed at various times from each flask. The samples were centrifuged at 5000 rpm for 5 min to separate the microparticles from the buffer. Protein concentration in supernatant was analysed to determine protein release. For buffer solutions at pH > 6.5, 200 µl HCl solution (pH = 1) was added to samples as they were taken to lower pH and prevent further particle dissolution and protein release. All of the experiments were replicated thrice.

3.6.3 Dissolution Test for Different Sized Microparticles

These tests were performed similarly but with different sized microparticles and only at pH of 6.6, 6.8, 7.1 and 7.4. The conical flasks were mixed at 37 °C for 10 min. The protein released from the microparticles as a function of the time was recorded. Each experiment was replicated three times.

4 RESULTS AND DISCUSSION

4.1 Introduction

Experiments were undertaken to encapsulate BSA in microparticles of ES 100, a pH sensitive polymer. The method of preparation was by O/O emulsification using sonication at 20 kHz at different amplitude levels, followed by solvent evaporation. A range of investigations were subsequently conducted:

- Observations of particle morphology and measurement of particle size by SEM;

- Measurement of the relationship between amplitude level and power input during sonication using a hydrophone and thermal energy dissipation in an adiabatic container;

- Validating the accuracy of a Lowry-type colorimetric assay, used to quantify protein release during dissolution experiments with the microparticles, in the presence of ES 100;

- Dissolution of the microparticles at alkaline pH to measure encapsulation efficiency; and

- For the selected microparticle sizes, measurement of protein release as a function of time during dissolution at various pH, which were selected to simulate pH conditions at different locations of the GI tract.

Based on the above results, the optimum conditions for manufacture of microparticles to encapsulate lactase for oral delivery to the small intestine were considered.

4.2 Sonication

4.2.1 Preliminary Observations

Figure 4.1 shows a picture of the tip-fluid interface during sonication of water at 70 % amplitude with the Branson 450 Digital Sonifier. The production of cavitation in the immediate vicinity of the tip and violent fluid motion further away can be clearly observed. The majority of output from the tip appears to emanate in a vertical direction, downwards.

(a) **(b)**

Figure 4.1: Pictures of sonication output produced at tip by Branson 450 Digital Sonifier at 70% amplitude in water: (a) observation of tip; (b) fluid motion.

4.2.2 Hydrophone Measurements

Figure 4.2 summarizes power output values at 20 kHz estimated for the sonicator by measurement of sound pressure with the hydrophone. The measurements were taken at different amplitude levels and hydrophone distances from the tip. The sound pressure levels were converted to energy outputs by Equation 3-1.

As can be observed in Figure 4.2, different energy output behaviours were obtained depending on the hydrophone position. However, the estimated output by this method should be the same regardless of hydrophone distance from the tip. Furthermore, the calculated outputs closer to the tip were much lower than further away.

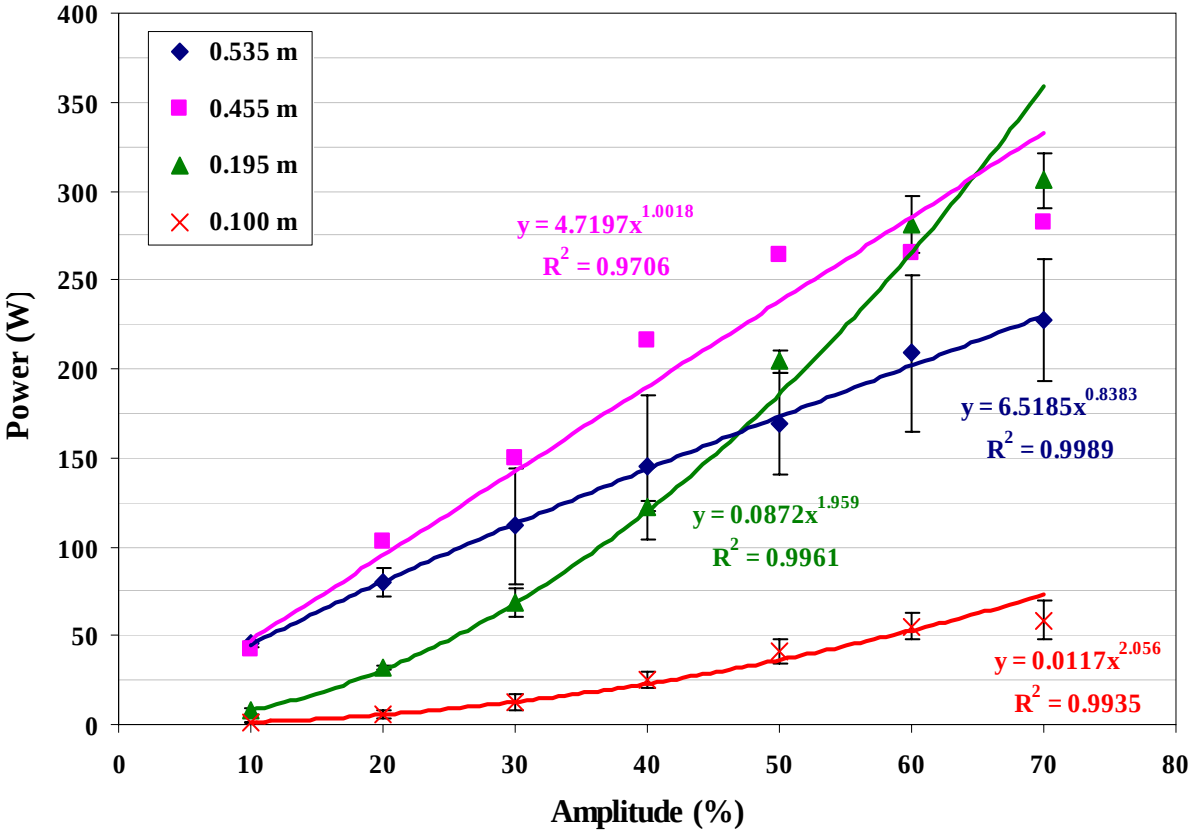


Figure 4.2: Acoustic power produced at different amplitudes.

(The legend in the figure shows the distance between hydrophone and tip; error bars show the standard deviation for replicates.)

Whilst not realized at the time of the measurements, this arose because the hydrophone was not ideally positioned. The hydrophone was located vertically below but not directly and was offset to the side. The value of this horizontal offset was constant regardless of vertical distance. As a consequence, when the hydrophone was nearer to the tip it was outside the primary zone of energy output, which emanated vertically and downwards. This output zone became broader and more diffuse further away from the tip. Hence, as the hydrophone position was adjusted further below the tip, it increasingly moved into this zone.

Additionally, the equation used to convert sound pressure measurements to energy output assumed sound pressure waves were produced equally in all directions (i.e. D_θ = 1). This was clearly not the case (i.e. D_θ > 1), and hence, energy output was overestimated. This problem was compounded by neglecting the reverberation field from the calculation (as previously mentioned in the Materials and Methods).

As a result, the values obtained from the hydrophone are probably in the right order of magnitude but are not accurate.

4.2.3 Adiabatic Bomb: Heat Dissipation Measurements

Figure 4.3 shows the power output from the sonicator estimated by measurements of heat dissipation in an adiabatic bomb. The output values are in the same order of magnitude as the hydrophone measurements but much lower in value. The output appears to increase exponentially with amplitude. Figure 4.3 suggests that an increase in sonicator amplitude from 10 to 70 % will increase energy output by between 15 and 20 times. Based on heat dissipation, the relationship between output power and amplitude of the sonicator in water may be conveniently represented by a power-law model:

$$y = 0.0355x^{1.7959} \quad \textbf{(4-1)}$$

where x is the amplitudes of 450 Digital Sonifier (%) and y is corresponding power (W).

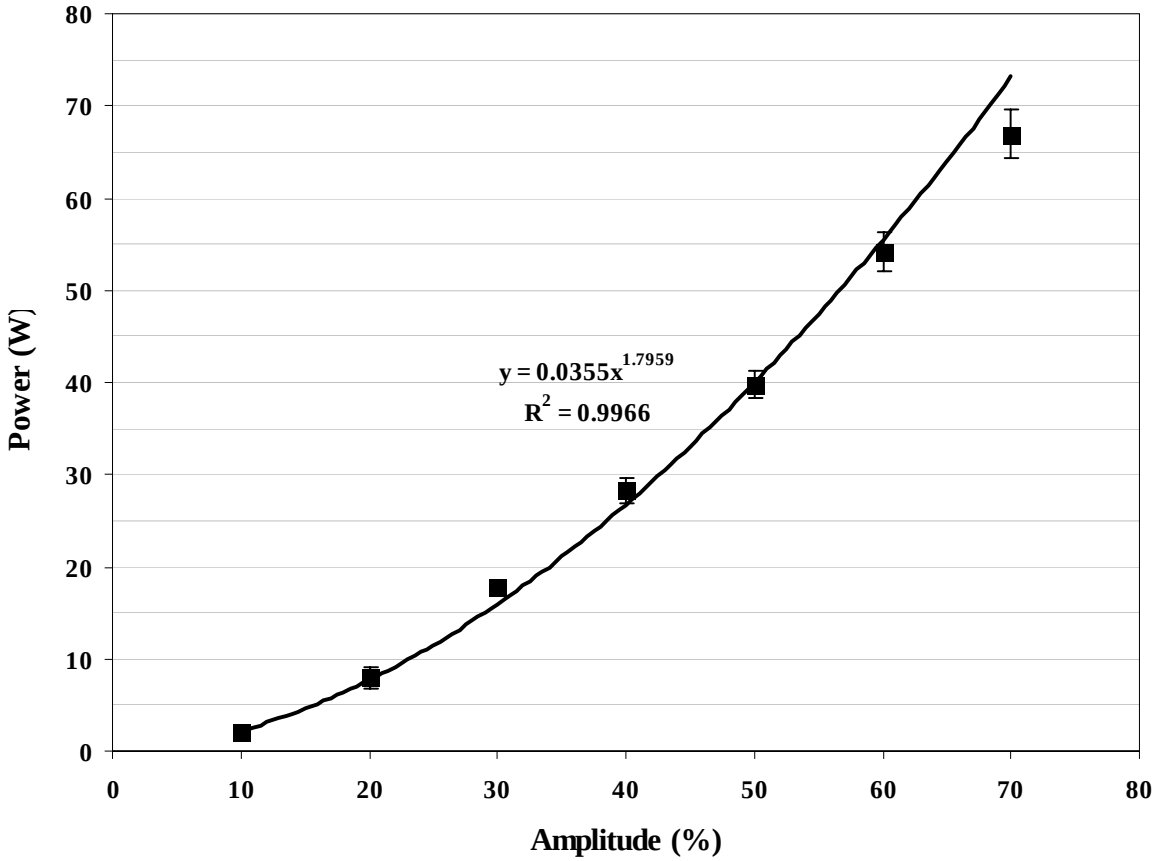
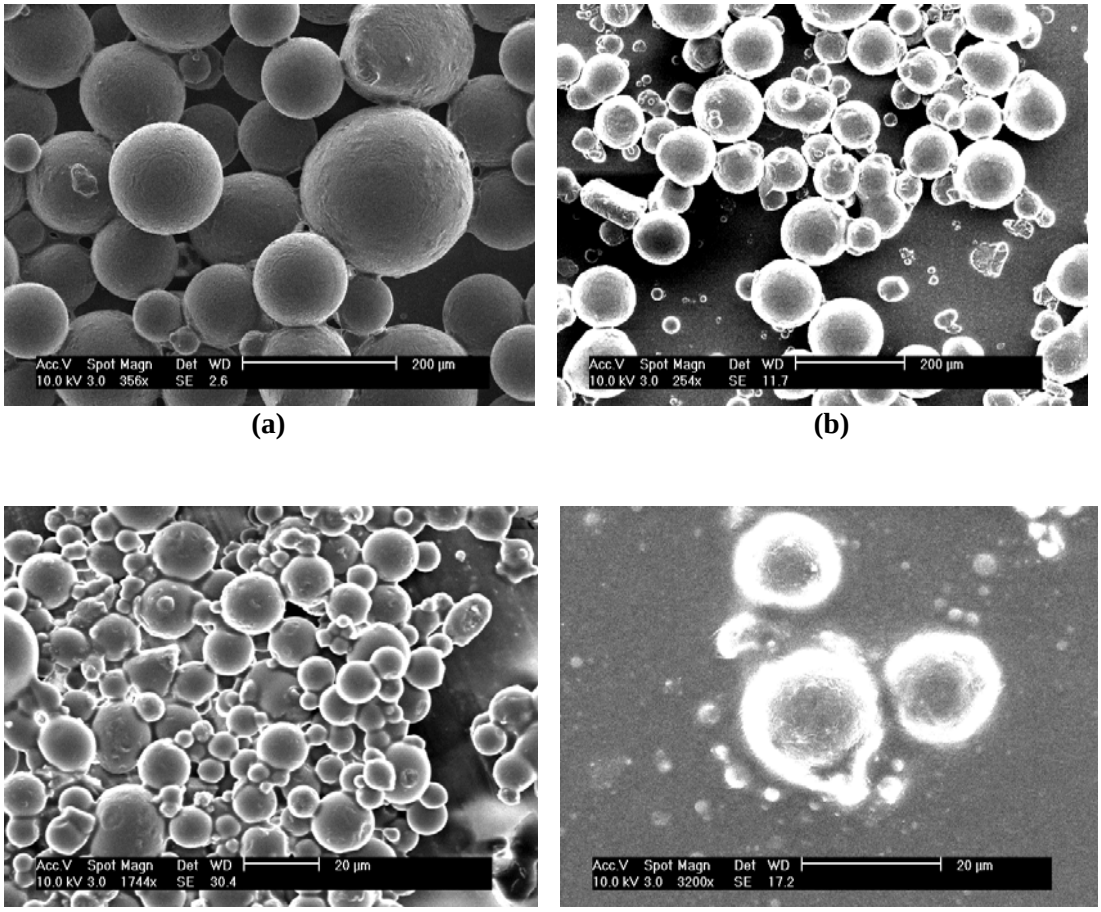


Figure 4.3: Power produced at amplitudes as estimated by thermal energy dissipation. (The error bars show S.D.)

4.3 Characterization of Microparticles

4.3.1 Particle Morphology

Figure 4.4 shows SEM images of ES 100 microparticles encapsulating BSA produced by sonication at 4 different amplitudes, 40%, 50%, 60% and 70% respectively. Note that Figure 4.6 (a) and (b) are at a different scale to (c) and (d).



(a) **(b)**

(c) **(d)**

Figure 4.4: SEM images of microparticles from different amplitudes: (a) 40% (Bar=200µm); (b) 50% (Bar=200µm); (c) 60% (Bar=20µm); (d) 70% (Bar=20µm).

The microparticles produced at different amplitudes all have uniform and similar morphologies, being of spherical-like shape. However, evidence can be observed of aggregation, where smaller particles have coalesced during the emulsification process.

Figure 4.5 displays a close-up image of an individual particle at 40% amplitude. The surface of the particle is relatively smooth and does not appear porous.

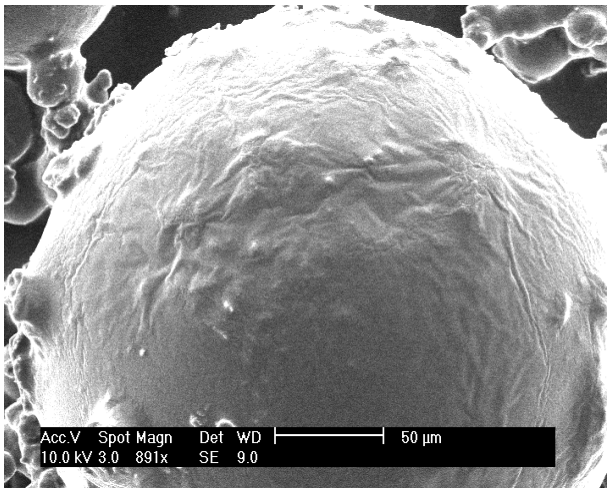


Figure 4.5: SEM images of microparticle surface at 40% amplitude (Bar=50µm).

Figure 4.6 compares an SEM image from the current work with those presented by other studies for ES 100 microparticles. Where possible, each image has been adjusted so that the sizes of particles appear approximately equal in scale. The particles in Figure 4.6 (b) are from Alavi *et al*. (2002), where the same chemicals and methods were employed except that high shear homogenization was used instead of sonication. The shape and surface characteristics of their microparticles do not seem significantly different to those observed in this study.

Figure 4.6 (c) shows microparticles obtained by Rodriguez *et al.* (1998). This study was almost identical to Alavi *et al.* (2002) but employed Span 85 instead of sucrose stearate as the surfactant during the emulsification step. Their microparticles are also similar in shape and surface characteristics to those observed in this study.

Figure 4.6 (d) displays a picture of microparticles produced by Amorim & Ferreira (2001). These microparticles appear to have a much rougher surface and a porous structure. Amorim & Ferreira (2001) used the same methods as Rodriguez *et al.* (1998), but applied a much lower ratio between ES 100 polymer and the encapsulated component. Their ratio was 2:4 (w/w) whilst Rodriguez *et al.* (1998), Alavi *et al.* (2002) and this study employed ratios of 5:1 (w/w), 5:1(w/w) and 10:1 (w/w) respectively.

Clearly, at low ratios there could be large occlusions of the encapsulated component present in the microparticle and at its periphery that could lead to this type of surface characteristic.

Figure 4.6 (e) and (f) show microparticles produced by Jani *et al.* (2005) and Lee *et al.* (2000), respectively. These studies used a different emulsification strategy: W/O/W and W/O/O, instead of O/O. The encapsulated polymer and component ratios were almost identical to those used in this study. The shape and surface characteristics in both cases are similar to those obtained in this study and by Rodriguez *et al.* (1998) and Alavi *et al.* (2002).

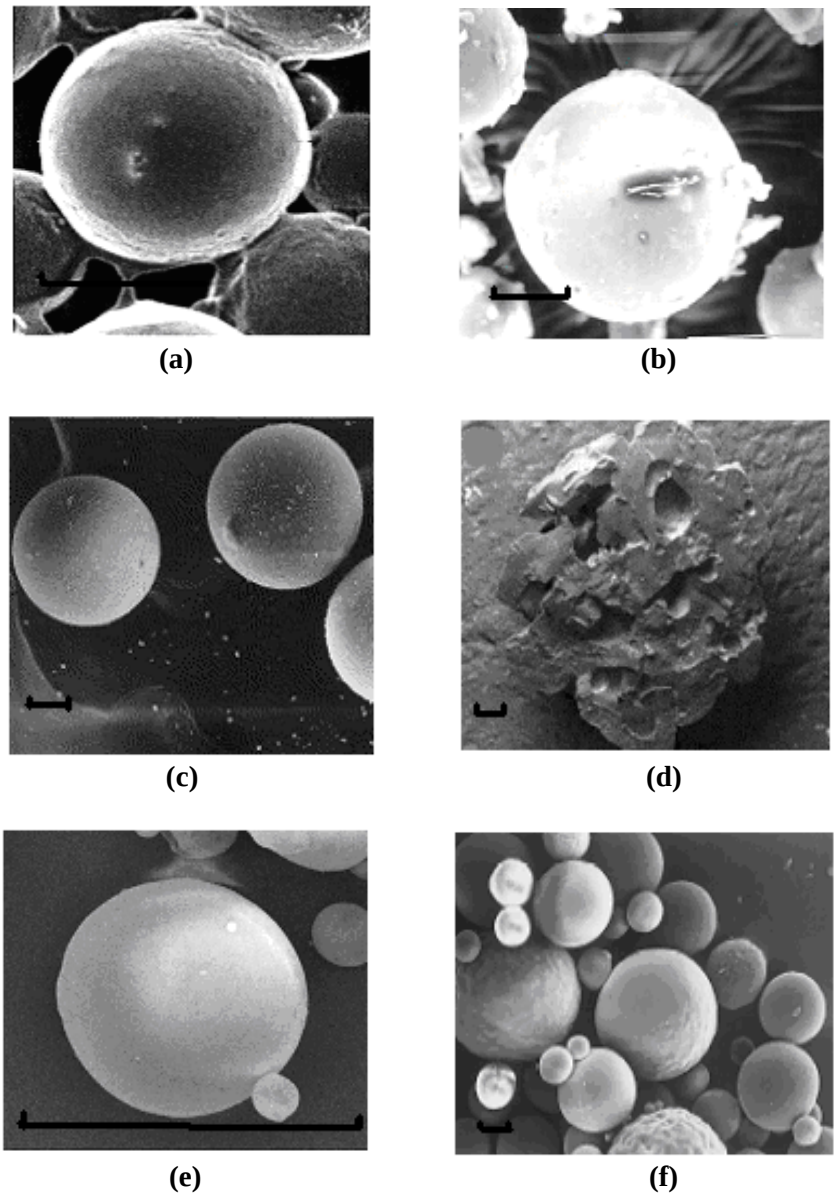


Figure 4.6: SEM graphs of ES 100 particle morphology: (a) this research at 40% amplitude; (b) Alavi *et al.* (2002); (c) Rodriguez *et al.* (1998); (d) Amorim & Ferreira (2001); (e) Jani *et al.* (2005); and (f) Lee *et al.* (2000) (Bar = 50 µm).

4.3.2 Particle Size Analysis

Figure 4.4 suggests that increasing sonicator amplitude during emulsification decreased the microparticle particle size. Figure 4.7 compares the frequency particle size distributions that were obtained by analysis of SEM images. Table 4.1 gives the median particle size, and 10^{th} and 90^{th} percentile values of the microparticles calculated from these particle size distributions.

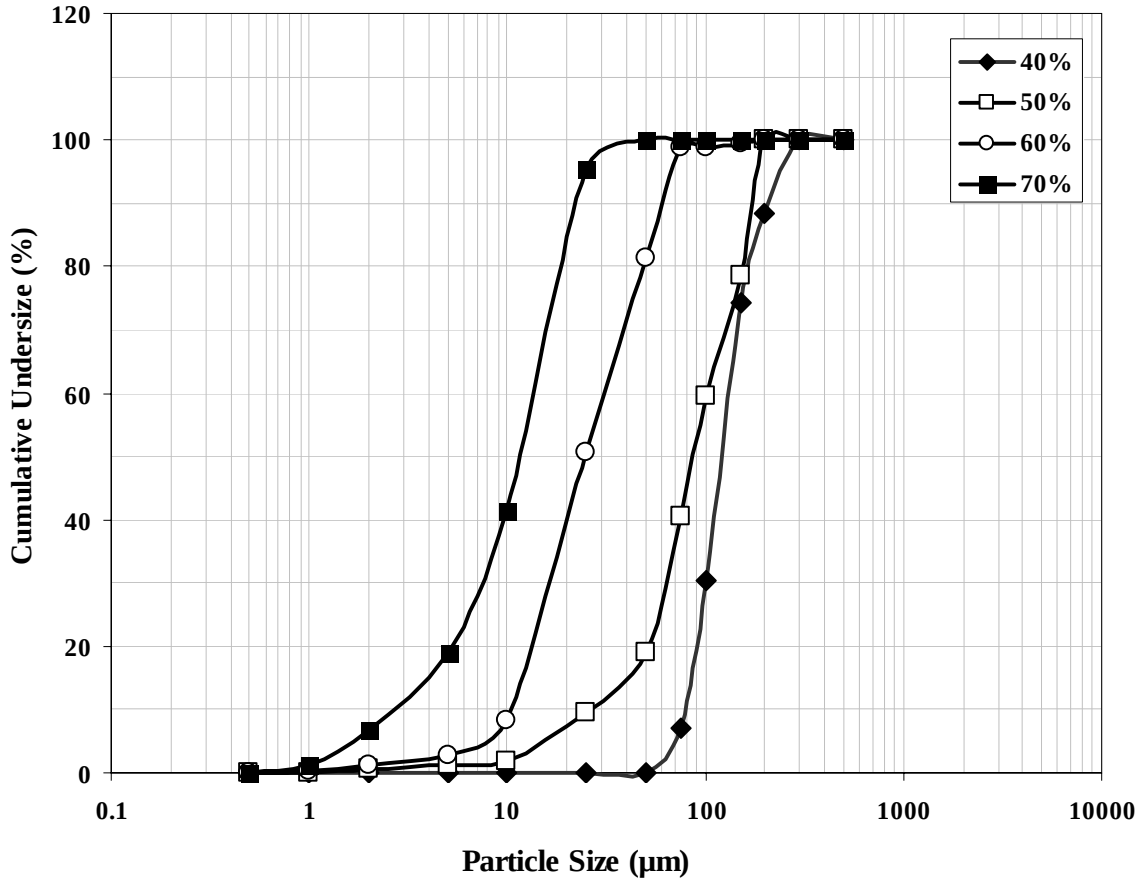


Figure 4.7: Frequency distributions of microparticles obtained at different sonicator amplitudes: (a) 40%, (b) 50%, (c) 60%, and (d) 70%.

(Sample size for frequency distributions was: n_p = 143 for 40% amplitude; n_p = 156 for 50% amplitude; n_p = 103 for 60% amplitude; n_p = 102 for 70% amplitude.)

Table 4.1: Median size of particles obtained from 40% to 70% amplitude.

Amplitude (%)	Median Particle Diameter (µm)	90% of produced particles (µm)	10% of produced particles (µm)
40	120	210	80
50	84	171	26
60	25	60	10
70	12	22	3

Table 4.1 confirms that increasing the amplitude decreased the median particle size. However, the ratio of the 90^{th} to 10^{th} percentile rose with increasing amplitude

The size of microparticles obtained in this study, particularly at the higher amplitudes of sonication, was much smaller than those obtained by previous studies. Amorim & Ferreira (2001) produced microparticles between 500 µm and 1mm by using stirring at 450 rpm for emulsification. Rodriguez *et al.* (1998) also used stirring but at 1000 rpm. The size of particles obtained was around 300 µm. Alavi *et al.* (2002) employed a high shear mixer allowing agitation at 6000 rpm. They obtained microparticles of approximately 195 µm. Unfortunately, relevant details about the conditions under which previous studies were performed are not available to estimate the power input that was provided during emulsification. However, the correlation of decreasing particle size with increasing agitation or mixing speed suggests that smaller particles are achieved by increased shear through higher energy input, as would be expected. At each of the amplitude settings used in this research, particles of even smaller size were obtained. Clearly, sonication appears able to provide a significantly higher energy input per unit volume and increased shear conditions for emulsification than previous studies.

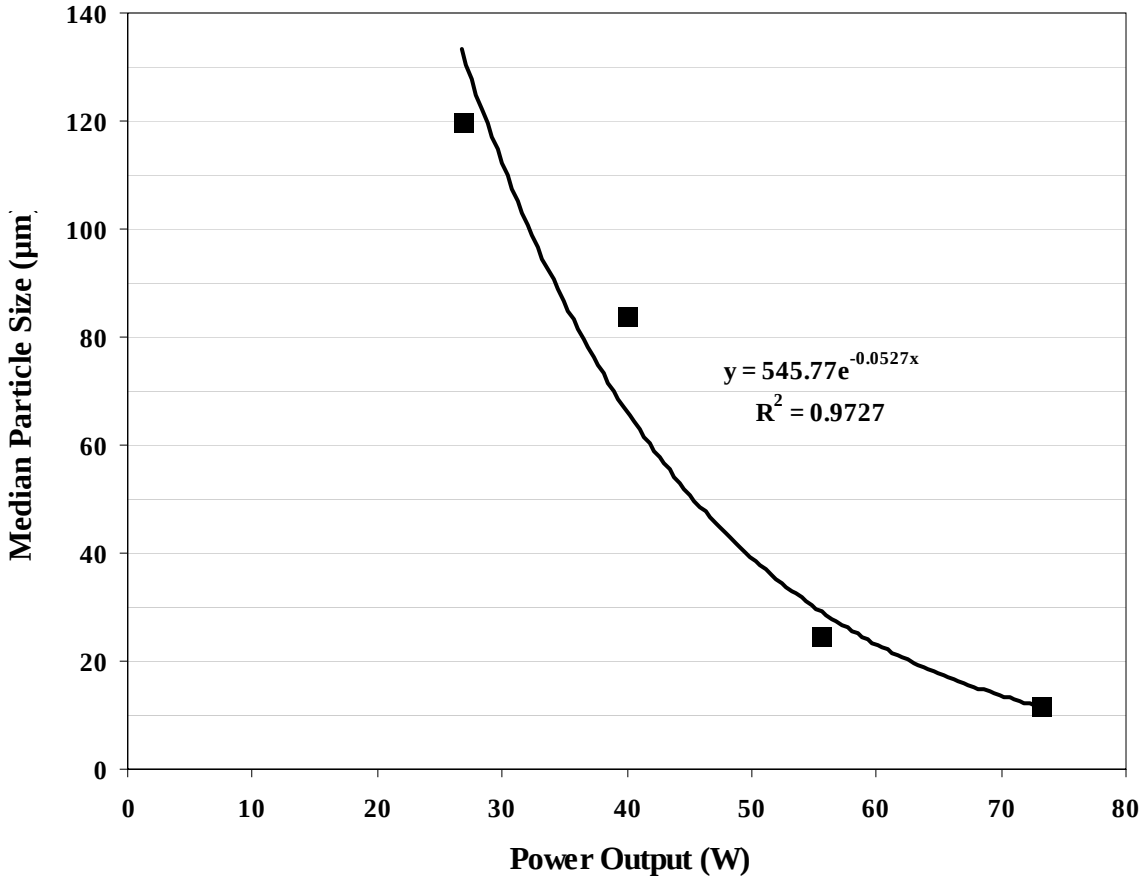


Figure 4.8: Relation between the output power and median particle size.

Figure 4.8 shows the relationship between the power output measured by thermal energy dissipation and the median particle size for each of the amplitude settings that were investigated. It displays a non-linear inverse relationship. It also suggests that the particle size may be starting to tend towards an asymptote with further increases in power input as would be expected.

4.4 The Effects of ES 100 in Absorbance: Statistical Analysis

Table 4.2 compares the response of the Bio-Rad DC protein assay in the presence or absence of ES 100 in a pH 6.5 buffer for the low and high concentration ranges, respectively. There are some slight differences in values at equivalent BSA concentrations but on average the presence of ES 100 does not appear to affect the assay response. This was confirmed by regression analysis using a dummy variable for presence of ES 100:

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \qquad \textbf{(4-2)}$$

where x_1 was the BSA concentration (mg/ml); x_2 was 0 or 1 corresponding to absence or presence of ES 100 polymer in the buffer; and β_0, β_1 and β_2 were the regression coefficients.

The regression analysis was performed on experimental data for both low and high assay concentration ranges. Table 4.3 summarizes results of the regression analyses, respectively. These suggest that in both cases the presence of ES 100 did not have a significant effect on the assay response. Furthermore, the linear regression between BSA concentration and absorbance was highly significant. Therefore, the relationship between BSA concentration and absorbance for the low concentration range of the assay could be represented by

$$\hat{y} = 2.14077\ x_1 + 0.08153 \qquad \textbf{(4-3)}$$

Likewise, for the high concentration range of the assay,

$$\hat{y} = 0.30115\ x_1 + 0.06008 \qquad \textbf{(4-4)}$$

These relationships were used to estimate the BSA concentration during dissolution experiments.

Table 4.2: Data for Bio-Rad DC protein assay.

Protein Assay	Presence of ES 100	Observation	BSA Concentration (mg/ml)	Absorbance
Low Concentration	With ES 100	1	0.01	0.0643
		2	0.06	0.2420
		3	0.12	0.3767
		4	0.18	0.4713
		5	0.25	0.6013
	Without ES 100	6	0.01	0.1160
		7	0.06	0.1870
		8	0.12	0.3413
		9	0.18	0.4863
		10	0.25	0.6043
High Concentration	With ES 100	1	0.20	0.1027
		2	0.50	0.2007
		3	0.80	0.2847
		4	1.10	0.3917
		5	1.40	0.4733
	Without ES 100	6	0.20	0.1103
		7	0.50	0.2397
		8	0.80	0.2980
		9	1.10	0.3790
		10	1.40	0.4780

Table 4.3: Analysis of variance for factors affecting absorbance.

		Coefficients	Standard Error	t_0	P-value
Low Concentration	β_0	0.08153	0.01784	4.57065	0.00257
	β_1	2.14077	0.10328	20.72716	1.52811E-07
	β_2	0.00414	0.01756	0.23580	0.82033
High Concentration	β_0	0.06008	0.01026	5.85762	0.00063
	β_1	0.30115	0.01026	29.36120	1.36909E-08
	β_2	-0.01038	0.00870	-1.19267	0.27185

***** $t_{0.025,\ 7} = 2.365$ (Montgomery, 2001)***

4.5 Encapsulation Efficiency

Table 4.4 presents results of encapsulation efficiency for BSA in ES 100 measured at the four amplitude settings 40%, 50%, 60% and 70%.

The encapsulation efficiencies achieved at amplitude levels of 50%, 60% and 70% lay between 70% and 80%. However, the encapsulation efficiency recorded at the 40% setting was much lower, around 40%.

Alavi *et al.* (2002) obtained maximum encapsulation efficiency for lactase of around 80%. Amorim & Ferreira (2001) obtained relatively high encapsulation efficiencies of 98% for casein and Apro. Rodriguez *et al.* (1998) was able to encapsulate 60% to 96% of ondansetron or budesonide in ES 100. As already mentioned, these studies were all performed at different energy inputs and shear conditions. High levels of agitation have been reported to decrease the encapsulation efficiency of microparticles (Rafati *et al.*, 1997). However, this does not seem to correlate with the trend observed in this study and from these other results. The slight difference in values obtained between this and other studies are probably because of the different components being encapsulated, and the method and techniques employed for preparation of the microparticles. In particular, solubility of a component in, and its partitioning between, the solvent and polymer phase will affect the amount which is successfully encapsulated (Kim *et al.*, 2002). Unfortunately, this does not explain why a lower value was consistently achieved at 40% amplitude.

Table 4.4: Encapsulation efficiency of microparticles made at different ratios of BSA and ES 100 (50% amplitude, n=2).

Amplitude (%)	W_{BSA} (mg)	$W_{ES\ 100}$ (mg)	W_{BSA}/ $W_{ES\ 100}$	EE (%) ± S.D.
40	22.5	225	1/10	42.03 ± 3.12
50	22.5	225	1/10	83.13 ± 2.74
60	22.5	225	1/10	69.96 ± 8.90
70	22.5	225	1/10	71.14 ± 3.68

**** W_{BSA} is the amount of BSA (mg); $W_{ES\ 100}$ is the amount of ES 100 polymer (mg); W_{BSA}/ $W_{ES\ 100}$ is the ratio between the amount of BSA (mg) and ES 100 polymer (mg); EE is the encapsulation efficiency (%).***

4.6 *In vitro* Release Studies

4.6.1 Release Amount: Influence of pH

The percentage release of BSA with time observed at different pH is shown in Figure 4.9. The microparticles were produced at 50% amplitude of sonication with a median diameter 84 μm. The rate and period of release display a strong functional dependence on pH. The relationship of BSA release with time exhibits exponential-type behavior. The approximate residence time in the GI tract is 2 hr.

Figure 4.10 summarizes the BSA release at 2 hr during dissolution experiments from Figure 4.9 as a function of pH. There was a slight leakage from the microparticles at low pH. However, significant release is not apparent until pH reaches 4.5. At pH 6.0, around 30% of encapsulated BSA was released. The release amount was approximately 40% and 70% for pH 6.6 and 6.8 respectively. Above pH 7, 100 % release was achieved within 2 hr. As pH increased further, the time for 100 % release decreased below 2 hr. Less than ½ hr was needed for total release at pH 8.1, the maximum pH value that was tested.

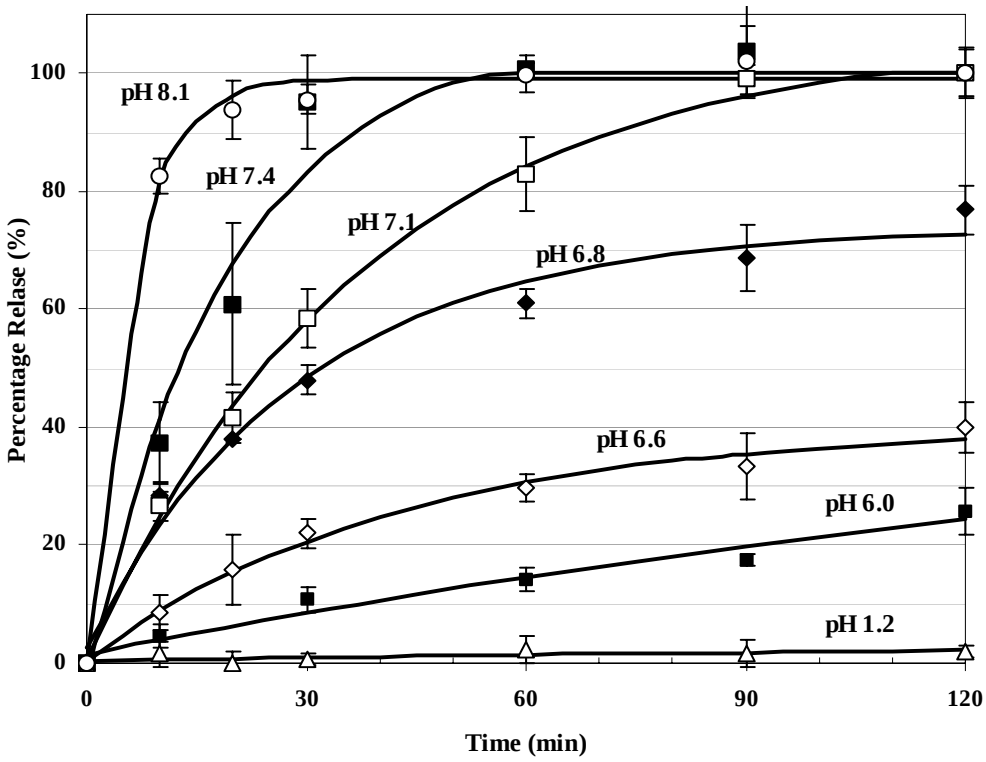


Figure 4.9: BSA release from ES 100 microparticles at selected pH values. (Points are raw data at different pH. The error bars show SD.)

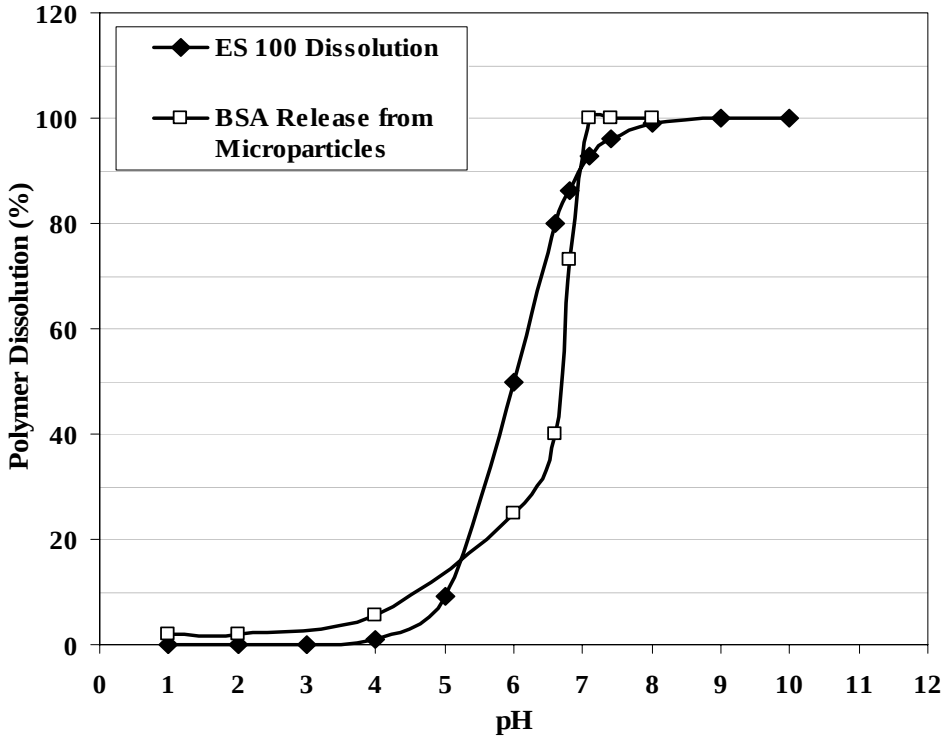


Figure 4.10: BSA release versus ES 100 microparticles.

Included in Figure 4.10 is the theoretical dissolution equilibrium of the ES 100 polymer (Nguyen & Fogler, 2005).

The manufacturer in its product literature for ES 100 indicates a dissolution threshold for ES 100 of 7.0. This is consistent with the theoretical dissolution behavior of the polymer and experimental data obtained in this study.

However, it is clear from Figures 4.9 and 4.10 that dissolution and/or protein release commences at much lower pH than the manufacturer's threshold. In fact, it seems that protein release from microparticles commences even before dissolution of the polymer is predicted theoretically. This could be because the theoretical predictions do not match the real behavior of ES 100 in the buffer solutions that were used in this study. However, the initial releases at low pH may simply be caused by leakage of BSA occluded in the ES 100 at or close to the microparticle surface. Furthermore, as pH rises, structural changes in the microparticle may occur prior to dissolution and permit additional solubilisation and leakage of BSA to occur. ES 100 polymer is of low permeability and nearly insoluble in acid medium. The low permeability results from high intermolecular attraction between its molecules. Hydrogen bonding between the hydroxyl groups of the carboxylic moiety and the carbonyl oxygen of ester groups increases the degree of compactness of the polymer and decreases its porosity and permeability (El-Kamel *et al.,* 2001). With increasing pH the carboxylic acid side groups of ES 100 start to become de-protonated. This may cause swelling (Bhagwat *et al.,* 2005), which may initially be localized at the microparticle surface. The swelling may allow leakage of encapsulated components even though the microparticle is not dissolving.

Similar observations of leakage from ES 100 at acidic pH have been made in previous studies, where significant release of encapsulated component from ES 100 prior to the specified dissolution pH threshold of 7.0 has occurred. Lee *et al.* (2000) measured a release of approximately 80% for acetaminophen and theophylline at a pH of 6.8 after 10 hr compared with 70% obtained for BSA in this study. The encapsulated components in their study were different kinds of drugs with MW< 500. The ratio of polymer to encapsulated component was 5:1 (w/w) and the particle size was approximately 200 μm. Amorim & Ferreira (2001) used a polymer and drug mixture ratio of 2:4 (w/w), and

encapsulated casein and Apro in microparticles of 500 μm to 1 mm. At pH 6.8, 100% of encapsulated drugs were released within 3 hours. Rodriguez *et al.* (1998) conducted release studies at pH=6.8 on microparticles of 300 μm made with a polymer and encapsulated component (CAB cores containing ondansetron or budesonide) ratio of 5:1 (w/w). Only 5% of an encapsulated drug was released after 2 hr.

One would expect that lower MW encapsulated components would be more easily solubilised and leaked or released prematurely than those of higher MW. Furthermore, most leakage would occur at a microparticle's surface. Hence, it would also be expected for an equivalent mass of polymer, smaller particles would generate greater leakage (Freiberg & Zhu, 2004). As well, higher polymer to encapsulated component ratios increase density of the polymer matrix and would be expected to decrease leakage. All of these factors are different in each of the above mentioned studies and it is impossible to analyze and observe such trends. However, the effect of polymer to encapsulated component ratio seems clearly evident in the study by Amorim & Ferreira (2001). Amorim & Ferreira (2001) employed a substantially lower polymer to encapsulated component ratio and produced larger microparticles than the other studies. SEM images of their particles (see Figure 4.6) even suggested they might be porous unlike those produced in this and other studies. Despite a much larger particle size, this may have been responsible for the 100 % release of casein (MW~26 kDa) and Apro (MW~ 6.5 kDa) they observed at pH 6.8.

4.6.2 Release Rate: Influence of Particle Size

Figure 4.11, 4.12, 4.13, and 4.14 compare the release rate of BSA with time observed from the various sized microparticles produced at different amplitude settings for pH of 6.6, 6.8, 7.1 and 7.4. As can be observed, particle size affected the release profile, with smaller particles increasing the rate of release. The explanation for this is essentially the same as given above in the previous section for the influence of particle size when comparing the results of this study with others (Freiberg & Zhu, 2004): smaller particle sizes present greater surface area at equivalent mass for leakage and/or dissolution.

However, particle size does not seem to influence the terminal value (i.e. as t tends towards infinity) of BSA release. All of the release profiles at each pH appear to tend towards the same asymptotic value of release.

From Figure 4.14, it can be seen that at a pH of 7.4 the release is completed in around 1 hr for particles of 120 µm size, and within half an hour for those with a size of 12 µm. Lee *et al.* (2000) and Rodriguez *et al.* (1998) also conducted release rate tests at this pH during their studies. The particle size in each of these studies was approximately 200 and 300 µm, respectively. For Lee *et al*. (2000), the release period required was 8 hours, whilst the release was completed in 5 hours for Rodriguez *et al.* (1998). This longer duration to achieve complete release is consistent with the larger particle size in these studies compared to the results obtained here.

Lee *et al.* (2000) also measured the release period for pH of 6.8. Over 10 hr was required compared with 1 hr for the 12 µm particles and just over 2 hr for the 120 µm microparticles in this study.

There is no other data available in the literature to compare release rates obtained in this study at other pH values.

There was no requirement for this study to model the process of microparticle dissolution. Hence, it cannot be established what the mechanism of dissolution was and how it should theoretically behave with particle size and pH. However, the experimental data in Figure 4.11, 4.12, 4.13, and 4.14 provide the necessary information to infer how these microparticles would behave during transit through the GI tract.

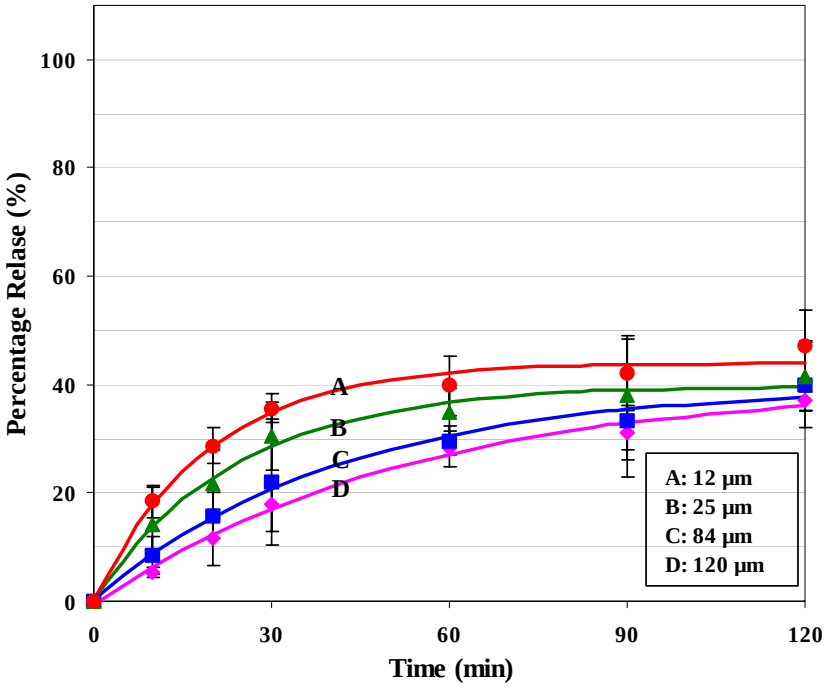


Figure 4.11: Release studies at PBS buffer (pH=6.6).

(Results are average ± S.D. of three batches. In those figures, ♦ stands for the raw results obtained from the microparticles with median particle size 120 µm; ■ stands for those with size 84 µm; ▲ stands for those with size 25 µm and ● for particles with size 12 µm.)

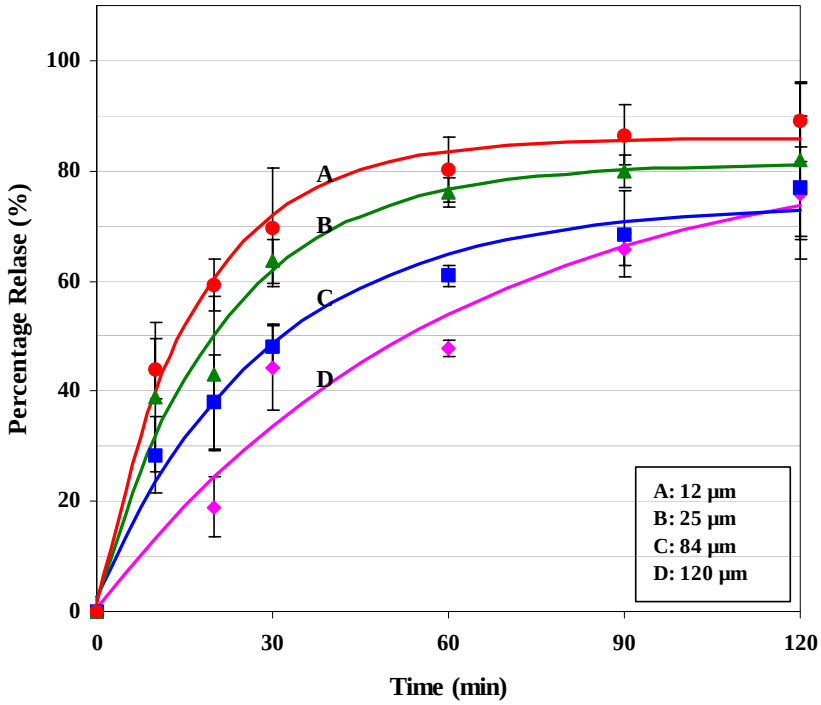


Figure 4.12: Release studies at PBS buffer (pH=6.8).

(Results are average ± S.D. of three batches. In those figures, ♦ stands for the raw results obtained from the microparticles with median particle size 120 µm; ■ stands for those with size 84 µm; ▲ stands for those with size 25 µm and ● for particles with size 12 µm.)

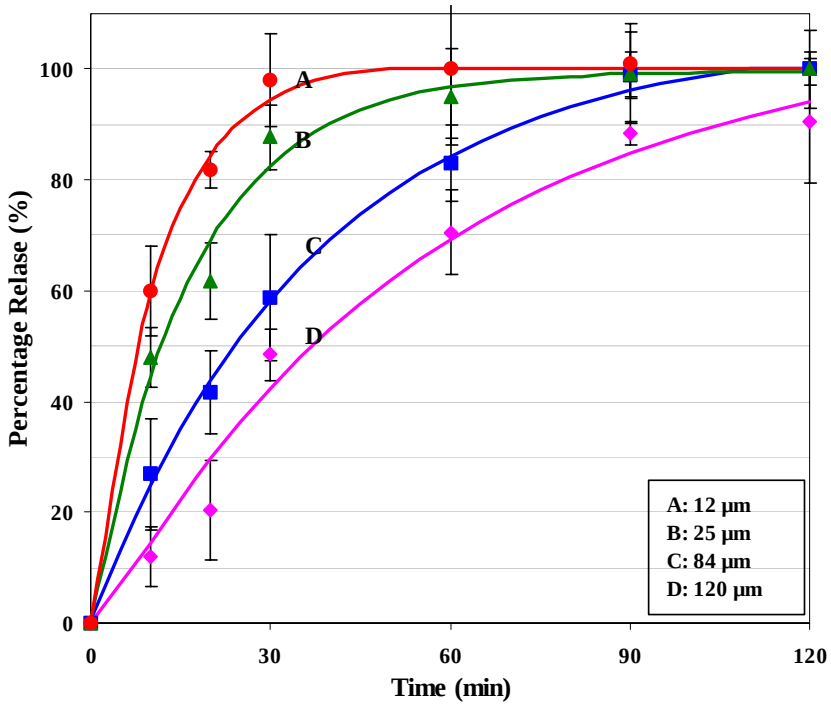


Figure 4.13: Release studies at PBS buffer (pH=7.1).

(Results are average ± S.D. of three batches. In those figures, ♦ stands for the raw results obtained from the microparticles with median particle size 120 µm; ■ stands for those with size 84 µm; ▲ stands for those with size 25 µm and ● for particles with size 12 µm.)

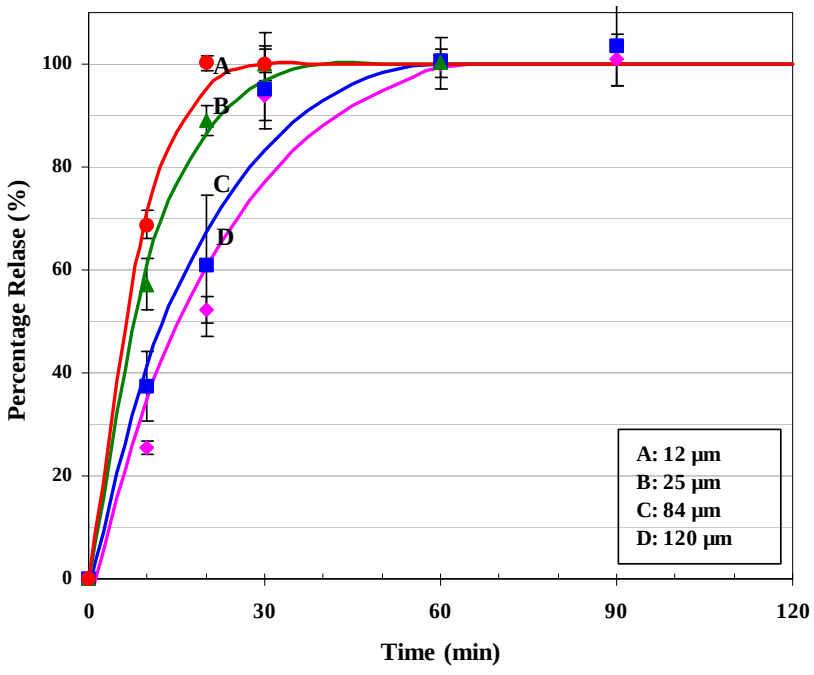


Figure 4.14: Release studies at PBS buffer (pH=7.4).

(Results are average ± S.D. of three batches. In those figures, ♦ stands for the raw results obtained from the microparticles with median particle size 120 µm; ■ stands for those with size 84 µm; ▲ stands for those with size 25 µm and ● for particles with size 12 µm.)

4.6.3 Projected Performance for Protein Release in GI Tract

As mentioned before, the aim of this research was to focus on the therapy for lactose intolerance (LI) that involves oral delivery of lactase enzyme. In particular, the microparticles must be able to survive the acidic pH of the stomach and effectively release the majority of the lactase within the transit time they will spend in the small intestine. This will ensure that lactose is broken down before it enters into the large intestine, as this is where bacteria ferment it, and can cause the abdominal cramps and gas, bloating and diarrhea – the symptoms of lactose intolerance (Pribila *et al.*, 2000).

The mean transit time for food in the GI tract is 2 hours in the stomach and around 3 hours in the small intestine (Coupe *et al*., 1991; Davis *et al.*, 1986; Davis *et al*., 1984;).

According to the Figures from 4.9 to 4.14, in the gastric juice of the stomach where pH may range from 1.0 to 2.5, there will be almost no release of protein from the microparticles, assuming similar behaviour between BSA and lactase. In the small intestine, the pH increases to pH 6.6 (±0.5) for the first hour of transit. At this pH, the microparticles produced in this study would release between 20 and 40 % of the encapsulated protein in 1 hour. pH of the small intestine then gradually increases to approximately 7.5 (±0.4) over the remaining 2 hours of transit. Based on this, the microparticles should be able to release the remaining protein well before they reach the terminal ileum and food passes into the large intestine. Particle size will affect how quickly the protein is released. It would probably be ideal to have particles that are at least less than 100 μm to ensure that release occurs in enough time to allow the lactase enzyme to act on the lactose in the food. In this respect, the kinetics of lactase is relatively rapid, in the order of 15 minutes (Zhou *et al.*, 2003). Therefore, the microparticles produced in this study, if lactase were substituted for BSA, should be able achieve the desired treatment objective.

5 CONCLUSION

This research focused on a method for dealing with LI. A microencapsulation technique for oral delivery of lactase enzyme was investigated. EUDRAGIT S 100 polymer was applied to encapsulate BSA, which was used as a surrogate for lactase enzyme. An O/O emulsification and solvent evaporation technique was used. Sonication was employed for emulsification.

The microparticles produced at different sonication amplitudes or power outputs all possessed uniform and similar morphologies, being spherical-like in shape. The surface of the particles was relatively smooth and did not appear porous. Microparticle size decreased with sonicator energy output from 120 µm to 12 µm with an amplitude change from 40% to 70%. The relationship between power output and median particle was non-linear and possibly asymptotic with further increases in power input.

The encapsulation efficiencies achieved at amplitude levels of 50%, 60% and 70% were between 70% and 80%. However, the encapsulation efficiency recorded at the 40% setting was much lower, around 40%. There was a slight leakage from the microparticles at low pH. At pH 6.0, around 25% of encapsulated BSA was released. The release amount was approximately 40% and 70% for pH 6.6 and 6.8 respectively. Above pH 7, 100 % release was achieved within 2 hr. Particle size affected the release profile, with smaller particles increasing the rate of release. Assuming there is a similar behavior between BSA and lactase, the microparticles should be able to survive the acidic pH of the stomach and effectively release the majority of the lactase within the transit time they will spend in the small intestine. This will ensure that lactose is broken down before it enters into the large intestine and LI symptoms occur.

6 RECOMMENDATIONS FOR FURTURE WORK

This study has focused on a microencapsulation method for lactase but using BSA as a surrogate protein. To further develop this method for potential commercial application, the following work should be undertaken.

- Experiments should be performed to encapsulate lactase in ES 100 and verify that similar performance will be achieved.

- Sonication might adversely affect protein activity during microparticle production. This should be investigated during the above recommended study.

- A second layer could be used to additionally encapsulate the ES 100 microparticles. This outer material could be utilised to preserve the particles in commercial products, such as milk. It would dissolve in the stomach releasing ES 100 particles, which would then travel onto the small intestine and deliver lactase enzyme. This would allow lactase delivery to occur as part of a food product and avoid the need for it to be taken separately prior to a meal.

APPENDIX A: CALIBRATION CURVES FOR HYDROPHONE

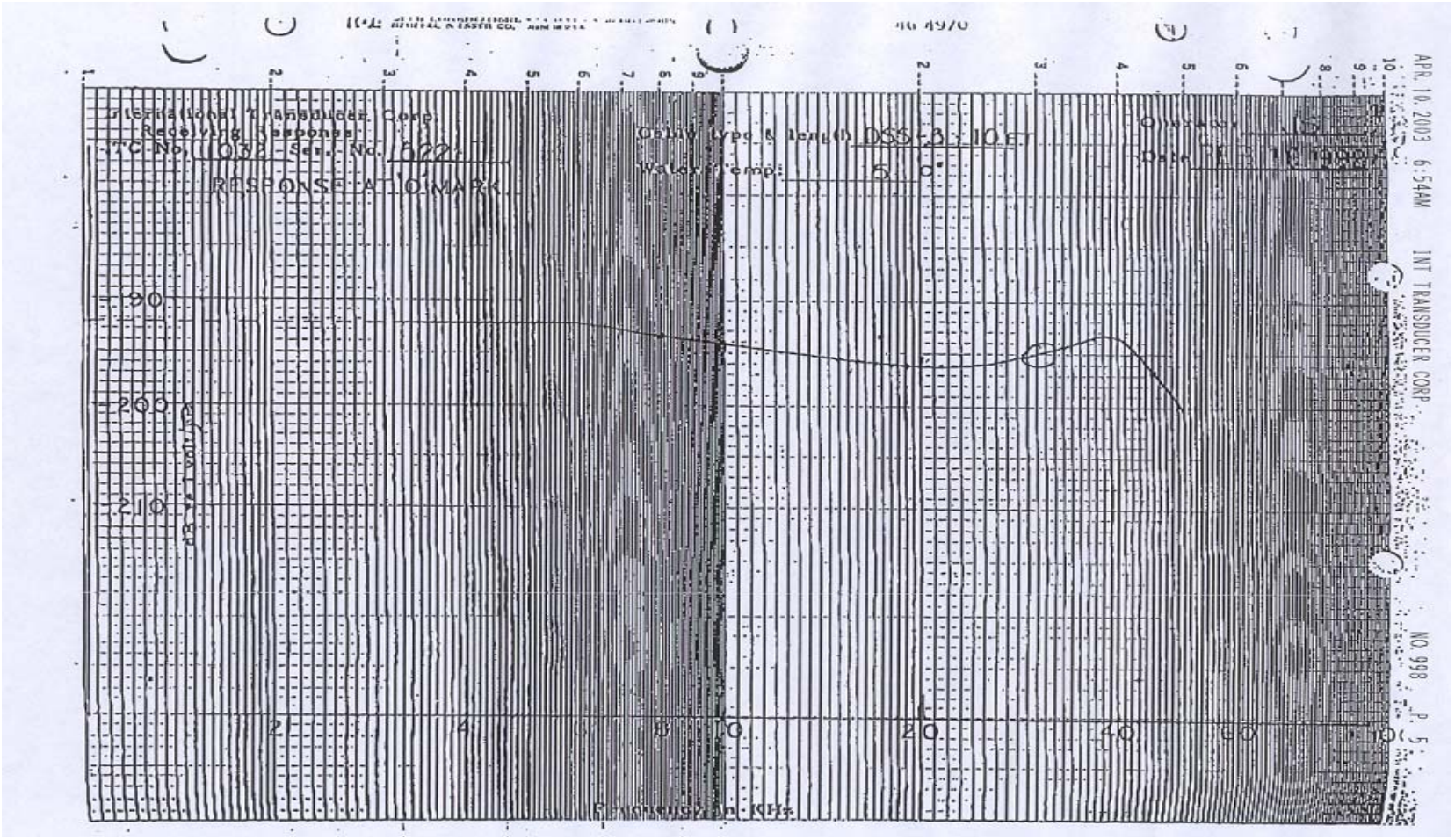


Figure A: Calibration curve obtained by School of Mechanical Engineering, the University of Adelaide.
(The calibration factor is 196 dBV/µPa at 20 kHz for hydrophone.)

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