University of Alberta

Barley Protein based Microcapsules for Nutraceutical Delivery

by

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ABSTRACT

Barley protein based microcapsules (1-5μm) incorporating fish oil/β-carotene were successfully prepared. Well suspended solid microcapsules, rather than emulsions, were able to form after high pressure emulsifying process. These wet-status microcapsules could be turned into dry powder by a spray drying process. The microcapsules demonstrated spherical shape and high loading capacity. Oxidative stability tests under accelerated conditions and in food formulations suggest barley proteins are effective microencapsulation materials to protect fish oil against oxidation. Microcapsule degradation and bioactive compound release behaviors were studied in the simulated gastro-intestinal tract. The data revealed that nano-encapsulations (20-30nm) were formed as a result of enzymatic degradation of microcapsule bulk matrix in the simulated gastric tract. These nano-encapsulations delivered β-carotene to a simulated human intestinal tract intact, where they were degraded by pancreatic enzymes and steadily released the β-carotene. These uniquely structured microcapsules may provide a new strategy to develop target delivery systems for nutraceuticals.

Keywords: barley protein; microencapsulation; β-carotene; fish oil; oxidative stability; controlled release

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In Aug. 2008, I came to Canada and began my new life and study. I knew it would be a challenge: new country, new language, new culture, new faces, everything is new. Then I thought, “Can I really get my graduate studies done successfully?” Well, it did deserve a try -- this was one of the right decisions I have made in my life.

Two years are long enough to forget some things but there are things I will never forget: the help, guidance, and training from my supervisor Dr. Lingyun Chen, and help and support from my families and friends.

My research didn’t go well at all at the beginning, which frustrated both me and my supervisor a lot. The bad situation lasted for almost a year until we finally got a clue. I actually almost gave up during that period. But Dr. Chen never thought about giving up on me, even though there are times when I disappointed her due to my poor experimental skills and immature academic thinking. She liked to encourage me saying, “I know this happens, but don’t worry, if you have any problem, I am ready to help.” I feel very grateful. She also personally demonstrated key lab skills, when I was in serious trouble with some lab operations. I have learned a lot in this way. She generously gave me all she could; she taught me experimental design, writing, how to give presentation and how to answer questions. No doubt, she was strict, but I am happy to have been trained well after two years. Thus I appreciated the help and guidance from her and also the difficulty I have experienced. They are all precious for my growth. I also want

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LIST OF ABBREVIATION

BE	barley endosperm protein
BG	barley glutelin
BGH	barley glutelin:hordein =1:1
BG1H2	barley glutelin:hordein =1:2
BG2H1	barley glutelin:hordein =2:1
BH	barley hordein
BWG	barley whole grain protein
CM	chylomicrons
CP_{BG}	coating protein from BG
CP_{BGH}	coating protein from BGH
CP_{BH}	coating protein from BH
DE	dextrose equivalent
DHA	docosahexaenoic acid
EDTA	ethylenediaminetetraacetic acid
EE	encapsulation efficiency
EPA	eicosapentaenoic acid
GI	gastro-intestinal
GRAS	generally recognized as safe
HMW	high molecular weight
HPLC	high performance liquid chromatography
LE	loading efficiency
LMW	low molecular weight

MD	maltodextrins
PBS	phosphate-buffered saline
PLA	ploy lactic acid
PLGA	poly lactic-coglycolic acid
PUFAs	polyunsaturated fatty acids
PV	peroxide value
RI	refractive index
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SP_{BG}	soluble protein from BG
SP_{BGH}	soluble protein from BGH
SP_{BH}	soluble protein from BH
TEM	transmission electron microscope
TGP	total grain protein
WHO	world health organization
WP	whey protein

Chapter 1 Literature Review

1.1 Nutraceutical and functional food

A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food (Health Canada, 2002). It is demonstrated to have a physiological benefit or to provide protection against chronic disease (Health Canada, 2002). The reported beneficial effects of nutraceuticals are growing steadily (Wildman, 2001). For example, vitamin D has been shown to decrease the risk of diabetes, vitamin E is found to decrease the incidence of cardiovascular disease, lycopene reduces the risk of prostate cancer, and experiments in animal models and tissue culture overwhelmingly support a protective effect of n-3 polyunsaturated fatty acids (PUFAs) against colon, prostate, and breast cancer (Beristain et al., 2008; Breemen and Pajkovic, 2008; Hamrick and Counts, 2008; Nishino et al., 2008). A functional food is similar in appearance to, or may be, a conventional food. It is consumed as part of a usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Health Canada, 2002). Examples include vitamin-enriched bread, omega-3-enriched dairy products, fermented food with live cultures, etc.

A report published by WHO (World Health Organization) in 2005 illustrates the impact of chronic disease in Canada. It points out the chronic diseases, including cancer, cardiovascular disease, chronic respiratory disease and diabetes

account for almost 90% of all deaths (WHO, 2005). The supplementation of certain nutraceuticals to avoid diseases is therefore considered necessary and receiving increasing recognition. In fact, it has been also reported that at least 80% of premature heart disease, stroke and type 2 diabetes and 40% of cancers could be prevented through healthy diets, regular physical activities and avoidance of tobacco products (WHO, 2005).

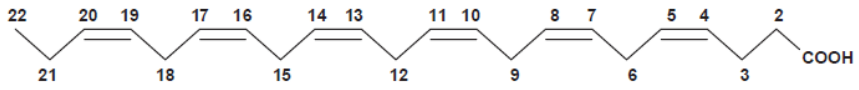
The addition of nutraceuticals to our food system provides a convenient way to develop novel functional foods that may contribute to a healthy diet and reduce the risk of chronic diseases (heart diseases, diabetes, cancer, etc.). (Chen et al., 2006; Nishino et al., 2008). The growing understanding of nutrition and health, increasing health-care costs, together with an aging population have created a market for functional foods and natural health products (Agriculture and Agri-Food Canada, 2007).

1.2 Challenges of incorporating nutraceuticals in food products

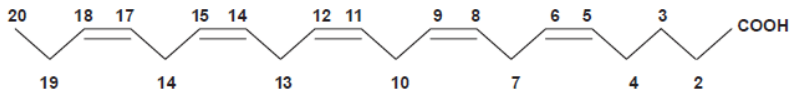
The effectiveness of nutraceutical products in preventing diseases depends on the bioavailability of the active ingredients (Chen et al., 2006). However, in many cases only a small portion of active molecules remain available for absorption after oral administration due to: (1) low permeability and/or solubility of molecules in the gut and insufficient gastric residence time and (2) instability during food processing (temperature, oxygen, light) or in the gastro-intestinal (GI) tract (pH, enzymes, presence of other nutrients).

1.2.1 Oxidation of omega-3 fatty acid

Omega-3 fatty acids have been regarded as important nutraceuticals because of their remarkable benefits for human health. They help to prevent cardiovascular disease, assist brain development, and have ameliorative effects on hypertension, inflammation, immune problems and other diseases (Curtis et al., 2008; Kagami et al., 2003; Lin and Su, 2007; Wang et al., 2006). Two examples of omega-3 fatty acids are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Figure 1-1). Their structures contain a carboxyl head group and an even numbered carbon chain (22 or 20 carbons) with two-or more (6 or 5) methylene-interrupted double (unsaturated) bonds. The term “omega 3” signifies that the first double bond exists as the third carbon-carbon bond from the terminal methyl (omega) end (n) of the carbon chain. The chemical structure of fatty acids is commonly abbreviated by a listing of the number of carbons, the number of double bonds, and the location of the first double bond from the methyl terminal. For example, DHA is represented as C22:6ω-3, indicating a carbon chain length of 22 with 6 double bonds; the first unsaturated bond is inserted at carbon 3 (SanGiovanni and Chew, 2005).



Docosahexaenoic Acid ($C_{22}H_{32}O_2$, 22:6ω-3, MW: 328.448)



Eicosapentaenoic Acid ($C_{20}H_{30}O_2$, 20:5ω-3, MW: 302.451)

Figure 1-1 Chemical structure of DHA and EPA

The high degree of unstauration in DHA and EPA suggests they are susceptible to oxidative deterioration. Oxidation usually encompasses a variety of chemical reactions between oils and ambient oxygen, including free radical generation, peroxide formation, hydroperoxide decomposition, scission, branching, polymerization, etc. And the formations of the various degradation products typically have an unpleasant smell and taste (Barrow et al., 2009).

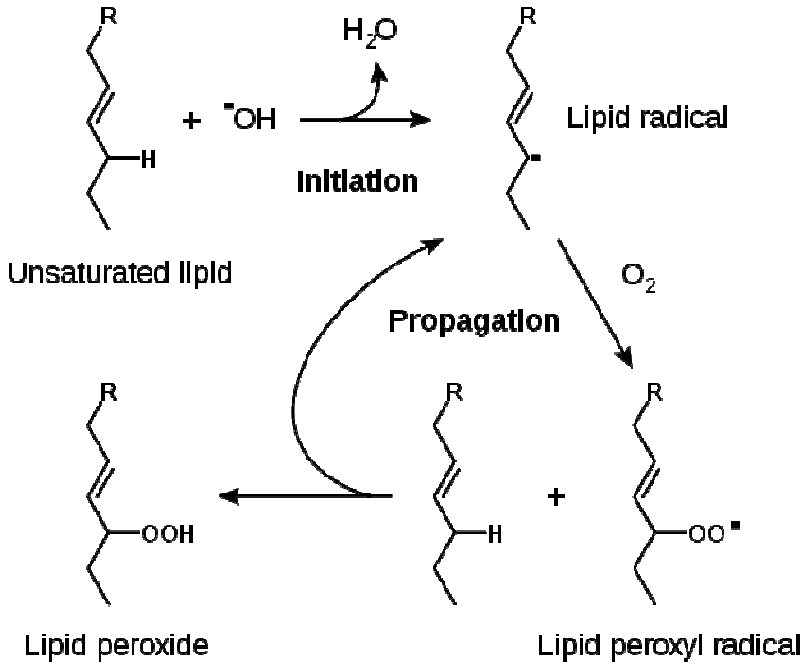


Figure 1-2 Mechanism of lipid peroxidation
http://en.wikipedia.org/wiki/Lipid_peroxidation (public domain)

Figure 1-2 shows the free radical mechanism of lipid peroxidation. Briefly, when a fatty acid radical is produced in an initiation step, it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This lipid peroxyl radical is very unstable and can easily react with another free fatty acid, producing a different fatty acid radical and a lipid peroxide or a cyclic peroxide if it has

reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way. This process is called “chain reaction mechanism” (Marnett, 1999; Trevisan et al., 2001). The chain reaction can also happen on allylic hydrogen and induce delocalization or saturation of the double bonds. Polyunsaturated fatty acids, which contain “bis-allylic hydrogens”, are much more vulnerable to be oxidized (Asadauskas et al., 2007).

1.2.2 Instability and limited absorption of beta-carotene

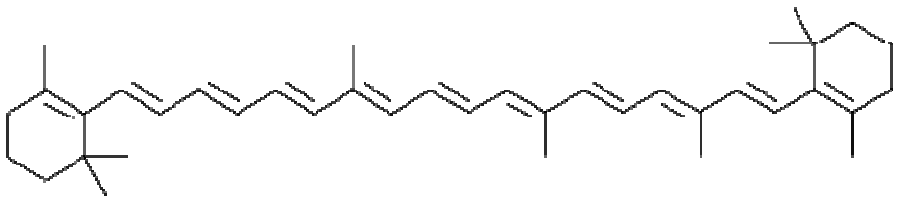


Figure 1-3 Chemical structure of beta-carotene

Beta-carotene is an organic compound and classified as a terpenoid (Figure 1-3). It cannot be synthesized by humans, but it can be provided by a diet enriched in fruits and vegetables (Granado et al., 2001). Beta-carotene has the highest provitamin A activity amongst the carotenoids (Delgado et al., 2000; Nishino et al., 2008) and is increasingly in demand, because of its reported anticancer (Giovannucci, 1999; Nishino et al., 2008; Trombino et al., 2009), free radical scavenging and other biological antioxidant activity (Murakoshi et al., 1999). The high degree of unstauration in the beta-carotene structure however suggests it is extremely susceptible to oxygen and the application of β-carotene is therefore limited by its sensitivity to light, heat, and air/oxygen and also by its extremely poor solubility in water (Sutter et al., 2007).

Limited absorption is another problem for beta-carotene, thus restricting its bioavailability in humans. As lipid soluble compounds, carotenoids follow the same absorptive pathways as other dietary lipids. Steps for carotenoid absorption (*in vivo*) include: (1) release of carotenoids from the food matrix, (2) solubilization of carotenoids into mixed lipid micelles in the lumen, (3) cellular uptake of carotenoids by intestinal mucosal cells, (4) incorporation of carotenoids into chylomicrons (CM) and (5) secretion of carotenoids and their metabolites associated with CM into the lymph (During and Harrison, 2004). The efficient digestion and absorption of dietary fat, as well as the presence of bile salt micelles, are essential for carotenoid absorption. Dietary fat serves at least two functions in carotenoid absorption. Firstly, it provides a hydrophobic domain within which carotenoids can be solubilized and secondly it also stimulates bile flow from the gall bladder (Furr and Clark, 1997). Failure to take in enough dietary fat will therefore limit the use of beta-carotene in human body. The presence of bile salt micelles is also obligatory, as absorption is minimal or nonexistent when intraluminal bile salts are below the concentration required for aggregation into micelles (Furr and Clark, 1997). Normal gastric secretions and their acidic effect on the pH of the upper small intestine were observed to improve absorption of β-carotene, whereas excessive acidity (pH < 4.5) reduced the solubility of carotenoids into bile salt micelles, thus markedly decreasing carotenoid absorption. The incomplete digestion of the food matrix and a negative influence from other components in food may also decrease the absorption of beta-carotene (Furr and Clark, 1997; Marisiddaiah and Baskaran, 2009).

Both environmental factors and drawbacks of nutraceuticals themselves are able to limit their activity and potential health benefits (Chen et al., 2006; Pothakamury and Barbosa-Cánovas, 1995). Therefore, protective mechanisms are necessary not only to guarantee a safe delivery, but also to ensure optimal dosage and efficient absorption of nutrients in human body (Chen et al., 2006; Gouin, 2004; Pothakamury and Barbosa-Cánovas, 1995).

1.3 Microencapsulation and controlled release

1.3.1 Microencapsulation

Microencapsulation is defined as a process in which tiny particles or droplets are surrounded by a coating, or embedded in a homogeneous or heterogeneous matrix, to give small capsules with many useful properties (Figure 1-4) (Gharsallaoui et al., 2007; Gibbs et al., 1999). Reasons for encapsulating an active ingredient include: (1) protection from chemical-physical agents like oxygen, humidity, acid pH, light and heat, (2) conversion from liquid to solid state of products, (3) reduction of potential gastric damage, (4) reduction of corrosiveness during storage, (5) taste-masking, (6) physical separation of the active principles from incompatible substances, (7) controlled release in the gastrointestinal tract and (8) rumen bypass (Desau and Park, 2005; Gharsallaoui et al., 2007; Gibbs et al., 1999).

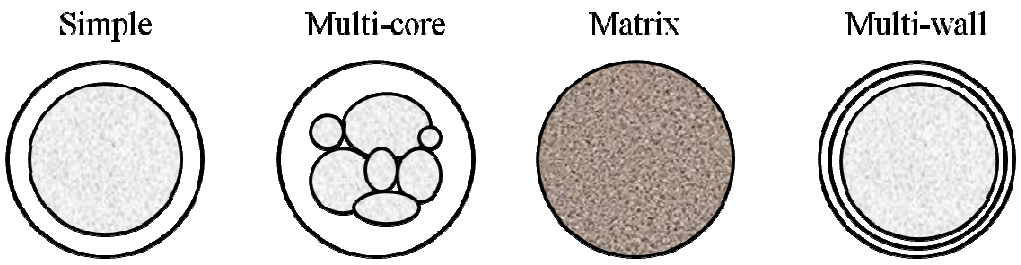


Figure 1-4 Morphology of different types of microcapsules

1.3.2 Controlled release

Controlled release is defined as a method by which one or more active agents or ingredients are made available at a desired site and time and at a specific rate (Pothakamury and Barbosa-Cánovas, 1995). It has been suggested that a very important feature for microcapsules is its capacity to control the release of the core components (Pothakamury and Barbosa-Cánovas, 1995). The food industry is taking advantage of the technology of controlled release for food additives including flavoring agents (flavor oils, spices, seasonings), sweeteners, colors, nutrients (vitamins, amino acids, minerals), essential oils, acids, salts, bases, antioxidants, antimicrobial agents, preservatives, ingredients with undesirable flavor and cross-linking agents (Pothakamury and Barbosa-Cánovas, 1995). Controlled release helps to overcome both the ineffective utilization and the loss of food additives during the processing steps. Mechanisms involved in controlled release include: diffusion, swelling, biodegradation, etc. (Pothakamury and Barbosa-Cánovas, 1995).

1.3.2.1 Diffusion controlled release system

Diffusion describes the spread of the active components through random motion from the polymeric matrix to the hydrophilic external environment (Nissim, 2008). The concept is tied to mass transfer driven by a concentration gradient, but diffusion can still occur when there is no concentration gradient. It is considered the most important mechanism used for controlled release (Siepmann et al., 1999). Figure 1-5 shows one diffusion system consisting of active agent (core) buried within a microcapsule matrix. The principal steps for the diffusion release include: (1) diffusion of the active agent within the matrix; (2) dissolution or partitioning of the active agent between the environmental fluid and the matrix barrier; (3) diffusion through the barrier into environmental fluid (Pothakamury and Barbosa-Cánovas, 1995).

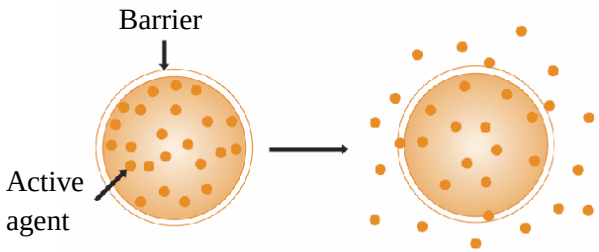


Figure 1-5 Diffusion controlled release system

The release rate from a matrix system depends on the thickness, the area and the permeability of the barrier. The desired release profiles can be achieved by adjusting the geometry and the dimensions of the system (Siepmann et al., 1999).

1.3.2.2 Swelling controlled release system

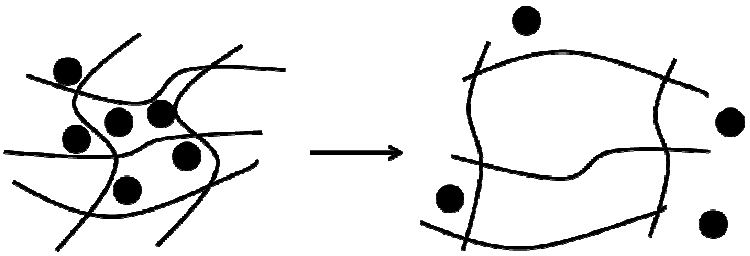


Figure 1-6 Swelling controlled release system

In swelling-controlled systems, the active agent, that is dissolved or dispersed in a polymeric matrix, is unable to diffuse to any significant extent within the matrix (Pothakamury and Barbosa-Cánovas, 1995). When the polymer matrix is placed in a thermodynamically compatible medium, the polymer swells owing to absorption of fluid from the medium. The active agent in the swollen part of the matrix then diffuses out (Figure 1-6). In diffusion-controlled systems the barrier or matrix is assumed to be unaffected during the release process, whereas in swelling controlled systems, the membrane undergoes a transition from a glassy to a gel state upon interaction with the fluid. The polymer chains in the gel state, being more mobile than those in the glassy state, allow the active agent to diffuse out of the matrix more rapidly. The release rate is determined by the glass-to-gel transition process (Pothakamury and Barbosa-Cánovas, 1995).

1.3.2.3 Biodegradation controlled release system

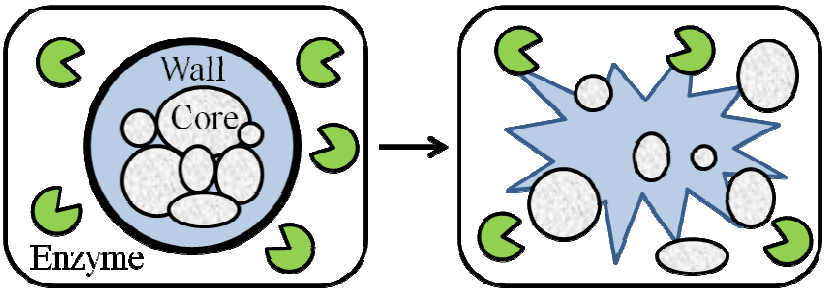


Figure 1-7 Biodegradation controlled release system

Biodegradation is the chemical breakdown of materials by a physiological environment. In a biodegradation controlled release system, the active agent within the polymer is released when the polymer degrades. As shown in Figure 1-7, after enzymes break down the microcapsule matrix, active agents are released.

The release rate of active agents may be controlled by diffusion, erosion or a combination of both (Pothakamury and Barbosa-Cánovas, 1995). Heterogeneous or homogeneous erosion commonly happens for erosion controlled release. Heterogeneous erosion occurs when degradation is confined to a thin layer at the surface of the delivery system, whereas homogeneous erosion is a result of degradation occurring at a uniform rate throughout the polymer matrix. The type of erosion, heterogeneous or homogeneous, depends on the hydrophobicity and morphology of the polymer. Heterogeneous erosion is more common with hydrophobic polymers, whereas homogeneous erosion is common with hydrophilic polymers (Pothakamury and Barbosa-Cánovas, 1995). Heterogeneous erosion is more desirable because it can lead to a constant release rate that is independent of the chemical and physical properties of the active agent.

Additionally, the release rate can be varied by changing the active agent loading, while maintaining the integrity, because the erosion is limited to the surface (Pothakamury and Barbosa-Cánovas, 1995).

The well designed coating may allow variable release rates of the active agents and the matrix can also be built to deliver these active agents to a preferential area. The timely and targeted release improves the effectiveness of bioactive compounds, broadens its application in various areas such as functional food, cosmetics, pharmaceuticals (intramuscular and subcutaneous injection) etc. (Brannon-Peppas, 1995; Gouin, 2004).

1.3.3 Technologies used in microencapsulation

Sophisticated technologies have been developed for microencapsulation (Gouin, 2004). The processes that are employed to form microcapsules include: spray drying, spray cooling, extrusion, coacervation, co-crystallization, molecular inclusion, etc. (Desau and Park, 2005; Gharsallaoui et al., 2007; Gibbs et al., 1999; Gouin, 2004).

1.3.3.1 Spray drying

Spray drying was first applied to encapsulate flavors using gum acacia as the wall material. Since then the process has been used for decades to provide flavor oils with protection against degradation/oxidation and to convert liquids to powders (Gouin, 2004). It is the most common technology for microencapsulation due to its low cost, available equipment and continuous production in plant. Many products such as instant food products, laundry detergents, pharmaceuticals,

ceramics and agrochemicals, can be produced by spray drying. The best known example is milk powder (Gharsallaoui et al., 2007). Figure 1-8 shows structure of a laboratory scale spray dryer.

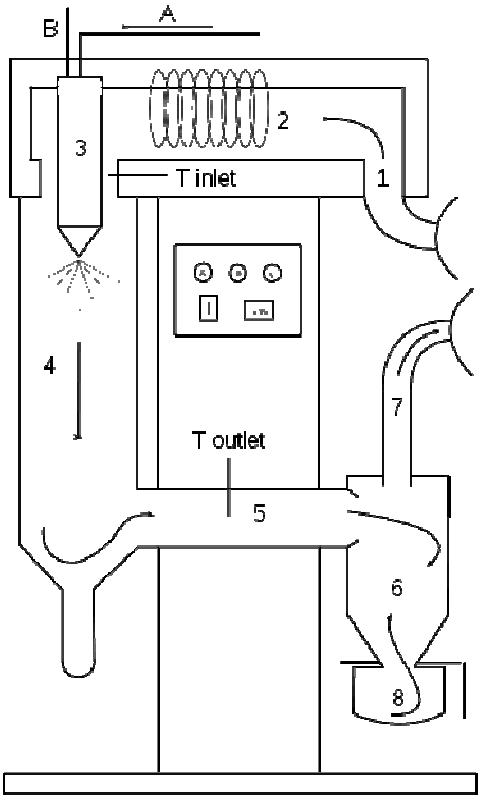


Figure 1-8 Structure of a laboratory-scale spray dryer
A: solution or suspension to be dried in; B: atomization gas in; 1: drying gas in; 2: heating of drying gas; 3: spraying of solution or suspension; 4: drying chamber; 5: part between drying chamber and cyclone; 6: cyclone; 7: drying gas is taken away; 8: collection vessel of product. http://en.wikipedia.org/wiki/Spray_dryer (with permission)

In a broad sense, three basic steps are involved in a spray-drying microencapsulation process: preparation of the dispersion or emulsion, atomization of the infeed emulsion, and dehydration of the atomized particles (Gharsallaoui et al., 2007).

Hydrophobic cores like various flavors and lipids are usually encapsulated in a comparably hydrophilic wall material (starch, dextrin, proteins, etc.) by the spray drying process. In these cases, emulsion is usually the first stage to stabilize core material in wall solutions. It can be prepared by dispersing the hydrophobic core into an immiscible solution of the coating agent. The dispersion must be homogenized, with or without the addition of an emulsifier, because some of the coating materials themselves have emulsifying interfacial activities (Gharsallaoui et al., 2007). Before the spray-drying step, the formed emulsion must be stable over a certain period of time. Oil droplets should be rather small and viscosity should be low enough to prevent air inclusion in the particle (Drusch and Schwarz 2006).

In the spray chamber the incoming emulsion is atomized by rotary wheel atomizers or pressure nozzles. The goal of atomization is to create a maximum heat-transferring surface between the dry air and the liquid in order to optimize heat and mass transfers. The choice of the atomizer configuration depends on the nature and viscosity of feed and the desired characteristics of the dried product. The higher the energy input is, the finer the formed droplets are. Given a constant energy input, the size of formed particles can be increased with increased feed rate. The size of particles also increases when both viscosity and surface tension of the initial liquid are increased (Gharsallaoui et al., 2007).

The atomized emulsion droplets will then be dried by the hot air (150-220ºC) introduced at the top of the chamber. The evaporation of solvent, usually water, occurs instantaneously, leading to the formation of microcapsules. The short time

exposure to heat together with instant evaporation, keeps the core temperature below 40ºC, thus limiting thermal degradation (Gharsallaoui et al., 2007). The rate of water diffusion from the droplet core to its surface is usually considered constant and equal to the surface evaporation rate. When the droplet water content reaches a critical value, a dry crust is formed at the droplet surface and the drying rate rapidly decreases and becomes dependent on the water diffusion rate through this crust. Drying is theoretically finished when the particle temperature becomes equal to that of the air. Most dense particles are recovered at the bottom of the collection vessel, while the finest ones pass through the cyclone to be separated from the humid air. In some cases, the powder particles leave the spray dryer at the bottom into the fluid bed, where further drying takes place.

Air inlet temperature and air outlet temperature are usually considered essential operation factors to obtain good quality microcapsules (Liu et al., 2004). Air inlet temperature is directly related to the microcapsule drying rate and the final water content. When the air inlet temperature is low, the low evaporation rate may cause the formation of microcapsules with high density membranes, high water content, poor fluidity, and a tendency for agglomeration (Gharsallaoui et al., 2007). However, a high air inlet temperature may cause excessive evaporation, resulting in cracks in the membrane, inducing subsequent premature release and degradation of the encapsulated ingredient or a possible loss of volatiles (Gharsallaoui et al., 2007). It is quite difficult to predict this outlet temperature for a given product, since it depends on the drying characteristics of the material. Contrary to the air inlet temperature, the air outlet temperature cannot be directly

controlled, since it is dependent on the air inlet temperature. The ideal air outlet temperature for microencapsulation of food ingredients, such as flavors, has been reported to be 50–80ºC (Gharsallaoui et al., 2007).

Since almost all spray drying processes in the food industry are carried out from aqueous feed formulations, the wall material must be soluble in water at an acceptable level. This limits the number of available wall materials for spray drying. Typical walls include gum acacia, maltodextrins, modified starch, whey proteins, soy proteins, and sodium caseinate (Gouin, 2004). Another disadvantage of spay drying is the energy waste due to the incomplete use of heat through the drying chamber (Gharsallaoui et al., 2007).

1.3.3.2 Spray cooling/chilling

Spray cooling/chilling is the least expensive encapsulation technology and is routinely used for encapsulating a number of organic and inorganic salts as well as textural ingredients, enzymes, flavors and other functional ingredients (Gouin, 2004). In spray-chilling and spray-cooling, the core and wall mixtures are atomized into the cooled or chilled air, which causes the wall solidification around the core. The coating material is typically some form of vegetable oil or its derivatives. However, a wide range of other materials may also be employed including fat, stearin, as well as hard mono- and diacylglycerols (Desau and Park, 2005). Unlike spray-drying, there is no evaporation involved in spray-chilling or spray-cooling, therefore these solidified microcapsules possess almost perfect sphere shape and smooth surface to give free-flowing powders. The formed

microcapsules are also insoluble in water due to the lipid coating. Consequently, these techniques tend to be utilized for encapsulating water-soluble core materials such as minerals, water-soluble vitamins, enzymes, etc (Desau and Park, 2005).

One drawback for the spray cooling/chilling system is that, this encapsulation process leaves a significant proportion of active ingredient lying on the surface of the microcapsules or sticking out of the fat matrix, thus having direct access to the environment (Gouin, 2004).

1.3.3.3 Extrusion

Encapsulation of food ingredients by extrusion is a relatively new process compared to spray-drying (Desau and Park, 2005). It has been used almost for the encapsulation of volatile and unstable flavors in glassy carbohydrate matrices (Gouin, 2004). Unlike extrusion used for cooking and texturizing of cereal-based products, the extrusion applied to flavor encapsulation is a relatively low temperature entrapping method, which involves forcing a core material into a molten carbohydrate mass, through a series of dies into a bath of dehydrating liquid (Desau and Park, 2005). Figure 1-9 shows a sectional view of an extrusion nozzle forming a microcapsule.

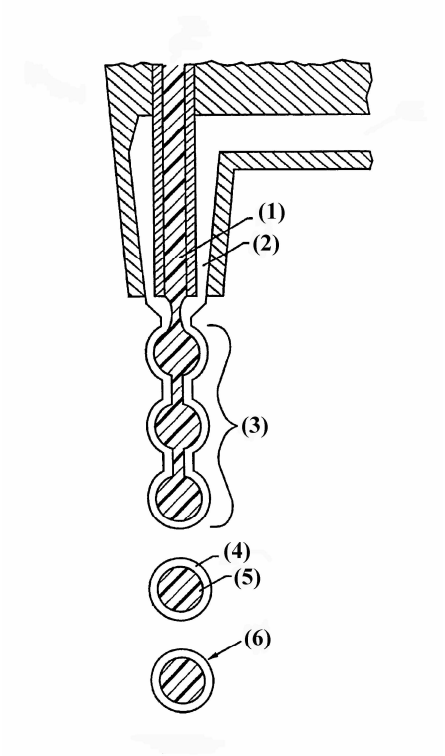


Figure 1-9 Sectional view of an extrusion nozzle forming a microcapsule

When the core materials (1) and shell materials (2) are extruded at different rates, the mode of compound drop formation changes. At low flow rates, drop formation is orderly and regular and the drops are uniform (6). At higher flow rates, connected drops (3) form. At still higher flow rates, the capsule size distribution is much broader, which is usually less desirable. If the nozzle is vibrated during the extrusion process, capsule size distribution can be controlled to give capsules relatively uniform diameter cores (5) and shells (4). When the capsule drops into the dehydrating liquid, the coating material hardens, and the encapsulating matrix forms to entrap the core material. The shell materials used may be composed of more than one ingredient, such as sucrose, maltodextrin, glucose syrup, glycerine, and glucose (Desau and Park, 2005).

The advantage of extrusion is that the core material is totally isolated by the wall material (Gibbs et al., 1999). This provides an excellent stability of the core against oxidation and therefore prolongs the shelf life (Desau and Park, 2005). Shelf life of up to 5 years has been reported for extruded flavor oils, compared to 1 year for spray dried flavors and a few months for unencapsulated oils (Gibbs et al., 1999). Carbohydrate matrices in the glassy states also have very good barrier properties to avoid gases diffusion due to the hydrophilic nature, thus further preventing oxidation by providing an almost impermeable barrier against oxygen (Gibbs et al., 1999). The payload in these systems, however, remain very low (8%~25%); whereas higher payloads led to unstable systems, leaking out and fast oxidation of the sensitive flavor oil. Such low payloads in flavor microcapsules are very unattractive, from an industrial point of view (Gouin, 2004). Another drawback is the rather large particles formed by extrusion (500–1000 µm), which restricts its application where mouth-feel is a crucial factor. The limited range of wall material is also an undesirable factor for extrusion (Gouin, 2004).

1.3.3.4 Coacervation

Coacervation can be defined as partial desolvation of a homogeneous polymer solution into a polymer-rich phase (coacervate) and the poor polymer phase (coacervation medium). The concept behind coacervation microencapsulation is the phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media (Gouin, 2004). The hydrocolloid shell can then be crosslinked using an

appropriate chemical or enzymatic crosslinker, if needed (Gouin, 2004). Coacervation is typically used to encapsulate flavor oils, but can also be adapted for encapsulation of fish oils nutrients, vitamins, preservatives, and enzymes (Curtis et al., 2008; Korus, 2001; Lamprecht et al., 2001).

Currently, two methods for coacervation are available, namely simple and complex processes. The mechanism of microcapsule formation for both processes is identical, except for the way in which the phase separation is carried out. In simple coacervation a desolvation agent is added for phase separation, whereas complex coacervation involves complexation between two oppositely charged polymers. Usually three steps are involved in a complex coacervation: (i) formation of three immiscible phases; (ii) deposition of the coating; and (iii) rigidization of the coating (Figure 1-10).

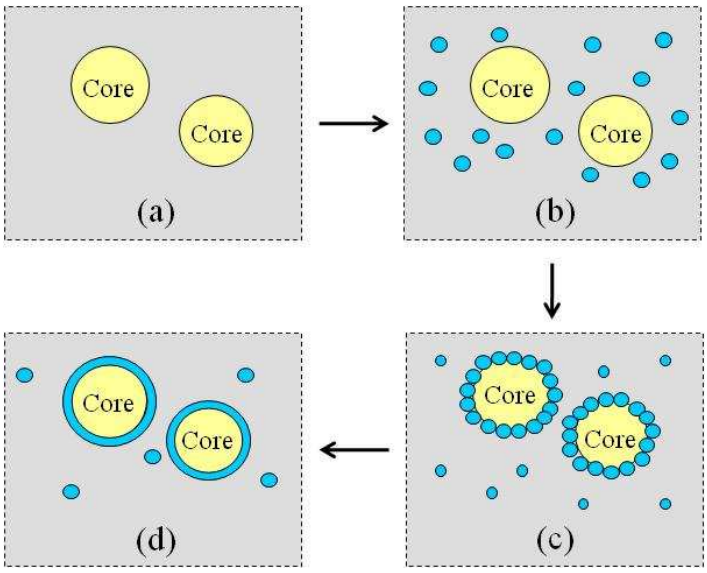


Figure 1-10 Schematic representation of the coacervation process
(a) Core material dispersion in solution of shell polymer; (b) separation of coacervate from solution; (c) coating of core material by microdroplets of coacervate; (d) coalescence of coacervate to form continuous shell around core particles.

The first step includes formation of three immiscible phases: liquid manufacturing vehicle, core material, coating material. The core material is dispersed in a solution of the coating polymer. The coating material phase, an immiscible polymer in liquid state is formed by (1) changing temperature of polymer solution, (2) addition of salt, (3) addition of nonsolvent, or (4) inducing polymer – polymer interaction, for example, interaction of gum arabic and gelatin at their iso-electric point. The second step includes deposition of liquid polymer upon the core material. Finally, solidification of the coating is achieved by thermal cross-linking, or desolventization techniques and forms a self-sustaining microcapsule. The microcapsules are usually collected by filtration or centrifugation, washed with an appropriate solvent, and subsequently dried by standard techniques such as spray- or fluidized-bed drying to yield free-flowing, discrete particles (Desau and Park, 2005).

Coacervation is a unique and promising microencapsulation technology, due to the very high payloads and desirable release control. However, this process is very expensive and rather complex, and cross-linking of the wall material usually involves glutaraldehyde, which must be carefully used according to the country's legislation. The problems related to harmful chemical cross-linkers, however, may be solved by using enzymatic cross-linkers instead (Gouin, 2004).

1.3.4 Wall materials used for microencapsulation

A variety of synthetic or natural biodegradable polymers have been developed for microencapsulation of nutraceuticals or drugs. Synthetic polymers

like poly (lactic acid) (PLA) and poly (lactic-coglycolic acid) (PLGA) are commonly used due to their superior biodegradability and regulatory physiochemical properties (Liu et al., 2005). However they cannot be used in food applications, where require compounds generally recognized as safe (GRAS) for consumption in large quantities (Chen and Subirade 2007). Natural polymers such as starch, lipid, polysaccharides and proteins, on the contrary, are usually considered safe and they demonstrate advantages like desirable absorbability and low toxicity of the end degradation products (Table 1-1) (Liu et al., 2005).

Table 1-1 Coating materials and technology used for microencapsulation

Category	Coating materials	Widely used methods
Carbohydrate	Starch, maltodextrins, chitosan, corn syrup solids, dextrin, modified starch, cyclodextrins carboxymethylcellulose, methyl cellulose, ethylcellulose, celluloseacetate-phthalate, celluloseacetate -butylate-phthalate	Spray- and freeze-drying, extrusion, coacervation, inclusion complexation coacervation, spray-drying, and edible films
Protein	Gluten, casein, gelatin, albumin, peptides	Emulsion, spray-drying
Gum	Gum acacia, agar, sodium alginate, carrageenan	Spray-drying, syringe method
Lipids	Wax, paraffin, beeswax, diacylglyerols, oils, fats	Emulsion, liposomes, film formation

The choice of wall material is very important. For example, in spray drying encapsulation, a suitable wall material should possess good emulsifying activity and stability, low viscosity, a tendency to form a fine and dense network during

drying and should not permit lipid separation from emulsion during dehydration (Desau and Park, 2005). The cost and the availability should also be considered.

1.3.4.1 Carbohydrates

Carbohydrates such as starches, maltodextrins and corn syrup solids are usually used in microencapsulation of food ingredients (Gharsallaoui et al., 2007). Due to their desirable drying properties and matrix forming abilities, they are preferred. However, the use of low molecular weight carbohydrates in microencapsulation is usually associated with problems of caking (formation of inter-particle bonds between adjacent particles when surface viscosity reaches a critical value) (Le Meste et al., 2002), stickiness (Adhikari et al., 2005), collapse and re-crystallization of the amorphous region. Crystallization of carbohydrates via glass transition can cause disruption of structural integrity of the wall matrix and induce agglomeration or caking of powders, thereby resulting in the release of some encapsulated oil and induction of lipid oxidation (Drusch et al., 2006&2007). For this reason it is better to use larger molecular weights of maltodextrins (MD) with low dextrose equivalent (DE) values, which have much higher glass transition temperatures and offer better physical stability to the wall matrix systems (Fuchs et al., 2006). However, some researchers showed contradicting results, for example, in Kagami's study, MDs with higher DE values have been reported to provide better oxidative protection compared to MDs with lower DE values (Kagami et al., 2003). Moreover, in Sheu's study, combinations of whey protein isolate with high DE value carbohydrates were more effective in limiting surface-dents formation than those with carbohydrates of low DE value

(Sheu and Rosenberg, 1998). Thus, the choice of different carbohydrates as wall materials needs further study and should be tried in specific situations. Some other negative comments on carbohydrate as wall include: caramelization properties, adherence to the surface of the spray-dryer (Bayram et al., 2005), and a rapid oxidation of microencapsulated oil (Drusch et al., 2006&2007), which may be due to the polarity difference between wall and core materials (Shaikh et al., 2006). Relatively, pectin (Monsoor, 2005) and gum arabic are reported to have better properties as wall materials in encapsulation. The good emulsifying properties of both pectin and gum arabic may result from the presence of the protein fraction (Dickinson, 2003; Leroux et al., 2003). But recent studies showed that gum arabic was not efficient as a wall material because of its limited barrier capacity against oxidation (Bertolini et al., 2001). Together with high cost, limited supply and quality variations, the use of gum arabic for encapsulation is restricted (Gharsallaoui et al., 2007). A reported alternative of gum arabic is mesquite gum, which can be used in the preparation of oil-in-water emulsions over a wide range of pH values. Cardamom-based oil microcapsules were successfully produced by spray drying using mesquite gum (Beristain et al., 2001). Soybean soluble polysaccharide was also found to be a superior emulsifier over gum arabic to retain microencapsulated ethyl butyrate during spray-drying (Yoshii et al., 2001).

1.3.4.2 Proteins

Wall materials that are based on carbohydrates have poor interfacial properties and must be chemically modified in order to improve their surface activity (Drusch and Schwarz, 2006; Kanakdande et al., 2007; Krishnan et al.,

2005; Soottitantawat et al., 2005). In contrast, proteins have an amphiphilic character that offers physicochemical and functional properties required to encapsulate any ingredient, whether hydrophobic, hydrophilic, or even microbial (Chen et al., 2006). Unique functional properties possessed by proteins, including the ability to form gels and emulsions, allow them to be an ideal coating material for encapsulation (Chen et al., 2006). The most commonly used protein for encapsulating food ingredients is milk (or whey) proteins (Kagami et al., 2003; Rosenberg and Sheu, 1996). Other animal proteins such as gelatin, casein, and some plant proteins such as soy protein, zein and wheat proteins have also been developed as efficient coating materials (Ezpeleta et al., 1996; Latha et al., 2000; Lazko et al., 2004; Liu et al., 2005; Paynea et al., 2002; Swatscheka et al., 2002).

Globular proteins such as whey proteins have the ability to denature, dissociate, and aggregate under different conditions of pH, ionic strength, and temperature to form particles with size ranging from 40 nm to 2 mm. These properties can be exploited to formulate active molecule-loaded particles of specific size (Chen et al., 2006). The ability to control the particle size of protein materials is of primary importance, not only for determining food product properties such as taste, aroma, texture, and appearance, but also for determining the release rates of the carried bioactive compounds and ultimately how much is absorbed into the body. In addition, owing to multiple functional groups in the primary sequences of polypeptides and the resulting diversity of chain folding structures, food proteins can be exploited to create different interactions with core compounds and subsequently form three-dimensional networks to incorporate and

protect these compounds in a matrix and deliver them to the site of action in a active form (Chen et al., 2006).

Despite numerous desirable properties, a protein wall matrix may prove unsatisfactory, due to possible oxidation of the encapsulated bioactive compounds. A wall matrix comprised only of protein (i.e. whey protein) has much higher oxygen permeability due to the similar hydrophobic polarity of oxygen (Kagami et al., 2003). The addition of hydrophilic carbohydrate might be one solution to improve the protective stability of wall systems (Kagami et al., 2003). Studies already revealed that adding hydrolyzed starches (glucose, lactose, corn syrup solids and maltodextrin as a secondary wall material to whey protein can improve drying properties of spray droplets by enhancing the formation of dry crust and to increase the oxidative stability of core material by reducing oxygen permeability of the wall matrix (Kagami et al., 2003). As a result, if a single encapsulating agent cannot provide all the ideal wall material properties, a combination is recommended to achieve maximum benefit.

The solubility of the target protein is the key prerequisite for many other functional properties like emulsion and gelation. Hydrophilic proteins that are highly soluble in an aqueous environment are therefore commonly considered first in many food applications. However, in a microencapsulation area, high solubility of the wall could also be a main drawback since a rapid solubilization means a fast core release profile (Liu et al., 2005). Chemical cross-linking procedures (e.g. glutaraldehyde and formaldehyde treatment) are usually applied to achieve a sustained core release (Latha et al., 1995; Sahin et al., 2002; Vandelli

et al., 2001). Unfortunately, in most cases the presence of residual cross-linking agents could lead to toxic side effects. Also, the unwanted reactions between the core and cross-linker could also result in the formation of toxic or inactivated derivatives (Liu et al., 2005). Natural hydrophobic protein systems, on the other hand, possess the potential to overcome the problems and can be considered a promising sustained release vehicle. For example, Xinming Liu et al. successfully developed microspheres using hydrophobic protein zein (alcohol soluble protein from corn) as coating material. They concluded that the desirable sustained release resulted from the hydrophobic property of zein. This might be attributed to the delay of water penetration consequently leading to retarded diffusion of the drug into the release medium (Liu et al., 2005). Another similar study reported nanoparticles formed by gliadin (alcohol soluble protein from wheat). These nanoparticles were found unnecessary for further chemical or physical solidification, owning to the low solubility of the proteins in water (Ezpeleta and Irache, 1996). In spite of the potential of hydrophobic protein as coating material, the research in this area is still limited.

1.4 Barley proteins

1.4.1 Barley

As the fourth most important cereal in the world after wheat, rice, and corn, barley is broadly grown in about 100 countries worldwide among which the European Union, Russia and Canada are the three main barley producers (Abdellatif et al., 2007). It is a very adaptable crop, popular in temperate areas

where it is grown as a summer crop and tropical areas where it is sown as a winter crop. In many western countries, 80-90% of barley production is for animal feeds and malt while the use of barley in human foods is very limited (Jadhav, 1998). In recent years, increased incorporation of barley into the human diet has been recommended, since it is unique among cereals that contain high concentrations of β-glucan, which is known to have cholesterol-lowering effects, regulating blood glucose levels and insulin response in diabetics (Cavallero et al., 2002).

1.4.2 Barley grain

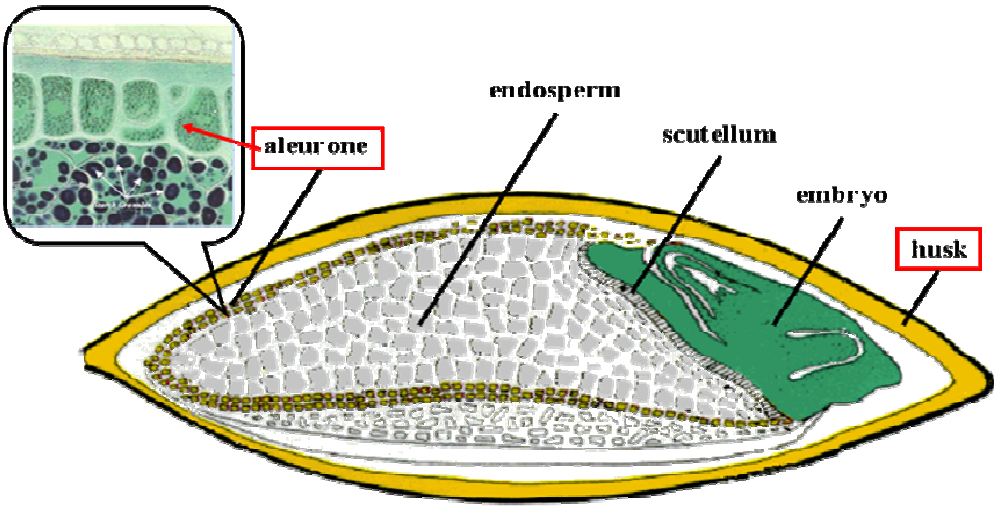


Figure 1-11 Barley grain structure
http://www.agoracosmopolitan.com/home/Frontpage/2007/01/03/01286.html

The barley kernels are spindle-shaped, thicker in the center, and tapered toward each end (Figure 1-11). The starch granules embedded in the protein matrix are encased within individual "endosperm" cells and are protected by a cell wall. Many of these cells are packed tightly within the cereal grains as "starchy endosperm", which is the white material found in the middle of the kernel. The

endosperm is protected by the aleurone layer that contains protein bodies and enzymes connected with endosperm digestion (Jadhav, 1998). Endosperm is the main source of nutrients for the developing embryo. The embryo is located at the attachment end of the caryopsis on its dorsal side. The entire structure is finally covered by the seed husk. From the plant's perspective, all of this packaging is absolutely critical for its survival and is designed to protect the plant embryo and its stored sources of energy and protein, to ensure it will be able to grow and survive for the first few days following germination. The enzymes in the aleurone layer play a very important role for brewing, because the amylase, protease, lipase etc. are able to loosen the packed grain and make it ready for further milling and efficient hydrolysis. The husk is also special, since it can act as an efficient filter separating the liquid part (fermentable sugar) and non-liquid part (non-soluble precipitates) in the following brewing process. Table 1-2 shows a proximate composition of barley grain (Jadhav, 1998). Not all barley grains have a similar composition; it varies according to the different environmental conditions like temperature, day length, water supply, and the availability of soil minerals.

Table 1-2 A proximate composition of barley grain

Component	Content (%, dry weight)
Starch	60-64
Arabinoxylans	4.4-7.8
Beta-glucans	3.6-6.1
Cellulose	1.4-5.0
Simple carbohydrates (glucose, fructose, sucrose, maltose)	0.41-2.9
Oligosaccharides (raffinose, fructosans)	0.16-1.8
Proteins	8-15
Lipids	2-3
Minerals (ash)	3-3

As with other cereals, starch is the main component in barley (60-64%) (Table 1-2). Normal barley starch contains 20 to 30% amylose (70 to 80% amylopectin), which may vary from less than 1% to 45% in waxy and high-amylose starches, respectively. Specialized uses of waxy starches are attributed to their high swelling power and colloidal stability, whereas high-amylose starches have unique gelling and film-forming properties (Jadhav, 1998).

Proteins are the second abundant component in barley (8–15%). They are one of the by-products of the barley starch production process (Andersson et al., 2001). Their amounts and compositions affect industrial usage of the barley grain (Yalcın and Celik, 2007). For example, the beer industry may choose low protein barley, while for animal feeding, barley with high protein content is preferred.

Barley has been recognized as an important contributor for beta-glucans. More consumption of barley as human food is therefore strongly recommended. The lipid or oil content of barley is relatively low (2-3%). However, the tocopherols and tocotrienols, that are found in barley and extracted with its oil, have biological value, including inhibition of lipid peroxidation in biological membranes, reduction of serum LDL cholesterol in human and protection against heart disease, etc (Vivekananthan et al., 2003).

1.4.3 Protein in barley

1.4.3.1 Protein content in barley grain tissues

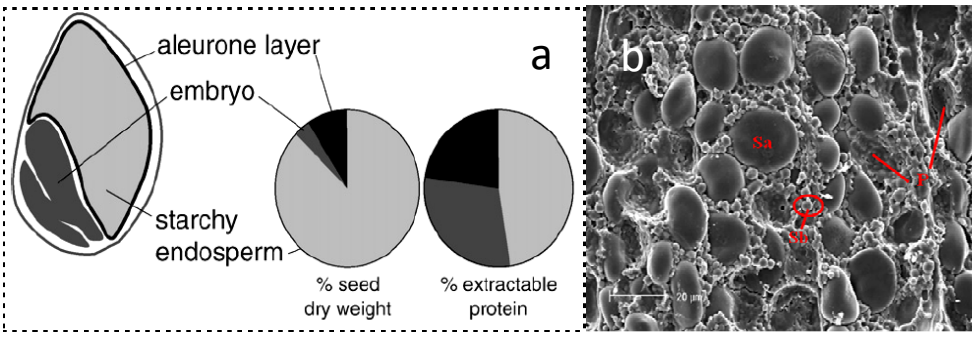


Figure 1-12 Protein content in barley grain tissues
(a) soluble protein contents in barley grain tissues (Finnie and Svensson, 2009). (b) a scanning electron micrograph of the endosperm of barley (website-public domain) (Black J.L., Quality Feed Grains, Warrimoo NSW 2774, Australia).

As shown in Figure 1-12a, the three main tissues of the barley seed are the aleurone layer, the embryo and the starchy endosperm which account for about 9%, 4% and 87% respectively, of the seed's dry weight and 23%, 30% and 47%, respectively, of the seed proteins, that are extractable in a low-salt buffer (Finnie and Svensson, 2009). However, the major endosperm storage proteins are alcohol soluble hordeins, which comprise 30–50% of the total grain protein. Figure 1-12b shows a scanning electron micrograph of the endosperm of barley. The starch consists of a mixture of large, lenticular granules (10 to 25 mm diameter) and smaller, irregular-shaped granules (<10 mm) which are known as A- (Sa) and B- (Sb) type granules, respectively. All these starch granules are then imbedded in a protein matrix. Most of these proteins are commonly water insoluble but can be extracted with alcohol or dilute acid/alkali solutions.

1.4.3.2 Protein classification and amino acid composition

Table 1-3 Osborne classification

Protein fraction	Definition
Albumin	Water soluble, coagulated when heat
Globulin	Water insoluble, soluble in dilute salt (0.5 M NaCl)
Prolamin	Soluble in 70% ethyl alcohol
Glutelin	Soluble in dilute acid (0.5 M acetic acid) or alkali (0.5 M NaOH)

As shown in Table 1-3, protein can be divided as albumin, globulin, prolamin and glutelin by sequential extractabilities as described by Osborne (Celus et al., 2006; Qi et al., 2006).

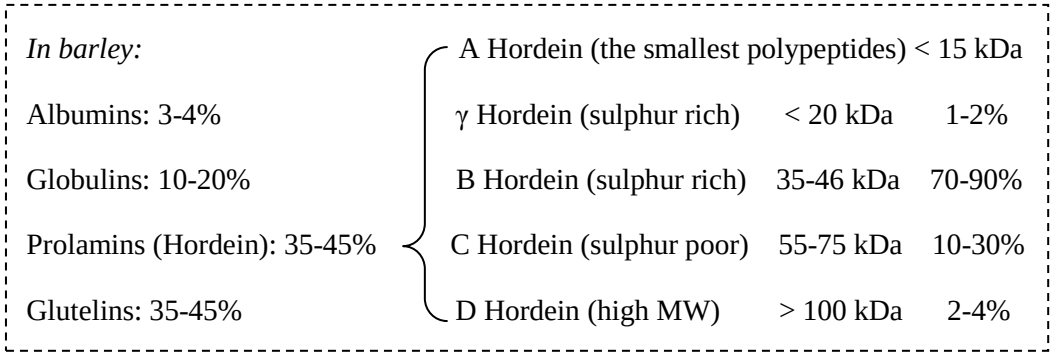


Figure 1-13 Barley protein fractions according to Osborne classification

Figure 1-13 shows barley protein fractions according to the Osborne classification and their individual properties (Qi et al., 2006). Hordein and glutelin, which together comprise 70-90% of the total grain protein (TGP), are known as the major endosperm storage proteins in barley. The hordein fractions (35-45% of the TGP), extracted with alcoholic media, can be further divided into five groups based on their electrophoretic mobilities and amino acid compositions: B hordein (sulphur-rich), C hordein (sulphur-poor), γ-hordein (sulphur-rich), D hordein (high molecular weight), and A hordein (the smallest polypeptides) (Celus et al.,

2006). In this context, the B hordeins account for 70–90% of the total hordein fraction (mol wt 35–46 kDa) (Shewry et al., 1985&1983). C, as well as some B hordeins, appear as monomers (sulphur-poor therefore contains limited disulfide bonds for monomer connections), while most B and D hordeins are linked by interchain disulfide bridges. Most γ hordeins are monomers with intrachain disulfide bonds, but polymeric types may also occur. The A hordeins, the smallest polypeptides, average molecular weight (MW) 15 kDa, may be alcohol-soluble albumins or globulins or breakdown products of larger hordeins rather than true hordeins (Celus et al., 2006). It has been hypothesized that in barley, HMW subunits form a backbone, which binds LMW subunits through disulfide bridges to form a gel-like aggregate. Hordein extraction with increasing concentration of sulphydryl reducing agents, revealed that D hordeins are extracted only at the highest 2-mercaptoethanol concentration, suggesting they form the gel protein 'backbone' (Celus et al., 2006).

Glutelin is defined as an alkali soluble protein after the hordein has been extracted. It has been reported impossible to prepare a glutelin fraction totally free of contamination from hordein. The non-hordein components tend to contain HMW and some LMW components (Celus et al., 2006). Few, if any of the barley glutelins, have been characterized in detail. Both hordein and glutelin fractions possess a high hydrophobic nature and therefore demonstrate low solubility in aqueous solutions.

Table 1-4 Barley protein amino acid composition (mol%)

Amino Acid	Albumin (mol%)	Globulin (mol%)	Hordein (mol%)		Glutelin (mol%)
			Insoluble in water	Soluble in water	
Ala	9.3	7.9	2.6	6.6	10.0
Asp	**10.6**	**10.5**	**1.8**	**5.2**	**11.5**
Cys	**6.5**	**5.6**	**2.4**	**/**	**/**
Glu	**19.2**	**14.5**	**35.0**	**30.2**	**21.0**
Gly	10.8	12.7	3.1	10.1	12.5
Ile	3.8	3.0	5.3	3.2	6.0
Leu	6.8	8.4	7.8	8.0	10.9
Lys	**3.4**	**7.8**	**0.6**	**1.5**	**0.8**
Met	2.5	1.6	1.2	1.6	1.9
Phe	3.6	4.4	6.8	4.9	5.3
Pro	**6.1**	**7.3**	**20.1**	**13.8**	**2.1**
Ser	6.7	7.9	5.8	7.1	9.7
Tyr	4.4	3.0	3.3	3.1	1.1
Val+Thr	6.4	5.5	4.3	4.8	7.2

Barley for feeding purposes has a shortage of essential amino acids, especially lysine (Lys), arginine (Arg), threonine (Thr), and methionine (Met), whereas an excess of proline (Pro) and glutamine (Glu) (Table 1-4) (Linko, 1989). It is reported that the first and second limiting essential amino acids for feeding pigs are lysine and threonine and for broilers they are methionine, lysine, and threonine (Abdellatif et al., 2007; Mette, 2007). As shown in the Table 1-4, an excess of proline and glutamine has been found in hordein fractions. This excess is actually one typical property for all alcohol soluble proteins and also the reason we call them “prolamin”, the combination of the two enriched amino acids.

Table 1-5 Amino acid composition for hordein

type (code)[a]	D (Q40054)	C (Q40055)	B (P06470)	γ (P17990)
ALA	3.2	1.5	2.6	2.1
ARG	1.6	0.9	2.6	1.8
ASX	1.5	1.5	0.7	2.4
CYS	1.5	0.0	2.9	3.5
GLX	28.0	38.8	32.1	30.1
GLY	15.7	0.6	2.9	3.1
HIS	3.0	0.6	1.5	1.4
ILE	0.7	3.4	4.4	3.8
LEU	4.1	8.6	8.0	7.0
LYS	1.2	0.9	0.7	1.8
MET	0.4	0.0	1.1	1.8
PHE	1.3	7.7	4.7	5.6
PRO	10.5	29.1	19.4	16.8
SER	10.5	2.5	4.7	5.6
THR	7.3	1.2	2.2	3.1
TRP	1.2	0.6	0.7	0.7
TYR	4.2	1.8	2.6	2.1
VAL	4.1	0.3	6.2	7.3

Table 1-5 shows the amino acid composition for different hordein fractions in barley (Mette, 2007). The C hordein of barley is lacking in cysteine and contains little or no methionine which two are the sulfur-containing proteinogenic amino acids. Their shortage is correlated with the fact that C hordein (sulphur-poor) appears as monomers with limited disulfide bonds. On the contrary, the C hordein is the main contributor for the high proline and glutamine content in hordein. As a monomer, the C hordein has been studied extensively compared to other relatively complex hordein fractions. Research has already revealed that the C hordein has an ordered conformation indicating the molecule is rod shaped, with dimensions varying from 360 × 17 A to 265 × 20 A. The circular dichroism spectroscopy also indicated its secondary structure rich in β-turns, but no α-helix or β-sheets. The results are compatible with the C hordein having a helical secondary structure based on repetitive β-turns (Field, 1986). Recent studies

proved the C hordein appears to consist predominantly of an octapeptide repeat motif (consensus Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln) with short non-repetitive N- and C-terminal domains of twelve and six residue respectively stiff coil – worm like chain (Kenneth, 1992). No other significant discovery, however, has been published so far referring to barley protein study except one paper that clearly defined the crystal structure of a barley lipid transfer protein (Bakan, 2009). Consequently, the structures of most barley proteins are still far from clear.

1.4.4 Barley protein as coating material in microencapsulation

Barley protein is one of the byproducts in brewing industry. Almost all protein fractions go to animal feeding with limited value-added application. The use of barley protein as a functional ingredient is restricted due to its high hydrophobic nature that comes from the high content of alcohol soluble protein (hordein) fractions. Many researchers have tried to use hydrolysis (enzymatic or chemical hydrolysis) to improve their solubility with the purpose of improving protein functionality such as emulsion, forming, gelling, and filming properties (Abdellatif et al., 2007). However, a rapid solubilization also means a fast permeation of the material in an aqueous environment, which may also result in the loss of their favorable properties when in wet or humid environments. For example, hydrophilic proteins and polysaccharides are mostly used as wall materials in microencapsulation; however they usually exhibit rapid diffusion of the core from the matrix which broadly limits their usage in liquid/semi-liquid systems. On the contrary, hydrophobic proteins that possess low solubility in aqueous environments may have the potential to maintain the matrix integrity

even in high humidity conditions and to achieve controlled release of the core due to the low permeation of the wall. Both hordein and glutelin from barley possess high hydrophobicity and recent research has revealed the excellent emulsifying and film-forming properties of these two fractions (Wang et al., 2010; Xia et al., 2010). Additionally, as the main storage protein in barley, hordein showed good oxygen barrier properties which may make it suitable as food coating/packaging material, since food degradation is often oxygen dependent (Gillgren and Stading, 2007). Studies also demonstrated the antioxidative and reducing activity of C hordeins, suggesting that protein fractions derived from barley grains could be potential natural antioxidants in preventing lipid peroxidation of polyunsaturated oils (Kawase et al., 1998; Wasaporn et al., 2009). However, the study on using barley protein as coating material in microencapsulation is currently unavailable.

1.5 Conclusion

Based on the hypothesis that barley protein may act as a promising protective vehicle for nutraceutical delivery, the purpose of this work is to:

(1) Test the feasibility of using barley protein to prepare microencapsulation;

(2) Study the impact of processing conditions on microcapsule characteristics (size, morphology, etc.) and optimize processing suitable for industrial production;

(3) Investigate the capacity whether these microcapsules are able to stabilize and controlled release nutraceuticals.

Canada is the third largest barley producer in the world. Alberta produces almost five million tonnes of barley per year, about half of Canada's annual crop. Nearly 80% of Alberta's barley crop is grown for livestock feed, particularly for the province's hog and beef sectors. About 15% is used for malt production and only 5% is used for human food. The success of this work may open a new route to enhance barley consumption by developing novel barley protein based microencapsulation system. This is also a way to develop value-added applications for barley.

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Chapter 2 Physical Characteristics and Oxidative Stability of Barley Protein Stabilized Fish Oil Microcapsules

2.1 Introduction

Microencapsulation has been widely used to protect fish oil from oxidation by forming a physical and impermeable barrier to oxygen diffusion (Shu et al., 2006). In addition, the barrier masks its unpleasant taste and forms free flowing 'dry' powders, improving patient acceptability and ease of handling (Barrow et al., 2009; Curtis et al., 2008). The type and physico-chemical properties of the wall material used are the most critical aspects that govern the functionality of microcapsule systems (Gharsallaoui et al., 2007). Carbohydrates such as starches, maltodextrins and corn syrup solids are usually used for microencapsulation (Gharsallaoui et al., 2007) due to their desirable drying properties as well as matrix forming ability. However, wall materials that are based on carbohydrates usually have poor interfacial properties and must be chemically modified in order to improve their surface activity (Kanakdande et al., 2007; Krishnan et al., 2005; Soottitantawat et al., 2005). In recent years, interest in using food protein based microencapsulation has increased owing to their excellent emulsifying, gel- and film-formation properties (Chen et al., 2006). Additionally, protein coatings are, in general, excellent oxygen and aroma barriers and degradable by digestive enzymes, thus can be used in developing microcapsules for controlled core release

in food applications (Chen et al., 2006). So far, whey proteins, caseinate and gelatins are the most common coating materials used to encapsulate fish oil by spray drying, spray cooling and coacervation methods, among which, spray drying is the most commonly used in the food industry due to its continuous production and easiness of industrialization (Gharsallaoui et al., 2007; Gibbs et al., 1999; Gouin, 2004; Shu et al., 2006). This process normally involves an initial step to form emulsions in which the protein wall material acts as a stabilizer for the core lipid, followed by a spray-drying to convert emulsions into free-flowing powders. Emulsions can be also solidified by adding a cross-linking reagent (e.g. transglutaminase), or coacervating with oppositely polysaccharides before spray-drying to reinforce the microcapsule structures. However, most research has focused on animal proteins (Curtis et al., 2008; Kagami et al., 2003; Keogh et al., 2001; Subirade and Chen, 2008), whereas, little attention has been paid to plant proteins, which are generally less expensive and reduce the risk of spreading diseases such as bovine spongiform encephalitis (mad cow disease).

Barley (*Hordeum vulgare L.*) is a very adaptable crop grown primarily for animal feeding and brewing (Eagles et al., 1995). Even in brewing industry, its by-products are also used as livestock feed. Barley grains and their brewing by-products are abundant and affordable plant protein source which contain 8-13% and 20-30% (w/w) protein, respectably (Yalçin et al., 2008). Hordein and glutelin are the major storage proteins enriched in barley endosperm which constitute 35-55% and 35-40% total barley endosperm proteins, whereas albumin and globulin are enriched in barley bran and germ (Finnie and Svensson, 2009). The hordein

fractions, extracted with alcoholic media, can be further divided into five groups based on their electrophoretic mobility and amino acid compositions: B hordein (sulphur-rich), C hordein (sulphur-poor), γ-hordein (sulphur-rich), D hordein (high molecular weight), and A hordein (the smallest polypeptides) (Celus et al., 2006). In this context, the B hordein (mol wt 35-46 kDa) and the C hordein (mol wt 55-75 kDa) account for 70–90% and 10-30% of the total hordein fraction (Shewry et al., 1985&1983). Glutelin is defined as an alkali soluble protein after hordein extraction. But it is not possible to prepare a glutelin fraction totally free of hordein contamination (Celus et al., 2006). Both hordein and glutelin fractions possess a high hydrophobic nature and recent research has revealed the excellent emulsifying and film-forming properties of these two major barley protein fractions (Wang et al., 2010; Xia et al., 2010). Thus we assume barley protein may function as a promising potential coating material for fish oil encapsulation. Plus, there are studies that have already demonstrated the antioxidative and reducing activity of the C hordeins, suggesting that protein fractions derived from barley grains could be potential natural antioxidants in preventing lipid peroxidation of polyunsaturated oils (Kawase et al., 1998; Wasaporn et al., 2009). In these studies, however, the C hordein powders were just mixed with unsaturated fatty acids. Microencapsulation, relatively, may provide better protective effect due to the physical shielding; however such work is currently unavailable.

The purpose of our research was therefore to test the feasibility of using barley proteins to encapsulate fish oil. The microcapsule preparation conditions were optimized and the particle size and morphology were carefully characterized.

The stability of encapsulated fish oil was evaluated in accelerated storage tests as well as in real food formulations. The roles of barley hordein and glutelin, contributing to microcapsule functionalities, were discussed.

2.2 Materials and methods

2.2.1 Material

Regular barley grains (Falcon) were kindly provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Barley protein content was 13.2% (w/w, dry status) as determined by combustion with a nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) calibrated with analytical reagent grade EDTA (a factor of 6.25 was used to convert the nitrogen to protein). Barley grains were first de-braned and milled into flour. Barley glutelin and hordein were extracted using alkaline and alcohol methods, respectively, according to our previous work (Wang et al., 2010). Endosperm protein was characterized as protein extracted from de-braned barley flour and whole grain protein was characterized as protein extracted from milled whole grain flour (with bran). The protein content (dry status) was > 85% (w/w) for all the extracted proteins.

Fish oil (Omega 30 TG Food Grade (Non-GMO) MEG-3^{TM} Fish Oil) was kindly donated by Ocean Nutrition Canada Limited (ONC) (Canada) with (EPA+DHA) content ~ 31%. Whey protein concentrate (80%) used for comparison was provided by Le Sueur Food Ingredient Company (USA). Fat free yogurt (Yoplait Vanilla, Yoplait USA, Inc) and fat free milk (Lucerne skim,

Safeway Inc) used for food formulation were purchased from a local superstore (Edmonton, AB, Canada). All chemicals were of reagent grade.

2.2.2 Microcapsule preparation

Aqueous solutions of the wall materials were prepared by dispersing them in water. Table 2-1 (page 63) shows different protein fractions/combinations used for wall materials. The barley protein powder was hydrated at pH 11.0 (adjusted with 3N NaOH) to form a 15% w/v solution. The solution was then adjusted back to pH 7.4 followed by an immediate mixing of fish oil to form a coarse emulsion using a homogenizer (30,000 rpm/min) (PowderGen, Fisher Scientific International, Inc., CA, USA). Microcapsules were then formed by passing the premixed emulsion through a high pressure homogenization system (Nano DeBEE, BEE international Inc. MA. USA) operated at 8000 psi. To prevent an increase in temperature of the final product, the emulsifying cell of the high pressure homogenizer was immersed in ice. The prepared microcapsules (wet status) were stored at 4^{o}C with 0.025% (w/v) sodium azide until further analysis. The wet microcapsules can be turned into free flow powder by a spray drying process (Büchi 190 Mini Spray Dryer, Büchi Labortechnik, Flawil, Switzerland). Three different air inlet temperatures (180^{o}C, 150^{o}C and 120^{o}C) were applied to study the impact of hot air on microcapsule morphology. The outlet temperature was controlled between 55-65^{o}C (Shu et al., 2006). The dried microcapsules (dry status) were stored in plastic bottles at 4^{o}C before analysis. The prepared samples were coded as shown in Table 2-1 and *W*- and *D*- stand for wet-status and dry-status, respectively. Whey protein microcapsules were prepared for comparison.

2.2.3 Microcapsule characterization

The size of the microcapsules in wet status was measured at room temperature by dynamic light scattering using a Zetasizer NanoS instrument (model ZEN1600, Malvern Instruments Ltd, UK). The protein refractive index (RI) was set at 1.45 and the dispersion medium RI was 1.33. The microcapsule suspensions were diluted in deionized water to a suitable concentration before analysis and data were averaged from at least three batches. The morphology of the spray-dried microcapsules was observed with a scanning electron microscope (SEM, S-2500, Hitachi, Tokyo, Japan) operating at 15 kV. The surfaces of the microcapsules were sputtered with gold, observed and photographed. The powders were also fractured carefully after frozen in liquid nitrogen, and the interior morphology was observed and photographed using the SEM.

2.2.4 Encapsulation efficiency, loading efficiency, and moisture content

Extraction of oil from barley protein microcapsules was based on the method described by Beaulieu et al. (Beaulieu et al., 2002). Dry status microcapsules (250 mg) were precisely weighed to the nearest 0.1 mg and added into 5 ml of pure ethanol. The mixture was shaken on a vortex mixer for 1 min, the sample was allowed to rest for 5 min, and then 5 ml of hexane was added. The mixture was shaken vigorously with a vortex mixer for 30 s and allowed to stand for 2 min. These mixing and standing procedures were repeated twice. Five milliliters of water was added, and the tube was inverted several times, and then sealed and shaken using a Multi-purpose rotator (Barnstead 2314, IA, USA) for 1 h. After

centrifugation (Beckman Coulter Avanti®J-E Centrifuge, CA, USA) at 8,000 × g for 15 min at 23°C, 4 ml of hexane was transferred to a tube and evaporated under nitrogen to remove the solvent. The remaining oil was weighed to the nearest 0.1 mg. The encapsulation efficiency (*EE*) and loading efficiency (*LE*) were calculated by the following equations*: EE (%)* = $W_{encapsulated\ oil}$ / $W_{total\ oil}$ × 100; where $W_{encapsulated\ oil}$ represents the weight of oil encapsulated in the microcapsule and $W_{total\ oil}$ represents the oil added initially in the particle formation mixture. *LE (%)* = $W_{encapsulated\ oil}$ / $W_{microcapsules}$ × 100; where $W_{microcapsules}$ represents the weight of the microcapsule encapsulating the oil inside. The moisture content of the microcapsules was measured gravimetrically by drying ~0.5g of the dry status samples in an air oven at 105°C for 12h.

2.2.5 Fish oil oxidative stability in accelerated storage test

The oxidative stability of the microencapsulated fish oil was tested at both dry status and in aqueous solutions (HCl-saline solution pH 2.0 and phosphate-buffered saline pH 7.4, presenting acidic and neutral environment, respectively) by the accelerated storage test at 40ºC for 8 weeks.

For stability test at dry status, approximately 5g (dry weight) of each sample was placed in pre-dried airtight glass and stored in an incubator at 40ºC. For stability test at wet status, approximately 5g (dry weight) freshly prepared microcapsules (without spray-drying) were suspended in pH 2.0 and 7.4 media, and incubated at 40ºC. The oxidative stability was monitored by measuring the peroxide value (PV) of the extracted oils. Approximately 100 mg (dry weight)

(Soottitantawat et al., 2005) of each sample was withdrawn from the bottle at weekly intervals. The oil extraction process was the same as indicated in 2.2.4.

The colorimetric method described by Bae et al. (Bae and Lee, 2008) was used to measure the PV of oils with some modifications. The extracted oil (40mg~50mg) was added to 9.8ml of chloroform/methanol (7:3, v/v) mixture in a glass tube, followed by the addition of 50µl each of ammonium thiocyanate and ferrous chloride solutions. The final mixture was then mixed and incubated for 5 min in a dimmed light at ambient temperature. After incubation, the absorbance was measured with a UV/vis spectrophotometer (model V-530, Jasco, CA, USA) at 505nm. Reagent and oil blank assays were also carried out. PV was quantified using a standard curve created from a series of hydrogen peroxide standard solutions and expressed as milliequivalents (meq) hydroperoxide per kg of oil.

2.2.6 Fish oil stability in selected food formulations (milk and yogurt)

The oxidative stability of the microencapsulated fish oil (wet status) was also tested in two food products. The microcapsule suspensions were mixed with milk or yogurt by stirring for 15 min to obtain homogeneous dispersions. This microcapsule-incorporated milk and yogurt was then pasteurized (80ºC, 30 min) and stored at 4ºC. Sodium azide (0.025%, w/v) was added as a bacteriostasis reagent. Samples were withdrawn weekly for fish oil stability analysis. The oil extraction process and the PV analysis were the same as that described in 2.2.4 and 2.2.5. Stability test was conducted for 4 and 5 weeks for milk and yogurt, respectively. Original fat free milk and yogurt was run as zero controls.

2.2.7 Statistical analysis

Each type of microcapsule was prepared in three independent batches. The microcapsule size and the moisture measurements as well as the quantification of the fish oil *EE* and *LE* values were done in duplicate for each batch. Data is represented as the mean of three batches ± SD. For each type of microcapsule, one batch of the sample was randomly selected for stability experiments. The PV data is the mean of three independent determinations ± SD. Statistical significances of the differences were determined by ANOVA and Student's t-test. The level of significance used was $p < 0.05$.

2.3 Results and discussion

2.3.1 Microcapsule preparation

Understanding of the roles of barley protein fractions for microencapsulation will be helpful for designing a wall material that can better protect core ingredient. In this context, barley glutelin (BG), hordein (BH) and their mixtures with glutelin to hordein ratio of 1:1, 1:2 and 2:1 (BGH, BG1H2, and BG2H1) were used as wall materials to prepare fish oil microencapsulation (Table 2-1). The feasibility of using barley endosperm protein (BE, composed of both hordein and glutelin) and whole grain protein (BWG, composed of albumin, globulin, hordein and glutelin) for fish oil microencapsulation was also investigated.

The emulsifying-stabilization process which has been widely used to prepare globular protein (whey and soy protein) based microcapsules (Subirade and Chen, 2008) was adapted in this work to prepare barley protein microencapsulation. This

process normally involves an initial step to form emulsions in which the protein wall material acts as a stabilizer for the core lipid. In the second step, the protein wall materials are solidified by adding a cross-linking reagent (e.g. glutaraldehyde, transglutaminase), or by coacervating with oppositely charged polymers. These stabilized microcapsules can then be converted into free-flowing powders using a spray-drying technique (Subirade and Chen, 2008). According to our preliminary work, the well suspended solid microcapsules, rather than emulsions, were able to form from all barley protein based pre-mixture after the high pressure treatment. Therefore, no solidification reagent or process was needed. The spray drying in this work was consequently used as a drying method to turn wet-status microcapsules into dry powders. This phenomenon is quite different from that observed for globular protein (whey and soy protein) stabilized emulsion systems, where the emulsions only form soluble aggregates via surface hydrophobic interactions after high pressure treatment (Beaulieu et al., 2002; Floury et al., 2002). The unique behavior of barley proteins to form solid particles during the high pressure homogenization process may be attributed to the hydrophobic nature of their molecular structures that are enriched with non-polar amino acids (~ 35-38%) including proline, alanine, valine, isoleucine, and leucine (Wang et al., 2010). This hydrophobic nature may allow barley protein to adhere and completely cover the oil droplets rapidly in the pre-emulsion process. The complexes would then tend to strongly aggregate due to the hydrophobic surface patches, to form thick unruptured coatings after high pressure treatment. The unique behavior is quite favorable from an industry point of view for the mass

production of micro-encapsulations. Processing can be simplified by removing the cross-linking or the coacervation process, and toxic or expensive cross-linking reagents are also not necessary.

A number of factors would likely affect the ability of barley proteins to function as microencapsulants, that is, protein structure and concentration, proportion of dispersed and dispersion phases and processing conditions.

Table 2-1 Availability of wet (*W*-) / dry (*D*-) status microcapsules prepared by different protein systems at a given concentration of fish oil

Code		Microcapsules except for water (wt%)			Availability Wet-status (*W*-)	Availability Dry-status (*D*-)
		Oil	Hordein	Glutelin		
BH	Hordein	33.3%	66.7%	0%	No	No
		50%	50%	0%	Yes	No
		66.7%	33.3%	0%	Yes	No
BG	Glutelin	33.3%	0%	66.7%	Yes	Yes
		50%	0%	50%	Yes	Yes
		66.7%	0%	33.3%	Yes	Sticky
BGH	G:H=1:1	33.3%	33.3%	33.3%	No	No
		50%	25%	25%	Yes	Yes
		66.7%	16.6%	16.6%	Yes	Sticky
		75%	12.5%	12.5%	Yes	Sticky
		80%	10%	10%	Yes	Very sticky
BG1H2	G:H=1:2	50%	33.33%	16.67%	Yes	Yes
BG2H1	G:H=2:1	50%	16.67%	33.33%	Yes	Yes
			Oil	Protein		
BE	Endosperm protein		50%	50%	Yes	Yes
BWG	Whole grain protein		50%	50%	Yes	Yes
WP	Whey protein		50%	50%	No	Yes

2.3.1.1 Impact of protein concentration and oil/protein ratio

The protein concentration and oil/protein ratio are important in microencapsulation system since a high protein concentration normally facilitates protein-protein interactions to form thick and viscoelastic layers at the oil droplet surface for a better encapsulation of the incorporated lipophilic compounds (Hogan et al., 2001). And a high oil/protein ratio generally leads to a high carrying capacity of the final microencapsulation products. In preliminary trials, the maximum protein concentration of 15% was achieved for barley protein microencapsulation. Further increasing the protein concentration led to formation of aggregated sticky substances rather than well dispersed microcapsules.

Trials were then carried out to vary the component of the wall material and oil/protein ratio in order to evaluate the effects of these two parameters on the microcapsule quality. The results are described in Table 2-1. The oil/protein ratio significantly impacted BH microcapsule formation as BH could be formed into good coarse emulsions only at oil/protein ratio $\geq$ 1.0 after homogenization treatment. We noticed that hordein tends to aggregate to form soft and viscous dough when dispersed in water, due to the strong surface hydrophobicity (Wang et al., 2010). This protein aggregation could be associated with a reduction in the emulsifying capacity of the hordein at the oil/protein ratio of 0.5. Increasing the oil/protein ratio $\geq$ 1.0, more protein molecules had an orientation of hydrophilic groups towards water phase and hydrophobic groups towards oil phase due to an increased dispersed phase volume, thus preventing protein aggregation and allowing formation of good coarse emulsions. After passing the high pressure

homogenizer, solid BH microcapsules (wet status) were formed at an oil/protein ratio of 1.0 to 2.0. BG microcapsule formation was unaffected by increasing the oil/protein ratio from 0.5 to 2. The formed BH and BG microcapsules were then spray-dried. BH microcapsule powders were hardly obtained they tended to adhere to the drying chamber wall surface due to sticky characteristic whereas free flowing BG microcapsules could be obtained at the oil/protein ratio of 0.5-1.0. Further increase of the oil/protein ratio (≥2.0) induced high apparent viscosity, owing to a high dispersed phase volume (Hogan et al., 2001) which made it difficult to be completely spray-dried, leading to clumping particulate substances. The optimized condition (15% protein concentration and an oil/protein ratio of 1.0) was therefore applied to prepare all microcapsules based on gluten and hordein mixture at BG/BH ratio of 1:1, 1:2 and 2:1, marked as BGH, BG1H2 and BG2H1, respectively as well as on endosperm protein (BE) and whole grain protein (BWG) (Table 2-1).

2.3.1.2 Impact of spray-drying inlet temperature

The spray drying inlet temperature is another major factor for microencapsulation since it will influence the microcapsule morphology. Figure 2-1 shows the SEM micrographs of the BGH microcapsules prepared at three different inlet temperatures (120ºC, 150ºC, and 180ºC). Irregular shaped microcapsules with surface indentations were obtained at the inlet temperature of 180ºC (Figure 2-1a). The surface indentations might be due to the rapid particle shrinkage during the early stage of the drying process (Shu et al., 2006). Such particle features suggest that a 180ºC inlet temperature may be too high for barley

protein microsphere preparation since high drying rates, associated with small particles, usually lead to a rapid wall solidification and thus smoothing cannot occur (Rosenberg and Sheu, 1996; Sheu and Rosenberg, 1998). Decreasing the inlet temperature to 150ºC resulted in microcapsules with a spherical shape, uniform size (3-5μm) and smooth surface (Figure 2-1 b-c) whereas, a further decrease of the temperature to 120ºC, resulted in agglomeration of powder particles (Figure 2-1d). This can be attributed to the relatively high water content in the particle wall material, resulting from inefficient drying. It has been reported that as an efficient plasticizer, water can decrease the glass transition temperature of the microsphere matrix. At the glass transition temperature, surface droplet viscosity and the stickiness of the powder particles increase, resulting in inter-particle bridge formations that finally lead to caking and the collapse of the particles (Beristain et al., 2002; Drusch et al., 2006&2007; Le Meste et al. 2002, Partanen et al., 2005).

The above results indicate that barley glutelin, hordein and their mixtures are able to be prepared into microencapsulation incorporating lipid compounds by a pre-emulsifying process followed by a high pressure homogenization treatment. The optimal condition for microencapsulation formation was determined to be 15% protein concentration and a 1.0 oil/protein ratio. The formed microcapsules suspension can then be converted into white, free-flowing powders by a spray-drying process at the optimum inlet temperature of 150ºC. Microcapsules prepared under the optimized conditions were used for their size and morphology characterizations as well as the oxidative stability test in the following steps.

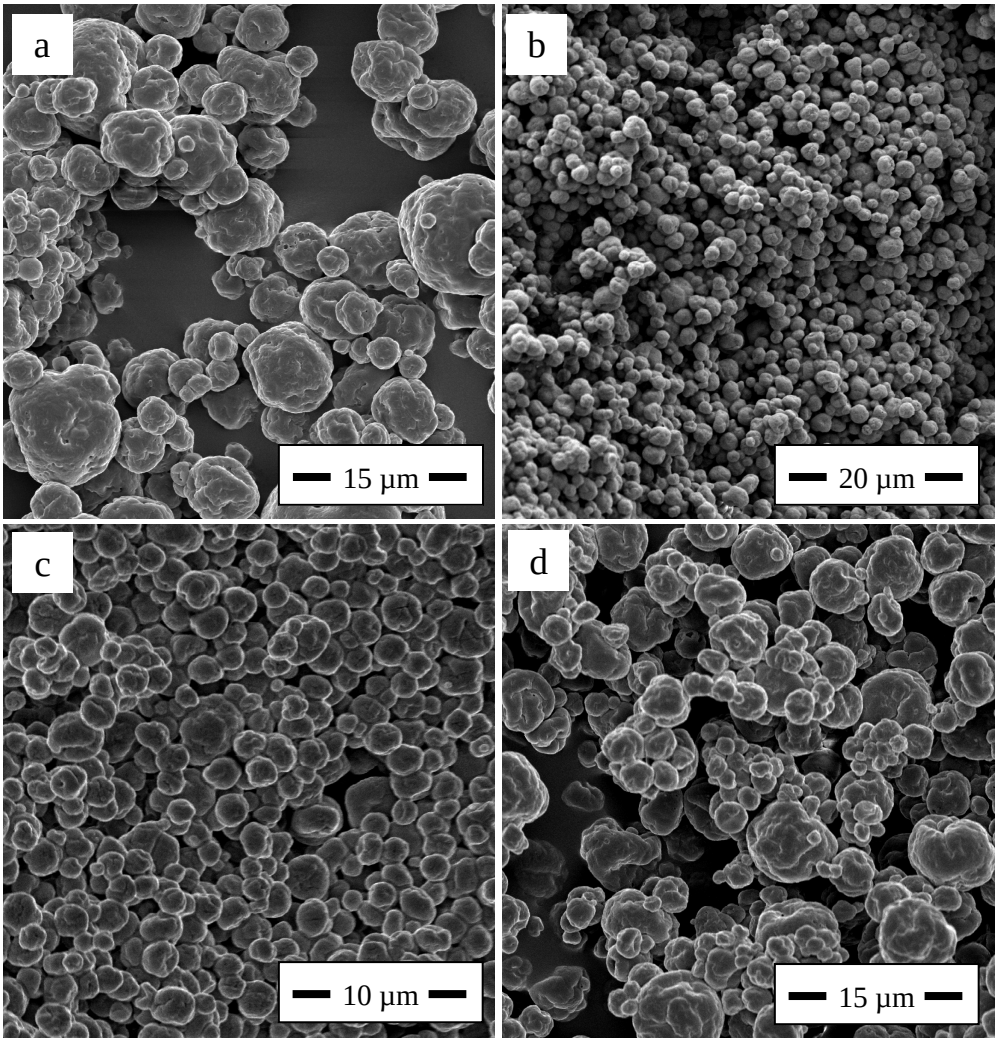


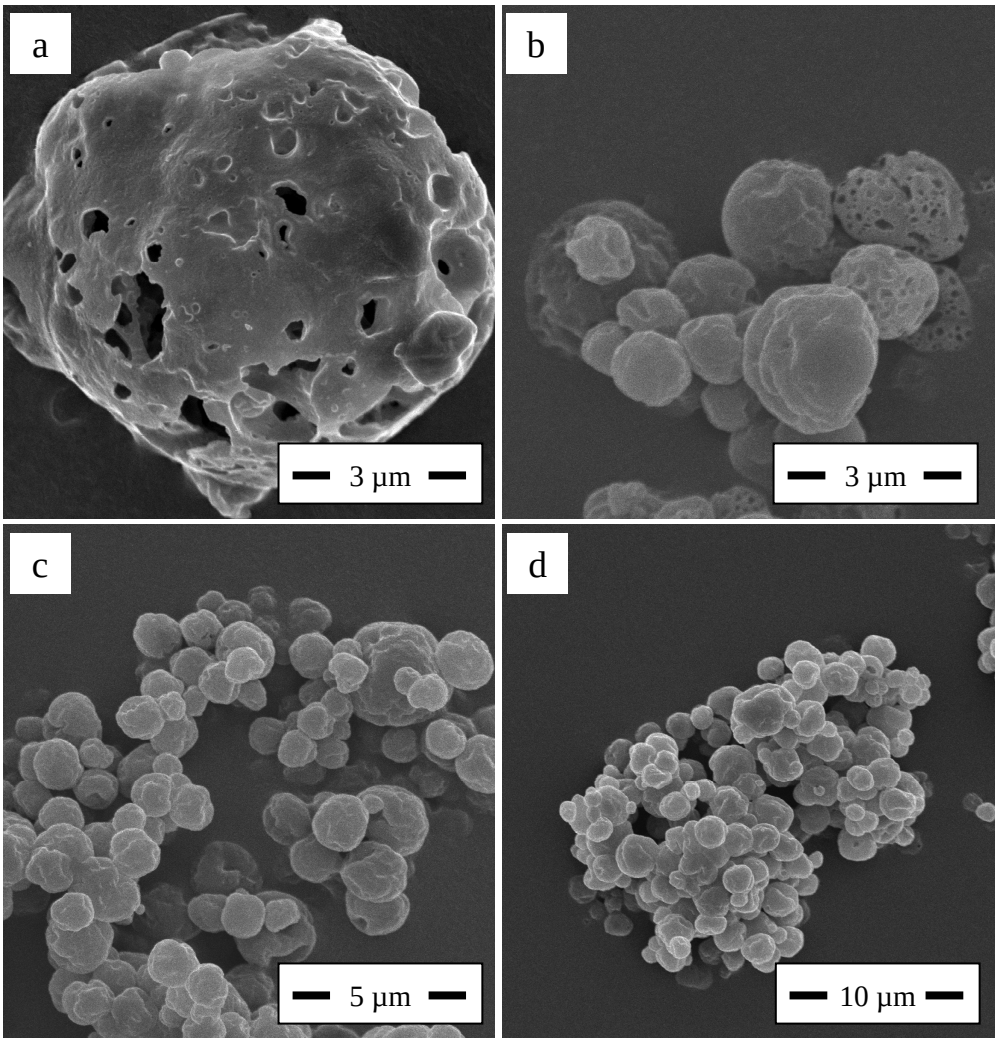
Figure 2-1 Surface morphology of spray dried BGH microcapsules with different inlet temperature by SEM
(a), 180°C; (b-c), 150°C; (d), 120°C.

2.3.2 Microcapsule size and morphology

The size and morphology obtained is typical for microcapsules intended for food application. The spray-dried microcapsules showed generally spherical shape with the diameters ranging from 1-5μm. There were no significant differences in the diameters of microcapsules made from different protein fractions. However, their surface morphology differed as assessed by SEM (Figure 2-2).

The presence of surface porous structure was inversely related to the proportion of included glutelin in the wall material. *D*-BH and *D*-BG1H2 microcapsules exhibited porous outer shell (Figure 2-2 a-b) whereas *D*-BGH, *D*-BG2H1 and *D*-BG microcapsules demonstrated dense, crack-free and smooth surfaces (Figure 2-1 b-c and Figure 2-2 c-d). As revealed in our previous work, the hordein matrix may have similar physical properties to wheat dough, and thus can expand or "balloon" when heated. During spray-drying, fast drying rates could lead to rapid hordein wall ballooning at an early stage of heating. This process was also accompanied by hordein denaturation and the loss of viscoelasticity (Cauvain, 2003). Thus, further expansion resulted in the breaking of coating networks, leading to a porous structure. BG does not exhibit viscoelastic characteristics, and therefore, could maintain a dense coating wall structure during the whole spray-drying process. *D*-BGH, *D*-BG2H1, *D*-BE and *D*-BWG (Figure 2-1 b-c; Figure 2-2 c ,e ,f) exhibited similar surface morphology as that of *D*-BG microcapsules (Figure 2-2d), suggesting that the coating wall surface may be mainly composed of glutelin, forming a dense outside structure that prevents hordein ballooning. This result indicates that addition of glutelin is important to maintain the integrity of the microcapsule coating during spray-drying. Remarkable sphere microcapsules with a smooth surface and little aggregation were found specifically for the *D*-BWG matrix while *D*-BE exhibited more aggregation, comparably (Figure 2-2 e-f). Whey protein (WP) microcapsules demonstrate less uniform size and shape and the dents on surface may result from the uneven shrinkage during the drying process (Figure 2-2g)

(Sheu and Rosenberg, 1998). Figure 2-2h shows the inner structure of the BGH microcapsules. The small pores inside indicate that oil droplets were well distributed/separated within the protein micron-matrix. Other barley protein microcapsules showed similar porous inner structures (data not shown). The dense, crack-free surface features together with the interior “bee-net” multiple emulsion structure of barley protein microspheres, may allow them to better withstand mechanical stresses and protect the incorporated ingredients against harsh environments (e.g. oxidation, light, low or high pH).



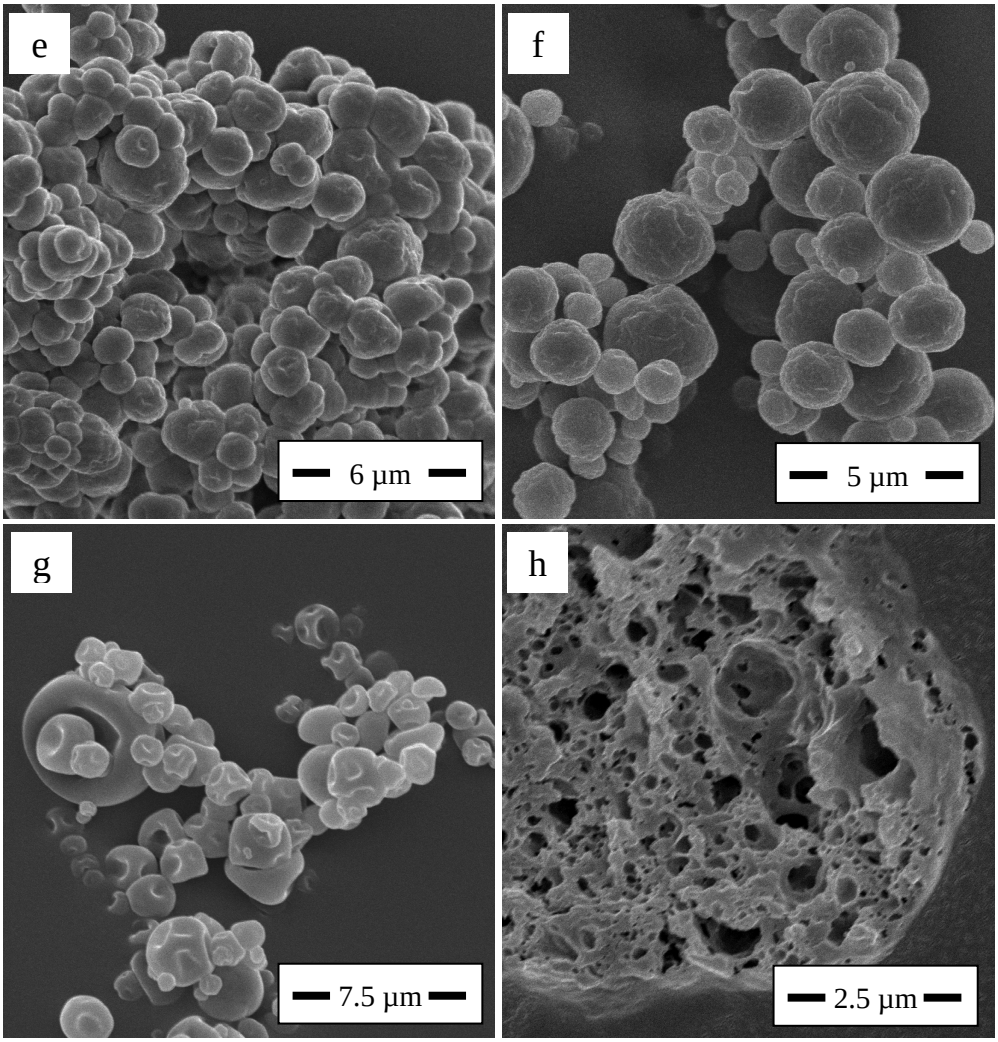


Figure 2-2 Surface morphology of spray dried microcapsules prepared by different wall systems by SEM
(a) *D*-BH; (b) *D*-BG1H2; (c) *D*-BG2H1; (d) *D*-BG; (e) *D*-BE; (f) *D*-BWG; (g) WP; (h) inner structure of *D*-BGH.

2.3.3 Encapsulation efficiency (EE), loading efficiency (LE) and moisture

Barley protein based wall materials were quite effective encapsulating agents as most of the microcapsules demonstrated rather high *EE* and *LE* values as shown in Table 2-2. This is probably related to barley protein's excellent emulsifying property (Wang et al., 2010) as well as its capacity to form solid microcapsule-coating-granule structures during high pressure treatment. In spite

of their porous structure, BH microspheres demonstrated similar *EE* and *LE* values compared to other barley protein microcapsules. This suggests that the hordein may have the capacity to bind oil droplets and keep them inside the microcapsule matrix, even when the surface integrity was destroyed to some degree during the spraying drying process.

The moisture content is very critical for formed microcapsules. High moisture will induce high viscosity and stickiness of powder particles, resulting in inter-particle bridge formations that finally lead to caking and collapse of the particles and the release/oxidation of the core (Drusch et al., 2006&2007; Beristain et al., 2002; Le Meste et al. 2002, Partanen et al., 2005). In this study, the moisture content of barley protein microcapsules maintained at very low levels ranging from 0.63 to 0.92% (w/w), significant different from that of whey protein microcapsules (5.57%, w/w). This is likely due to the hydrophobic nature of both barley glutelin and hordein enriched with hydrophobic amino acid groups including proline, leucine, alanine and valine. A slight decrease of moisture was observed with the increase of hordein content in the wall material. As an alcohol soluble protein, hordein has been reported to have higher percentage of non-polar amino acid groups compared to glutelin (Wang et al., 2010). It is therefore reasonable to deduce that increasing the hordein content may be an efficient way to decrease water content in the wall system and therefore somehow limits lipid core oxidation.

Table 2-2 Encapsulation efficiency (*EE*), loading efficiency (*LE*), and moisture of fish oil microcapsules prepared by different protein systems

	(± SD)		
	Encapsulation Efficiency (%)	*Loading Efficiency (%)*	*Moisture (%)*
BH	93 ± 1.67	46 ± 0.83	/
BG	97 ± 2.96	49 ± 1.47	0.90 ± 0.0167
BGH	96 ± 2.61	48 ± 1.30	0.86 ± 0.0636
BG1H2	100 ± 2.12	50 ± 1.06	0.75 ± 0.0319
BG2H1	97 ± 2.22	49 ± 1.11	0.77 ± 0.0700
BE	95 ± 2.83	45 ± 3.42	0.92 ± 0.0258
BWG	98 ± 1.89	50 ± 1.42	0.63 ± 0.0320
WP	95 ± 2.50	48 ± 1.25	*5.57 ± 0.0345

2.3.4 Oxidation stability

2.3.4.1 Stability of dry status microcapsules in accelerated storage test

The oxidative stability of the encapsulated fish oil was analyzed under the accelerated condition of 40ºC because low and ambient temperatures often require a long period of time. The oxidation of unsaturated oil usually results in a variety of compounds including free radicals and hydroperoxides (Firestone, 1993). Peroxide value (PV) is an index to quantify the amount of hydroperoxide in fat and oil. It is commonly chosen as a useful index to control food safety and quality since it indicates the initial stage of fat and oil deterioration. Figure 2-3 shows the PV changes of the encapsulated fish oil in dry microcapsules in an accelerated storage test (40ºC, 8 weeks). The unencapsulated bulk fish oil was also tested as a control under the same conditions. The oil blank (crude fish oil without any processing treatment and contains no antioxidant) demonstrated desirable stability

within 2 weeks of storage (< 10 meq peroxide/kg oil), but its PV value started rising sharply after 5 weeks and reached a maximum level at almost 350 meq peroxide/kg oil in the 8th week. On the contrary, the PV values of fish oil encapsulated in barley protein microcapsules gradually increased initially and reached maximum levels of 45-76 meq peroxide/kg oil in the 3-4 weeks and then declined to 6.6-15 meq peroxide/kg oil in the 8th week. The relatively higher initial PV was attributed to the oxidation of surface/near surface oil of the microcapsules when exposed to oxygen, light and heat during microencapsulation processing. Research has revealed that the auto-oxidation of the encapsulated and non-encapsulated core material already happened during the spray drying process (Drusch and Berg, 2008; Drusch and Schwarz, 2006). The peroxide values in oxidized oil are usually unstable and are themselves oxidized to other compounds. In this study, at the beginning of oxidation, peroxides increased but they were eventually oxidized to aldehydes and ketones and therefore the peroxide levels fell in the later stages of oxidation (Drusch et al., 2006&2007; Firestone, 1993; Naohiro and Shun, 2006). After the deep oxidation of surface/near surface oil, no further increase of the PV value was detected, suggesting the inside oil was well protected in the microcapsule matrix. There were studies discussing that in some cases, wall matrix comprised of only protein may have much higher oxygen permeability due to the similar hydrophobic polarity of oxygen and the matrix; if so, this might be one reason contributing to the immediate oxidation of parts of the fish oil. Hydrophilic carbohydrates such as lactose or hydrolyzed starch can be considered as a secondary wall material with the purpose of reducing oxygen

permeability and at the same time limiting the diffusion of the hydrophobic core through the wall, consequently leading to a better oxidative stability of the encapsulated core (Gharsallaoui et al., 2007; Kagami et al., 2003; Moreau and Rosenberg, 1996).

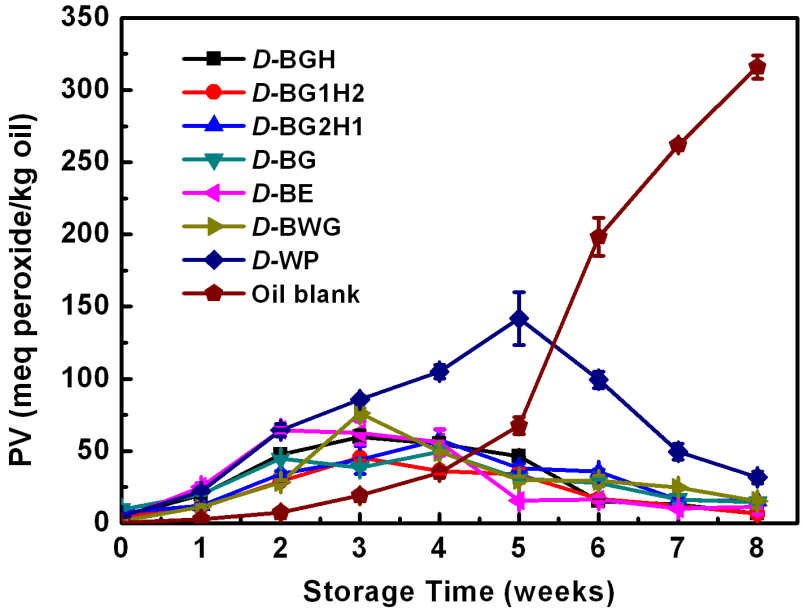


Figure 2-3 Peroxide value (PV) changes for encapsulated fish oil in dry status (*D*-) microcapsules prepared by different wall systems in accelerated storage test
Oil blank stands for crude/unprocessed fish oil.

For barley protein microcapsules, the PV reached a maximum level around 40-80 meq peroxide/kg oil after 2-3 weeks of treatment. Whereas, for whey protein microcapsules, PV increased more rapidly, reaching a maximum level of ~150 meq peroxide/kg oil in the 5th week. This result suggested barley protein microcapsules may provide better protective ability against fish oil oxidation compared to whey protein microcapsules. One the one hand, barley protein

microcapsules may possess less surface/near surface oil than whey protein microcapsules because solid barley protein microspheres were able to form directly just after high pressure homogenization processing. This may allow barley protein microcapsules to better protect the encapsulated oil against oxidation during the spray drying process. One the other hand, less favorable morphology of whey protein microcapsules was already observed by SEM (Figure 2-2g) and their high moisture (Table 2-2) may also contribute to the less protective ability of whey protein microcapsules.

Among barley protein microcapsules, the BG1H2 matrix demonstrated desirable protective ability and generally, those with a high content of hordein all demonstrated the lowest PV values, suggesting that hordein may play an important role in preventing oil oxidization. This might be attributed to the unique structure of hordein with abundant hydrophobic amino acids (Leu, Val, Phe and Tyr) which may better bind encapsulated oil (Wang et al., 2010). It was also reported that C-hordein (one major hordein fraction) consists almost entirely of repeats based on the octapeptide motif Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln and has demonstrated conformational transitions between poly-L-proline II-like and βI/III turn structures. The repetitive domain seems to form a helical secondary structure rich in β-turns and the entire molecule of takes on a rod-like conformation with dimensions of about 30nm × 2nm. Such a unique structure may form a “cage” to better hold lipid molecules (Kawase et al., 1998) inside the protein matrix against oxidation. Additionally, as stated before, the antioxidative and reducing activity of C hordeins could also be one reason contributing to the better protective ability of

the high-hordein-content barley protein matrix in preventing lipid peroxidation (Kawase et al., 1998; Wasaporn et al., 2009).

2.3.4.2 Stability of wet status microcapsules in accelerated storage test

The PV level of encapsulated fish oil in wet status microcapsules was measured to evaluate the potential of using barley protein microcapsules in aqueous solutions. Buffers with a pH of 7.4 and 2.0 were chosen as representatives for neutral and acidic environments, respectively. No leakage of oil was observed for any barley protein microcapsule suspensions even after storage for more than six months at 4ºC, indicating the integrity of microcapsules was well maintained. No oil release was found even for *W*-BH microspheres, suggesting the particle integrity was preserved after passing the high pressure homogenizer, confirming the porous structure of *D*-BH was formed during the spray-drying process.

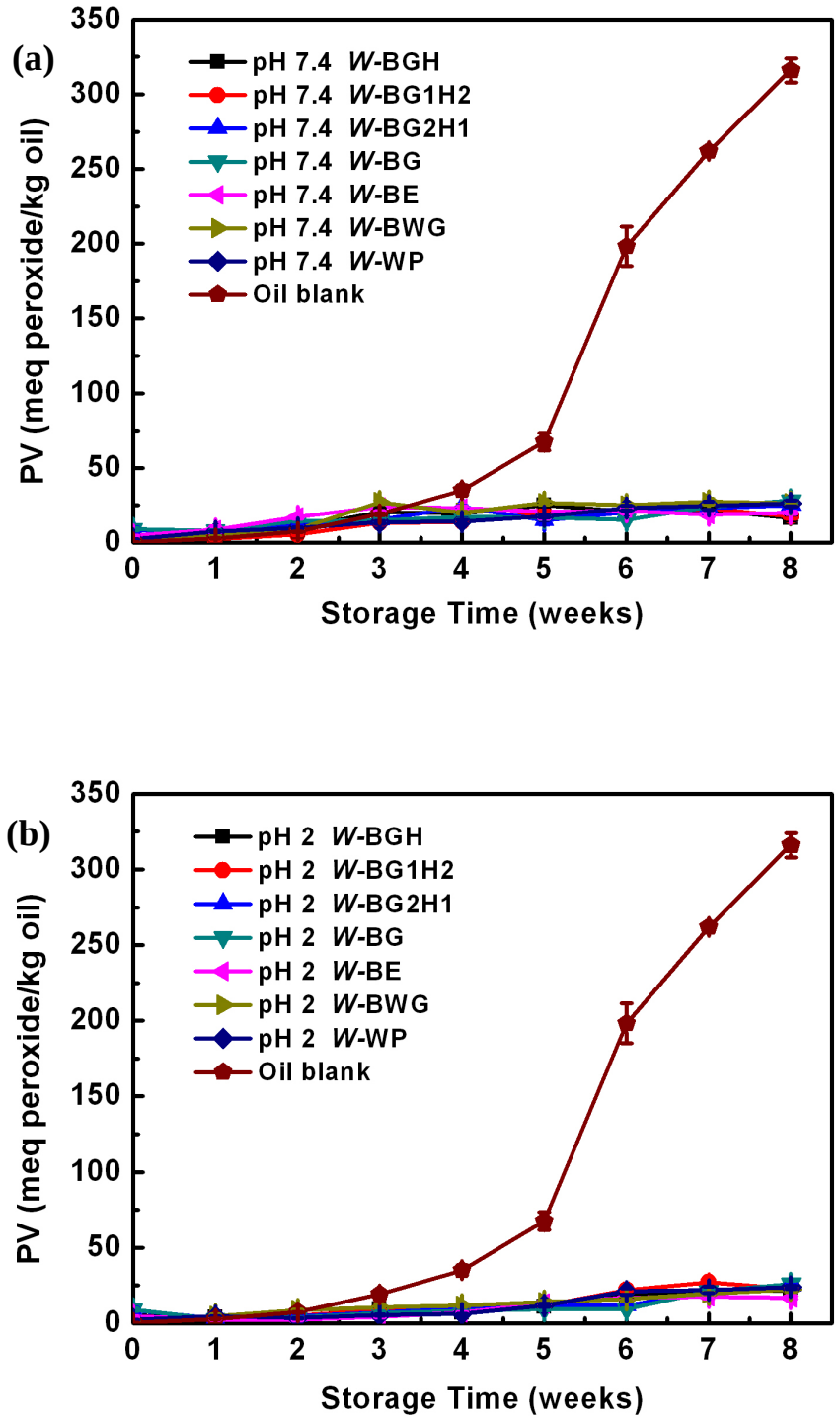


Figure 2-4 Peroxide value (PV) changes for encapsulated fish oil in wet status (*W*-) microcapsules prepared by different wall systems in accelerated storage test
(a) in pH 7.4 buffer; (b) in pH 2 buffer.

Figure 2-4 shows the PV changes for encapsulated fish oil in wet status microcapsules in the accelerated storage test. Since the emulsion was formed from the whey protein solution mixed with fish oil only after high pressure homogenization processing, the emulsion complex was used directly in this test in comparison to wet status barley protein microcapsules. All barley protein microcapsules showed low oxidative levels of encapsulated oil (PV < 30 meq peroxide/kg oil) even after 8-week of storage. No significant difference was found in terms of different matrixes in the storage test at both pH 7.4 and pH 2 media (Figure 2-4 a-b). This result suggests barley protein microcapsules (wet-status) might be suitable to be used in liquid/semi-liquid food system. The much lower PV value observed at wet status compared to that at dry status confirms that the lipid oxidation at dry status was trigger by spray-drying process. This process might also lead to leakage of the encapsulated oil to the exterior of the microcapsules which resulted in acceleration of oxidation changes and higher peroxide values after the process.

2.3.4.3 Stability of wet status microcapsules in food formulation

W-BG1H2 and *W*-BWG microcapsules were selected as representatives for stability test in two food formulations: fat free milk and yogurt. The PV values of the encapsulated fish oil were measured weekly for 4 and 5 weeks, respectively, which correspond to the average shelf life of fat free milk and yoghurt. Both milk and yogurt with microcapsules added was pasteurized (80ºC, 30min) in order to simulate the real industrial situation. As shown in Figure 2-5, PV values of the encapsulated fish oil remained low (PV < 20 meq peroxide/kg oil) in both milk

and yogurt during the 4-5 weeks of storage. The fish oil microcapsules were especially stable in yogurt as PV levels were below 5 meq peroxide/kg oil even after 5 weeks of storage. It has been recommended that PV levels shouldn't exceed 30 meq peroxide/kg oil in an edible food product (Naohiro and Shun, 2006). Thus, the above result confirms the applications of barley protein stabilized fish oil microcapsules being used in liquid and semi-liquid food systems.

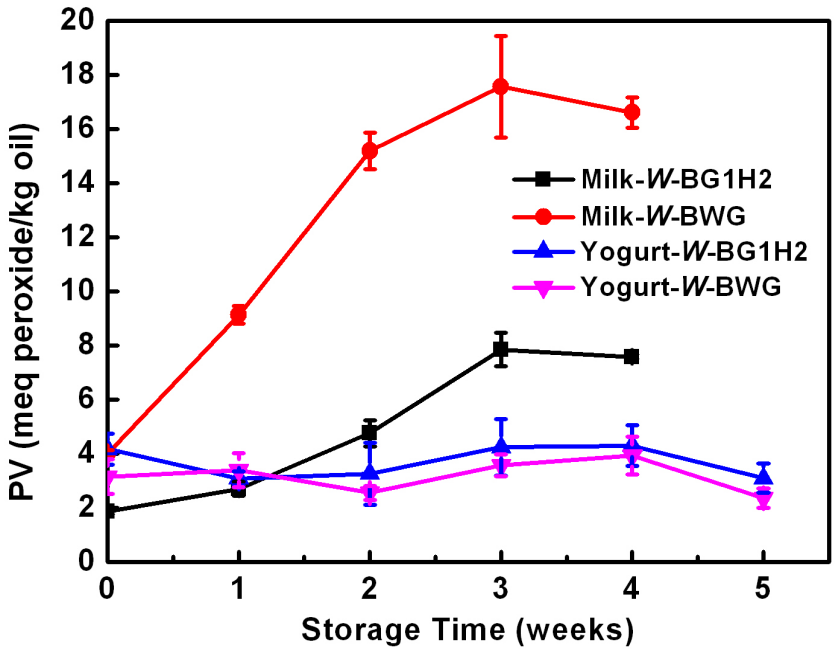


Figure 2-5 Peroxide value (PV) changes for encapsulated fish oil in *W*-BG1H2 and *W*-BWG microcapsules in real food formulation test (milk and yogurt)

2.4 Conclusion

Barley protein stabilized fish oil microcapsules (1-5μm) were successfully prepared by a pre-emulsifying process followed by a high pressure

homogenization treatment. The optimal conditions for microencapsulation formation were determined to be 15% protein concentration and a 1.0 oil/protein ratio. The formed microcapsules can be converted into white, free-flowing powders by a spray-drying process at the optimum inlet temperature of 150ºC. These microcapsules exhibited high oil encapsulation efficiency. Barley hordein and glutelin play different roles in contributing to the microcapsule properties. The existence of glutelin is important to maintain the integrity of the microcapsule coating during spray-drying, thus allowing formation of microcapsules with a dense and smooth surface; whereas, microcapsules enriched with hordein demonstrate a comparably higher capacity to prevent oil oxidization. Both the accelerated experiment and the stability test in food formulations suggest that barley protein microcapsules, especially those enriched with hordein, have great potential to be used in liquid and semi-liquid food systems.

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Chapter 3 Nano-encapsulations Liberated from Barley Protein Microcapsules for Oral Delivery of Bioactive Compounds*

3.1 Introduction

Oral administration is by far the most convenient way for delivery of bioactive compounds, especially when repeated or routine administration is necessary (Chen and Langer, 1998). However, this route is restricted for many bioactive compounds that have poor solubility, poor permeability, and/or poor stability in the gastro-intestinal environment (Sahana et al., 2008). Polymeric nanoparticles are promising candidates for oral delivery of bioactive compounds since they can adhere to the intestinal membrane and can increase residence of included compounds. Furthermore, ‘‘M-cells’’ in the Peyer’s patches can absorb polymeric nanoparticles by receptor-mediated endocytosis to directly deliver bioactive compounds into the circulation. Some uptake of polymeric nanoparticles can also occur through transcellular and paracellular pathways (Desai et al., 1996; Florence, 1997; Norris et al., 1998). In order to preserve functionality, nanoparticles must survive the harsh gastric conditions of low pH and pepsin digestive enzymes. A major drawback of these dispersions is their tendency to decrease their interfacial surface area and then aggregate (Li and Kaner, 2006). Strategies for preventing aggregation include coating particles with foreign

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capping agents and/or tailoring the particle surface charges to create separation through electrostatic repulsion (Elbadawy et al., 2010; Medina-Ramı´rez et al., 2009). For example, polyethylene glycosylated nanoparticles have greater *in vitro* stability due to a steric stabilization mechanism (Hinrichs et al., 2006). Also, some surfactants can improve the stability of solid lipid nanoparticles during storage (Freitas and M¨uller, 1998; Kim et al., 2005; Mehnert and Mäder, 2001; Olbrich and Müller, 1999). Despite various surface modifications to increase nanoparticle stability, their shelf life is still often limited (Hinrichs et al., 2006). Once released into the human gastrointestinal system, the stability of the nanoparticles is largely influenced by pH, proteases, and the presence of other food compounds (e.g. polysaccharides and lipids).

Research using natural biodegradable polymers, like proteins, as delivery systems continues to be an area of active research interest despite the advent of synthetic biodegradable polymers (Park et al., 2005; Torres-Lugo and Peppas, 2000). Aside from being a vital macronutrient in food, proteins possess unique functional properties including their ability to form gels, films and emulsions, offering the possibility of developing delivery systems for both hydrophilic and lipophilic bioactive compounds (Chen et al., 2006; Liu et al., 2005; Picot and Lacroix, 2003; Weinbreck et al., 2004). In past decades, gelatin, casein, whey protein, soy protein, zein and gliadin have been prepared into gels, micro- and nano-particles incorporating drugs, unsaturated fatty acids, vitamins, probiotics as well as bioactive peptides (Subirade and Chen, 2008). Hydrophilic compounds release from a protein matrix by diffusion, whereas lipophilic compounds are

released mainly by enzymatic degradation of the protein matrix in the GI tract (Chen, 2009). Barley proteins are an abundant and affordable plant protein source (Yalçin et al., 2008). Recent research has revealed the excellent emulsifying and film-forming properties of two major barley protein fractions: hordein and glutelin (Wang et al., 2010; Xia et al., 2010).

Our objective was to develop barley protein-based emulsion microcapsules for oral administration of lipophilic bioactive compounds. This may provide a new approach for targeted and controlled delivery of nano-encapsulations in the human gut by avoiding nanoparticle aggregation and degradation during storage or in stomach conditions. This research describes the preparation, characterization, and evaluation of emulsion microcapsules based on barley glutelin and hordein. Microcapsule degradation and bioactive compound release behaviours were studied using *in vitro* systems and are presented together with a discussion of a proposed encapsulation release mechanism.

3.2 Materials and methods

3.2.1 Materials

Regular barley grains (Falcon) were kindly provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Barley protein content was 13.2% (w/w, dry status) as determined by combustion with a nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) calibrated with analytical reagent grade EDTA (a factor of 6.25 was used to convert the nitrogen to protein). Barley glutelin and hordein were extracted using alkaline and alcohol

methods, respectively, according to our previous work (Wang et al., 2010). The protein content (dry status) was 85% (w/w) for the extracted glutelin and 90% (w/w) for the extracted hordein. Canola oil used for the emulsification was purchased from a local supermarket. Unstained standard protein molecule marker for SDS-PAGE was purchased from Bio-RAD (Richmond, CA, USA). Beta-carotene, pepsin (from porcine gastric mucosa, 424 units/mg) and pancreatin (from porcine pancreas) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemicals were of reagent grade.

3.2.2 Microcapsule preparation

Three types of emulsion microcapsules were prepared using barley glutelin, hordein and a 1:1 (w/w) mixture of glutelin and hordein. The barley proteins acted as coating materials and emulsion microcapsules were prepared by an emulsifying-stabilization method. Firstly, a premixed emulsion was prepared by mixing 15% (w/v) aqueous protein suspension with canola oil containing 0.05% (w/v) β-carotene (bioactive compound model) at the protein/oil ratio of 1:1 (w/w) using a homogenizer (PowerGen, Fisher Scientific International, Inc., CA, USA). Microcapsules were then formed by passing the premixed emulsion through a microfluidizer system (M-110S, Microfluidics Co., USA) operated at 350 bar. To prevent an increase in the temperature of the final product, the pipe components of the Microfluidizer were immersed in a bath of cold water. The prepared microcapsules were stored at 4^{o}C with 0.025% (w/v) sodium azide until used *in vitro* for release and degradation studies. A portion of the microcapsules were spray-dried using a mini-spray dryer (Büchi 190 Mini Spray Dryer, Büchi

Labortechnik, Flawil, Switzerland) at an air inlet temperature of 150^{o}C and an air outlet temperature of 55-65^{o}C for oil payload evaluation and particle morphology observation. The prepared samples were coded as BG, BH and BGH, corresponding to microcapsules prepared from glutelin, hordein and their 1:1 (w/w) mixture, respectively.

3.2.3 Microcapsule characterizations

The size of the microcapsules in wet status was measured at room temperature by dynamic light scattering using a Zetasizer NanoS instrument (model ZEN1600, Malvern Instruments Ltd, UK). The protein refractive index (RI) was set at 1.45 and dispersion medium RI was 1.33. The microcapsule suspensions were diluted in deionized water to a suitable concentration before analysis and data were averaged from at least three batches. The morphology of the spray-dried microcapsules was observed with a scanning electron microscope (SEM, S-2500, Hitachi, Tokyo, Japan) operating at 15kV. The surfaces of the microcapsules were sputtered with gold, observed and photographed. The powders were also fractured carefully after being frozen in liquid nitrogen, and the interior morphology of the microcapsules was studied and photographed using the SEM (Xu et al., 2007). The interior morphology of the wet microcapsules was also observed using a transmission electron microscope (TEM, JEOL 2100 EX, Tokyo, Japan) at an accelerating voltage of 120 kV. Microcapsules were fixed in 2.5% glutaraldehyde in Millonig's buffer (pH 7.2) for 1.5 h and postfixed in 1% Osmium in the same buffer for 2 h. After dehydrating in a series of ethanol solutions of different concentrations, the samples were then replaced with

propylene oxide for two changes for 15 min each. The sample was then embedded in Araldite and polymerized at 60^{o}C for 48h. The ultrathin section was stained in 2% uranyl acetate and 0.2% lead acetate and photographed (Leung et al., 2005).

3.2.4 Oil payload in the microcapsule

Extraction of oil from barley protein microcapsules was based on the method described by Beaulieu *et al.* (Beaulieu et al., 2002). The spray-dried microcapsules (250mg) were precisely weighed to the nearest 0.1mg and added into 5ml pure ethanol. The mixture was shaken on a vortex mixer for 1 min, the sample was allowed to rest for 5 min, and then 5ml of hexane was added. The mixture was shaken vigorously with a vortex mixer for 30s and allowed to stand for 2 min. These mixing and standing procedures were repeated twice. Five millilitres of water was added, and the tube was inverted several times, and then sealed and shaken using a Multi-purpose rotator (Barnstead 2314, IA, USA) for 1h. After centrifugation (Beckman Coulter Avanti®J-E Centrifuge, CA, USA) at 8,000 × g for 15 min at 23^{o}C, 4ml of hexane was transferred to a tube and evaporated under nitrogen to remove the solvent. The remaining oil was weighed to the nearest 0.1mg. The encapsulation efficiency (*EE*) and loading efficiency (*LE*) were calculated by the following equations: EE (%) = $W_{encapsulated\ oil}$ / $W_{total\ oil}$ × 100; where $W_{encapsulated\ oil}$ represents the weight of oil encapsulated in the microcapsules and and $W_{total\ oil}$ represents the oil added initially in the particle formation mixture. LE (%) = $W_{encapsulated\ oil}$ / $W_{microcapsules}$ × 100; where $W_{microcapsules}$ represents the weight of the microcapsules encapsulating the oil inside.

3.2.5 In vitro release

Release profiles of the microcapsules were studied by incubating them in four different release media: HCl-saline solution (pH 2.0); phosphate-buffered saline (pH 7.4) or PBS; simulated gastric fluid (SGF) (pH 2.0) with 0.1% pepsin (w/v); and simulated intestinal fluid (SIF) (pH 7.4) with 1.0% pancreatin (w/v). Three batches were tested for each medium. For each batch, microcapsule samples were added into 8 separate tubes filled with the same release medium for incubation while continuously shaking using the Multi-purpose rotator at 37^{o}C. Each tube contained ~250mg (dry weight) microcapsules and 25ml release medium. The tubes were withdrawn at different time intervals. Digestive enzymes were inactivated by heating the release medium at 95^{o}C for 3 min. Quantitative analysis of the released β-carotene was based on the colorimetric method of Pan *et al*. (Pan et al., 2007). The β-carotene content in the hexane was determined by measuring the absorbance at 450nm with a UV-visible spectrophotometer (model V-530, Jasco, CA, USA). Blank SGF and SIF solutions were run as zero controls.

3.2.6 In vitro protein matrix degradation

The *in vitro* protein matrix degradation assays were conducted as described above for the *in vitro* release experiments. After digestive enzyme inactivation and oil removal, the degraded soluble proteins were then separated from other substances using an ultra-centrifuge (Optima Ultracentrifuge, MAX-130K, Beckman Coulter Inc. USA) at 50,000 × g for 25 min at 23^{o}C. The supernatants were filtered through Whatman No. 1 filter paper and the filtrates were then

freeze-dried (FreeZone 6 Liter Console Freeze Dry System, Labconco Corporation, Kansas City, MO, USA) and weighed to the nearest 0.1mg. Blank SGF and SIF with digestive enzymes were also run as controls. The percent protein matrix degradation was calculated by the following equations: *degradation* (%) = $W_{degraded\ protein}$ / $W_{microcapsule\ protein}$; where $W_{degraded\ protein}$ represents the weight of degraded soluble protein in the release medium, and $W_{microcapsule\ protein}$ represents the total protein in the microcapsules. Changes in microcapsule morphology after incubating in SGF and SIF were observed using the TEM. The samples were prepared by coating a copper grid with a thin layer of digestive suspension and then staining with 1% (w/v) phosphotungstic acid. Excess liquid was blotted from the grid, and then samples were air dried and examined using the TEM at an accelerating voltage of 120 kV.

3.2.7 Characterization of the protein layer stabilizing nano-encapsulations

After the *in vitro* degradation of BGH, BG and BH microcapsules in SGF with pepsin (section 3.2.6), the liberated nanoparticle precipitates were isolated using an ultra-centrifuge at 50,000 × g for 25 min at 4^{o}C and washed thoroughly with distilled water followed by hexane to remove the remaining oil. Both soluble fractions and the precipitates were freeze-dried before analysis. SDS gel electrophoresis (Mini-PROTEIN Tetra Cell, BIO-RAD, Hercules, CA, USA) was performed to study the subunits of the protein layer isolated from the nanoparticles and the digested soluble proteins in comparison to the original

barley glutelin and hordein. The protein sample was mixed with loading buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.5% 2-mercaptoethanol and 1% bromophenol blue (w/v)) and then heated at 100^{o}C for 5 min. After cooling, 12µl samples (5mg/ml) were loaded on 5% stacking gel and 15% separating gel and then subjected to electrophoresis at a constant voltage of 75V. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue-R-250 in water - methanol - acetic acid (4:5:1, v:v:v) for 30 min and destained with water -methanol-acetic acid (8:1:1, v:v:v).

For amino acid analysis, the isolated protein layer was hydrolyzed under vacuum in 4 *M* methanesulfonic acid with 0.2% (w/v) tryptamine according to the method of Simpson et al. (Simpson et al., 1976) with slight modifications. Glass sample tubes (6 × 50 mm) were used in the reaction vial assembly, which was then placed to the Work Station (Waters, Milford, MA, USA). After treating as suggested in the Work Station manual, where the contents were hydrolyzed at 115^{o}C for 24h, the pH was adjusted to neutral with 3.5 *M* NaOH. Amino acid analysis was performed using the Waters ACCQ-Tag method. The high-performance liquid chromatography (HPLC) system (Agilent series 1100, Palo Alto, CA, USA) consisted of an autosampler and a binary pump, a control system with a column heater maintained at 37°C, and a UV detector set at a wavelength of 254nm. A reversed-phase AccQ.Tag 150 × 3.9 mm C18 column with a solvent system consisting of a three-eluent gradient (AccQ.Tag eluent, acetonitrile, and water) was used at a flow rate of 1.5ml/min. Data acquisition was controlled by ChemStation software.

3.2.8 Statistical analysis

Each type of microcapsule was prepared in three independent batches. The microcapsule size measurements and quantification of the β-carotene *EE* and *LE* values were done in duplicate for each batch. Data are represented as the mean of three batches ± SD. For each type of microcapsule, one batch of the sample was randomly selected for the *in vitro* release and degradation experiments. The release and degradation data are the mean of three independent determinations ± SD. Statistical significances of the differences were determined by Student's t-test. The level of significance used was $p < 0.05$.

3.3 Results and discussion

3.3.1 Microcapsule preparation

The emulsifying-stabilization process is widely used to prepare globular protein (whey and soy protein) based microcapsules. This process normally involves an initial step to form emulsions in which the protein wall material acts as a stabilizer for the core lipid. In the second step, the protein wall materials are solidified by adding a cross-linking reagent (e.g. glutaraldehyde, transglutaminase), or coacervating with oppositely charged polymers. These stabilized microcapsules can then be converted into free-flowing powders using a spray-drying technique (Subirade and Chen, 2008). In this work, the emulsifying-stabilization process was adapted to prepare BGH, BG and BH microcapsules. A high protein concentration in particle mixture normally facilitates protein-protein interactions to form thick and viscoelastic layers at the oil droplet surface for a

better encapsulation of the incorporated lipophilic compounds (Hogan et al., 2001). Our preliminary experimental data demonstrated that the maximum protein concentration of 15% and an oil/protein ratio of 1:1 can be achieved for barley protein microcapsule formation. Further increasing the protein concentration and oil/protein ratio led to formation of aggregated sticky substances, rather than well dispersed microcapsules. Therefore, a protein concentration of 15% and oil/protein ratio of 1:1 were applied to prepare BGH, BG and BH microcapsules in this research.

Since barley glutelin and hordein have low solubility in aqueous solution at neutral pH (Wang et al., 2010), they were initially hydrated and dispersed in a pH 11 solution adjusted using 3 *N* NaOH at 23^{o}C. The dispersion pH was then reduced to 7 by adding 1 *N* HCl, followed immediately by the pre-emulsion process. Such processing allowed the formation of relatively stable barley protein suspensions at neutral pH without apparent precipitation, facilitating the emulsification process. The stable formed pre-mixed emulsions were then passed through a microfluidizer system (Wang et al., 2010). Interestingly, the well suspended solid microcapsules, rather than emulsions, were formed from all three types of coating materials immediately after the high pressure treatment. This phenomenon is different from that observed for globular proteins (whey and soy protein) stabilized emulsion systems, where the emulsions only form soluble aggregates via surface hydrophobic interactions after high pressure treatment (Beaulieu et al., 2002; Floury et al., 2002). The unique behaviour of barley proteins to form solid particles during the microfluidization process may be

attributed to the hydrophobic nature of their molecular structures that are enriched with non-polar amino acids (~ 35-38%) including proline, alanine, valine, isoleucine, and leucine (Wang et al., 2010). Additionally, the hydrophilic amino acid residues in barley proteins are probably buried in the core, whereas the hydrophobic amino acid residues are exposed on the outside (Zhao et al., 2010). Barley protein's surface hydrophobic nature may explain its tendency to adhere and completely cover the oil droplets rapidly in the pre-emulsion process. These complexes would tend to strongly aggregate due to hydrophobic surface patches to form thick unruptured coatings after high pressure treatment. This unique behaviour is quite favourable from an industry point of view for the mass production of micro-encapsulations. Processing can be simplified by removing the cross-linking or the coacervation processing, and toxic or expensive cross-linking reagents are not necessary.

3.3.2 Microcapsule characterization

All three types of microcapsules can be converted into white and free-flow powders by spray-drying. SEM photographs of the spray-dried BGH, BG and BH microcapsules are shown in Figure 3-1. These particles demonstrated diameters ranging from 3-5 μm with a spherical shape; however, their surface morphology differed. BGH and BG microcapsules were dense, crack-free and possessed smooth surfaces (Figure 3-1a and b) with many small pores homogeneously distributed inside (Figure 3-1d). A porous structure was observed for BH microcapsules (Figure 3-1c). As revealed in our previous work, hordein forms soft and viscous dough when dispersed in water (Wang et al., 2010). Hordein may

have similar physical properties as wheat dough and can expand or “balloon” when heated. During spray-drying, high drying rates associated with small particles can lead to rapid hordein wall ballooning at an early stage of heating. This process is also accompanied by hordein denaturation and the loss of viscoelasticity (Cauvain, 2003). Thus, further expansion can result in the breaking of coating networks, leading to a porous structure. BG dose not exhibit viscoelastic characteristics, and therefore can maintain a dense coating wall structure during the whole spray-drying process. The small pores inside the microcapsules indicate that oil droplets were well separated within the protein micron-matrix. Similar surface morphology of BGH microcapsules and BG microcapsules suggests that the coating wall surface was mainly composed of glutelin, forming a dense outside structure that prevented hordein ballooning. Figure 3-1e shows the internal morphology of the BGH microcapsule without spray-drying observed by a TEM. Unlike hydrophilic proteins which form a thin layer membrane around oil droplets that stabilize the emulsion, barley protein formed solid granules coating oil droplets with sizes ranging from several hundred nanometers to around 1μm. These granules then associated to form a microcapsule. BG and BH microcapsules (without spray-drying) showed a similar interior morphology compared to BGH microcapsules (figures not shown). Such dense, crack-free surface features and interior microcapsule-coating-granule structures may allow BGH and BG microcapsules to better withstand mechanical stresses and protect the incorporated ingredients against harsh environments (e.g. oxidation, low or high pH).

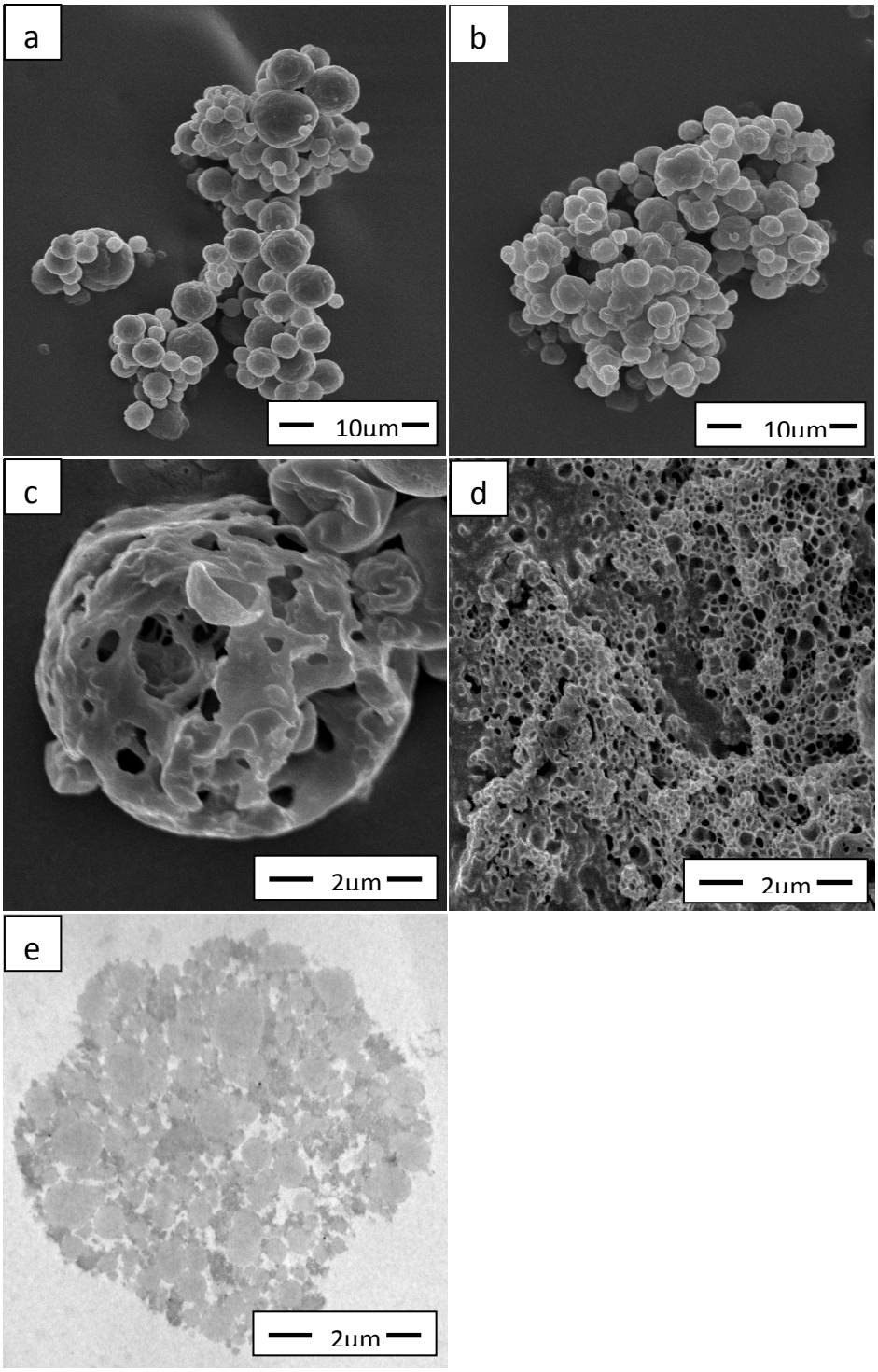


Figure 3-1 Surface morphology of spray-dried microcapsules
Prepared from (a) BGH, (b) BG, (c) BH, and (d) interior morphology of spray-dried BGH microcapsules by SEM, as well as (e) interior morphology of BGH microcapsules (without spray-drying) by TEM.

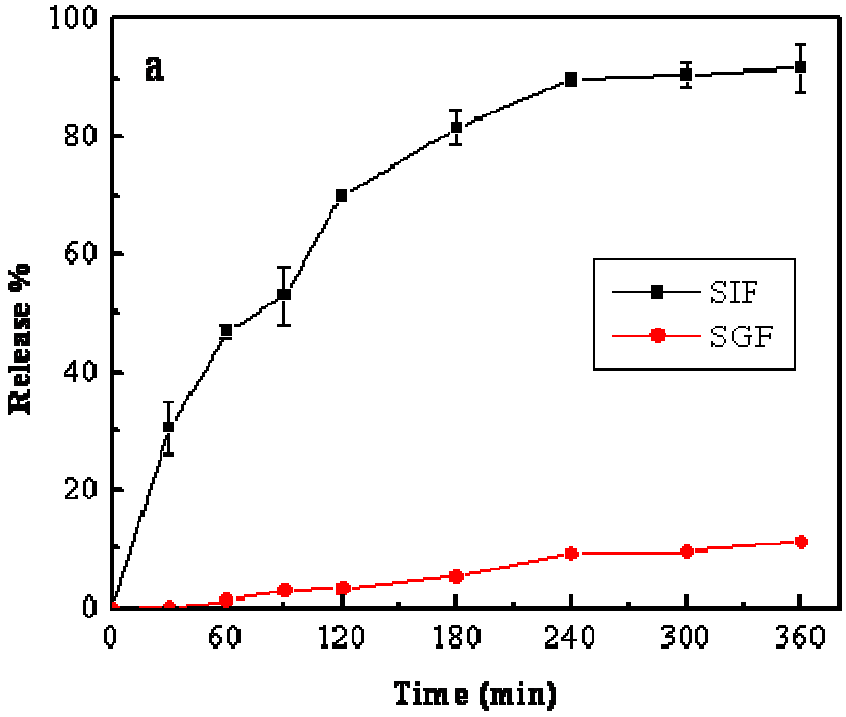
3.3.3 Microcapsule loading and in vitro release

Beta-carotene was selected as the model bioactive compound, since this precursor of vitamin A is well recognized as a disease-preventing antioxidant. Although abundantly available in vegetables and fruits, only a small proportion of β-carotene is bioavailable from its natural plant matrix when taken orally (Rich et al., 2003; Tyssandier et al., 2003). Incorporating β-carotene into micro-encapsulated emulsions provides a convenient method to enhance its oral absorption in the human GI tract. As shown in Table 3-1, three types of barley protein microcapsules all demonstrated very high *EE* (92.9-97.0%) and *LE* (46.5-48.5%) values, indicating most of the added β-carotene was encapsulated in the barley protein microcapsules. This is probably due to barley protein's excellent emulsifying properties (Wang et al., 2010) as well as its capacity to form solid microcapsule-coating-granule structures during high pressure treatment which means the oil droplets are well retained inside the particle matrix during the particle formation and spray-drying processes. In spite of their porous structure, BH microcapsules demonstrated similar *EE* and *LE* values to those of BGH and BG microcapsules. This suggests that the BH matrix may bind oil droplets, which prevents their leakage during the spray-drying process.

Table 3-1 Encapsulation efficiency (*EE*) and loading efficiency (*LE*) for barley protein microcapsules

Microcapsules	*EE (%)*	*LE (%)*
BGH	95.5 ± 2.6	47.8 ± 1.3
BG	97.0 ± 3.0	48.5 ± 1.5
BH	92.9 ± 1.7	46.5 ± 0.8

The release properties of the three types of microcapsules were investigated in the simulated gastric and intestinal fluids with and without digestive enzymes. A control experiment verified that β-carotene cannot be released from BGH, BG and BH microcapsules without digestive enzymes in pH 2.0 and 7.4 buffers, indicating that the integrity of the microcapsules was well maintained. Thus only β-carotene release profiles in SGF with pepsin and SIF with pancreatin were described in Figure 3-2. In SGF with pepsin, β-carotene was slowly released from BGH microcapsules and less than 5% β-carotene was detected in the release medium after 2h of the test. This corresponds to the usual time for food and drugs to pass through the stomach to the small intestine (Figure 3-2a). Even after 6h of the test, only 11.3% β-carotene was released. Interestingly, in SIF with pancreatin, β-carotene was steadily released from the BGH microcapsules at almost zero-order release kinetics (r^2 = 0.97) in the first 2h. Over time the release curve levelled off gradually, until after 6h when 91.6% of the β-carotene had been released. Similar β-carotene release profiles were observed for BG and BH microcapsules in the simulated GI tract, except for BH microcapsules in SGF with pepsin, where β-carotene release rates increased rapidly after 3h and 60.5% of the β-carotene was released after 6h (Figure 3-2b and c).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Time (min)
SIF
SGF
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40
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80
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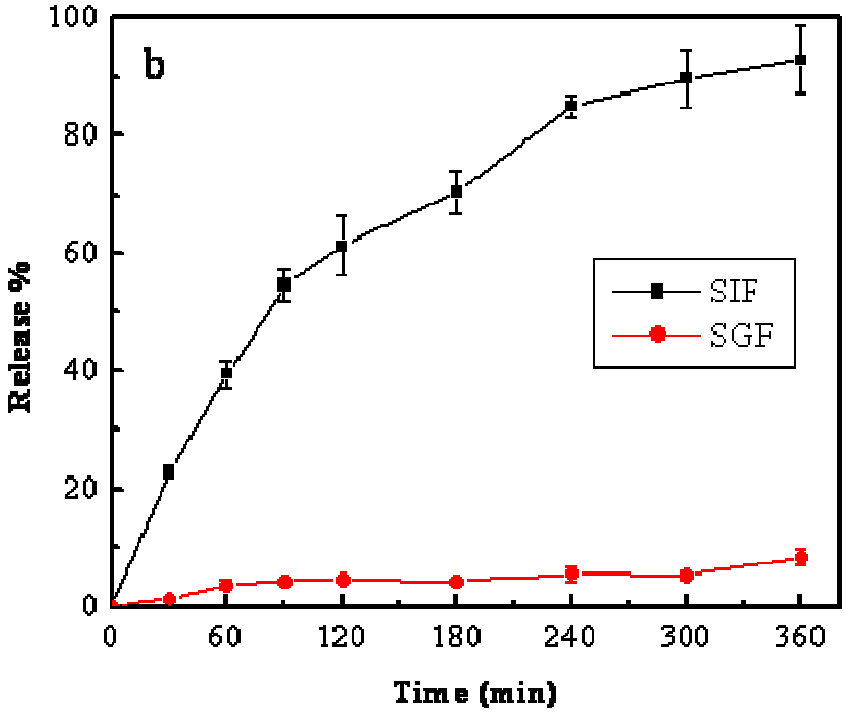
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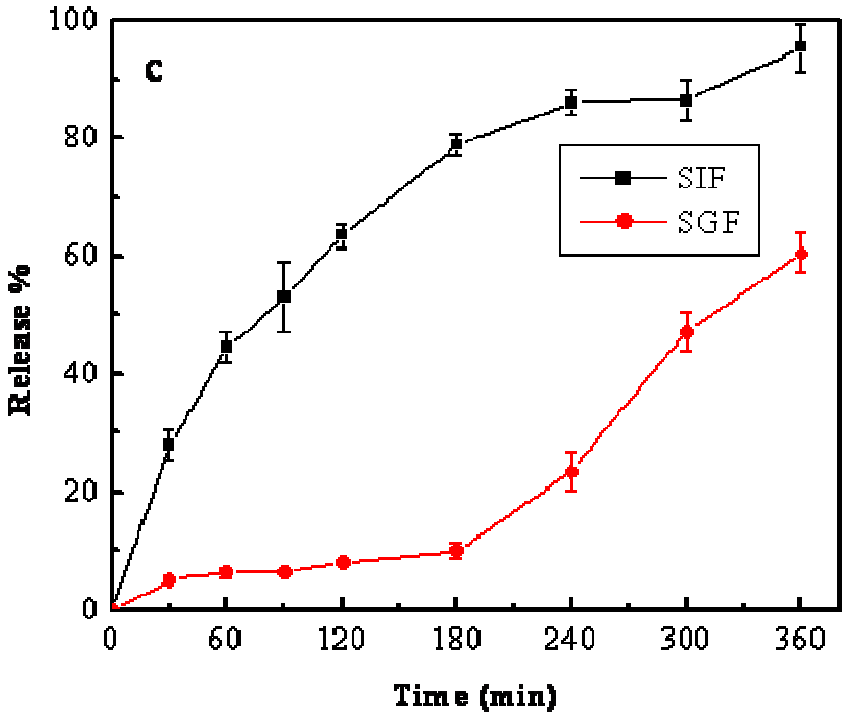


Figure 3-2 Release profile of β-carotene in the simulated gastric (SGF) and intestinal (SIF) fluids with digestive enzymes
From (a) BGH, (b) BG and (c) BH microcapsules.

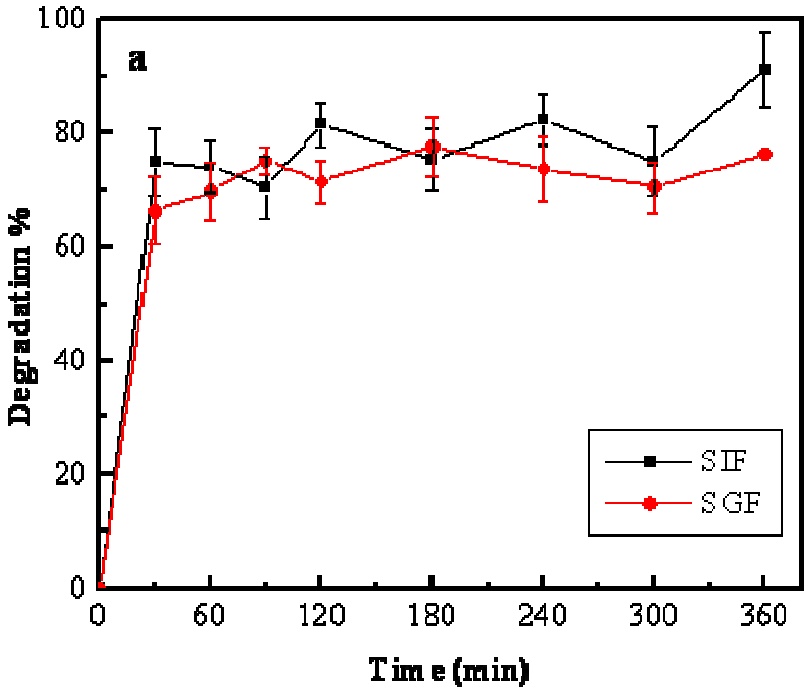
All three types of microcapsules had good stability in both neutral and acidic aqueous solutions, likely due to their surface hydrophobicity that formed a strong barrier to prevent permeation of environmental fluids. According to our previous work, microcapsules made from hydrophobic proteins are generally not sensitive to pH changes and swell little in aqueous media (Chen and Subirade, 2009). No leakage of oil was observed even after storage of these three types of microcapsule suspensions for more than six months at 4^{o}C in different pH buffers (without digestive enzymes). This storage test demonstrates their excellent potential to encapsulate bioactive compounds for use in liquid and semi-liquid

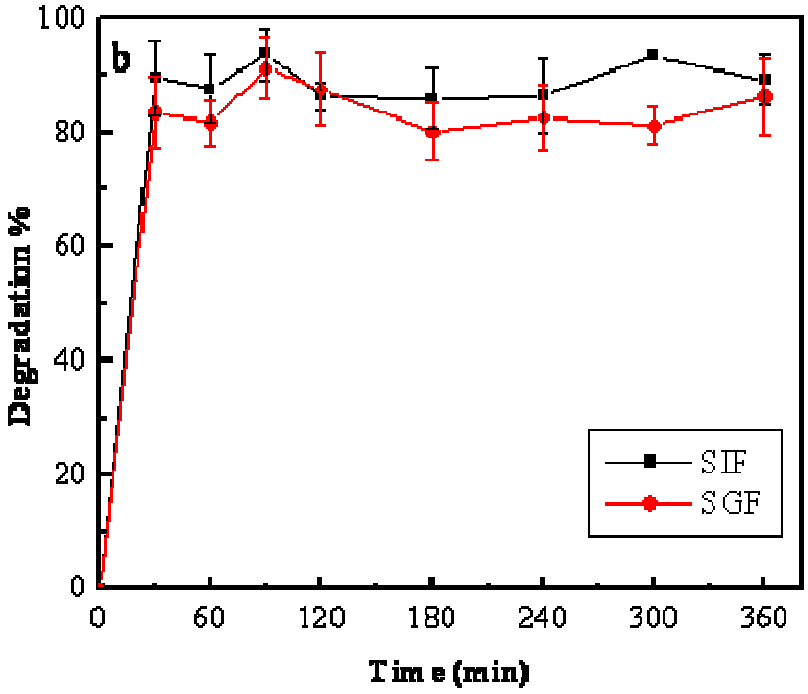
food and drug formulations. The stability of BH microcapsules in aqueous media also indicates that the particle integrity was preserved after passing the microfluidizer, confirming that the porous structure was formed during the spray-drying process. The nature of these barley protein microcapsules to retard β-carotene release in SGF for 2h increases the likelihood of bioactive compounds reaching the intestine for absorption in an intact and active condition. Moreover, the near zero-order release kinetics of β-carotene in SIF in the first 2 hours would enhance their absorption in the small intestine. Similar release profiles were also observed for dry status barley protein microcapsules (data not shown). Comparably, the dried microspheres seemed to show more retarded release than the wet-status microcapsules, which may be attributed to the reinforcement of particle surface after spray drying process. The hydrolysis therefore took more time and finally led to more sustainable release.

3.3.4 In vitro degradation

Protein degradation studies of BGH, BG and BH microcapsules were conducted in SGF and SIF media. Since no soluble protein was detected in pH 2.0 and 7.4 buffers without digestive enzymes according to the control experimental data, only protein degradation profiles in SGF and SIF with enzymes were described in Figure 3-3. Surprisingly, all three types of microcapsules very rapidly degraded in SGF with pepsin and SIF with pancreatin. Most of the protein (65-90%) was converted to soluble protein hydrolysates after 30 min of the test. The degradation curve then levelled off in the following hours. No significant differences in degradation profiles were observed among these three types of

microcapsules (p>0.05). The rapid protein degradation in the simulated GI tract with enzymes indicates that all three types of barley protein microcapsules are vulnerable for digestive enzyme attack, the same as microcapsules based on globular proteins (Chen et al., 2010). The overall protein network degradation and β-carotene release over time were poorly correlated. In spite of rather quick matrix degradation for all three types of barley protein microcapsules, seldom was β-carotene released in SGF within 2 h. Beta-carotene release rates in SIF were much slower than protein matrix degradation rates as well.





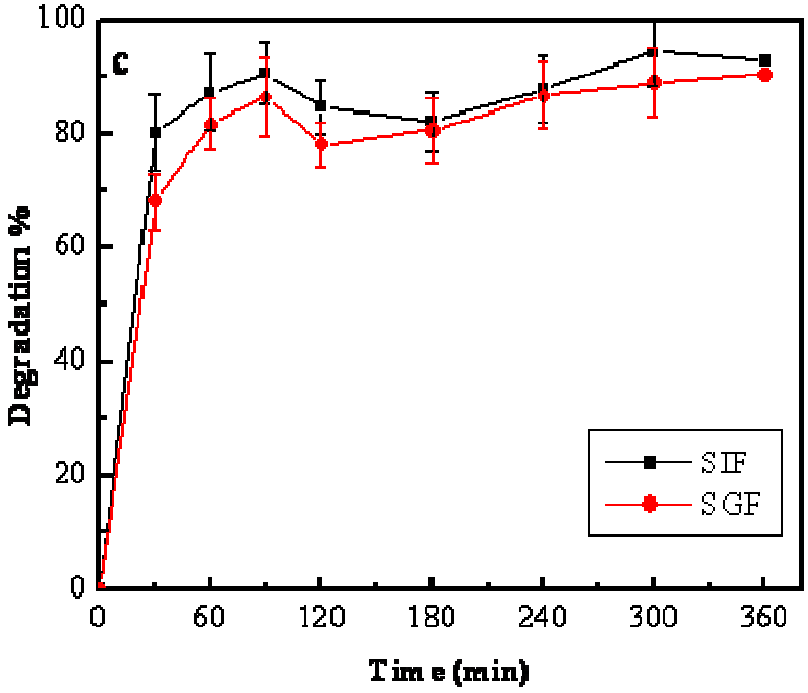


Figure 3-3 Protein degradation profile
(a) BGH, (b) BG and (c) BH microcapsules in the simulated gastric (SGF) and intestinal (SIF) fluids with digestive enzymes.

The degraded barley protein microcapsules were then observed with a TEM after samples had been incubated in simulated GI tract with digestive enzymes. Figure 3-4 shows the morphology changes of BGH microcapsules in SGF and SIF. Nanoparticles with average sizes between 20-30 nm predominated as a result of microcapsule bulk matrix degradation when incubated in SGF for 30 min (Figure 3-4a). After 1 h of incubation, bulk matrices disappeared with mono-dispersed nanoparticles remaining in the release medium (Figure 3-4b). In order to test whether these nanoparticles could be transferred into the simulated intestinal tract without aggregation, their stability was further studied in pH 7.4 buffer without pancreatin. The liberated nanoparticles were still well-dispersed in pH 7.4 buffer within 30 min as observed by TEM (figure not shown). Some aggregation did occur after 2h of incubation in pH 7.4 buffer; however, most of the particles exhibited a size of 50-250 nm (Figure 3-4c). Interestingly, in SIF with pancreatin, both liberated nanoparticles and the original BGH microcapsules were degraded within 6h of incubation, leaving well dispersed nano-emulsions in the SIF medium. Figure 3-4d shows emulsions released from nanoparticles. These released nano-emulsions were probably stabilized by the soluble protein hydyrolysates in the release media, which may improve the absorption of the incorporated β-carotene in the small intestine (Rich et al., 2003). The same phenomenon was observed for BG microcapsules, except a more rapid degradation occurred for BG microcapsules in SGF with pepsin. After 10 min of incubation, the protein matrix disappeared completely and mono-dispersed nanoparticles of 20-30 nm were found in the release medium (figure not shown).

Nanoparticles were also formed from pepsin degradation of the BH microcapsules, however, these nanoparticles aggregated quickly once released, resulting in formation of precipitates.

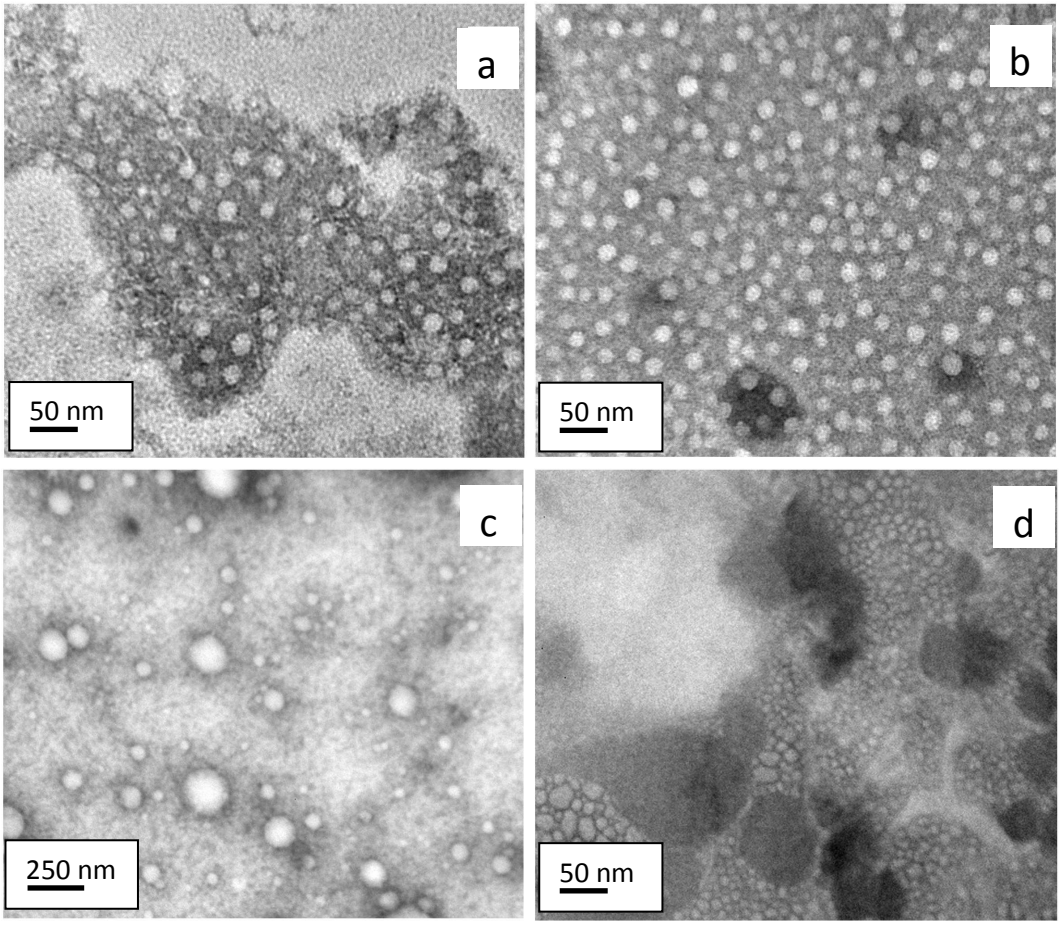


Figure 3-4 Morphology changes of BGH microcapsules in the SGF and SIF by TEM
Nanoparticles observed after incubating microcapsules in SGF with pepsin (a) for 30 min and (b) for 1 h, and nanoparticles incubated in SIF (c) without pancreatin for 2 h, and (d) with pancreatin for 6 h.

This phenomenon of obtaining nanoparticles from the degradation of a protein matrix has never been reported previously. The unique degradation behaviour of barley protein microcapsules is likely related to the special structure of the protein layer directly coating the nanoparticles. This protein resists pepsin

degradation and stabilizes incorporated oil droplets, which explains the limited β-carotene release in SGF. When transferred into SIF, this protein layer was hydrolyzed by pancreatin to release the nano-emulsions containing β-carotene.

3.3.5 Characterization of the protein layer coating nanoparticles

Since only the thin layer of protein directly coating the nanoparticles imparted resistance to pepsin degradation, the structure of this layer of protein may be more important than the protein matrix as a whole in providing resistance against hydrolysis. These surface layers of protein were studied by separating them from the degraded soluble proteins by precipitating the nanoparticles liberated from BGH, BG and BH microcapsules using an ultracentrifuge (labelled as CP_{BGH}, CP_{BG} and CP_{BH}). Their SDS-PAGE patterns were compared with those of the hydrolyzed soluble proteins from BGH, BG and BH microcapsules (SP_{BGH}, SP_{BG} and SP_{BH}), and the original barley glutelin and hordein (Figure 3-5). Four subunits of hordein were identified of molecular weights 85-90; 55-80; 30-50 and < 15 KDa, corresponding to D, C, B and A hordeins, respectively (Figure 3-5 line b). B (S-rich) and C hordeins (S-poor) are the major components which account for 70–80% and 10-20% of the total, respectively (Celus et al., 2006). The 85-90 KDa molecular weight band was weak, suggesting that only a small portion of D hordeins were extracted when ethanol was used as the sole extraction agent. D-hordeins are regarded as the high molecular weight storage proteins of barley consisting of polypeptides linked together with intermolecular disulfide bonds (Celus et al., 2006). They can be extracted by alcohol solution in the presence of a high concentration of reducing reagent to break the inter-chain disulfide bonds.

The barley glutelin fraction showed four major bands at molecular weights of 85-90; 35-55; 25 and <20 KDa, respectively (Figure 3-5 line a). The band at 85-90 KDa was assigned to D-hordeins, which have limited solubility in ethanol alone, but can be extracted in alkaline solution. The broad band at 35-55 KDa may be a contamination of B-hordeins in the glutelin fraction, because it was not possible to prepare an undenatured glutelin fraction free of hordein contamination (Celus et al., 2006). In general, barley glutelin has not been investigated as extensively as hordein, thus information about its subunits is limited. All the major bands disappeared in the SDS-PAGE patterns of the hydrolyzed soluble proteins after incubating BGH, BG and BH microcapsules in SGF with pepsin for 2 h. Instead, new broad bands appeared at the bottom of lane (Figure 3-5, line c-e), confirming that most of the proteins in BGH, BG and BH microcapsules were rapidly hydrolyzed to form peptides with molecular weight smaller than 10 KDa. The SDS-PAGE patterns for CP_{BGH}, CP_{BG} and CP_{BH} showed a broad band at 40-50 KDa (Figure 3-5, line f-h). This confirms that the protein layers coating on nanoparticles can resist pepsin degradation when incubated in SGF. According to SDS-PAGE patterns, these protein layers could be one part of B-hordein subunits or peptides resulting from partial hydrolysis of C or D-hordeins that were resistant to further pepsin digestion in SGF.

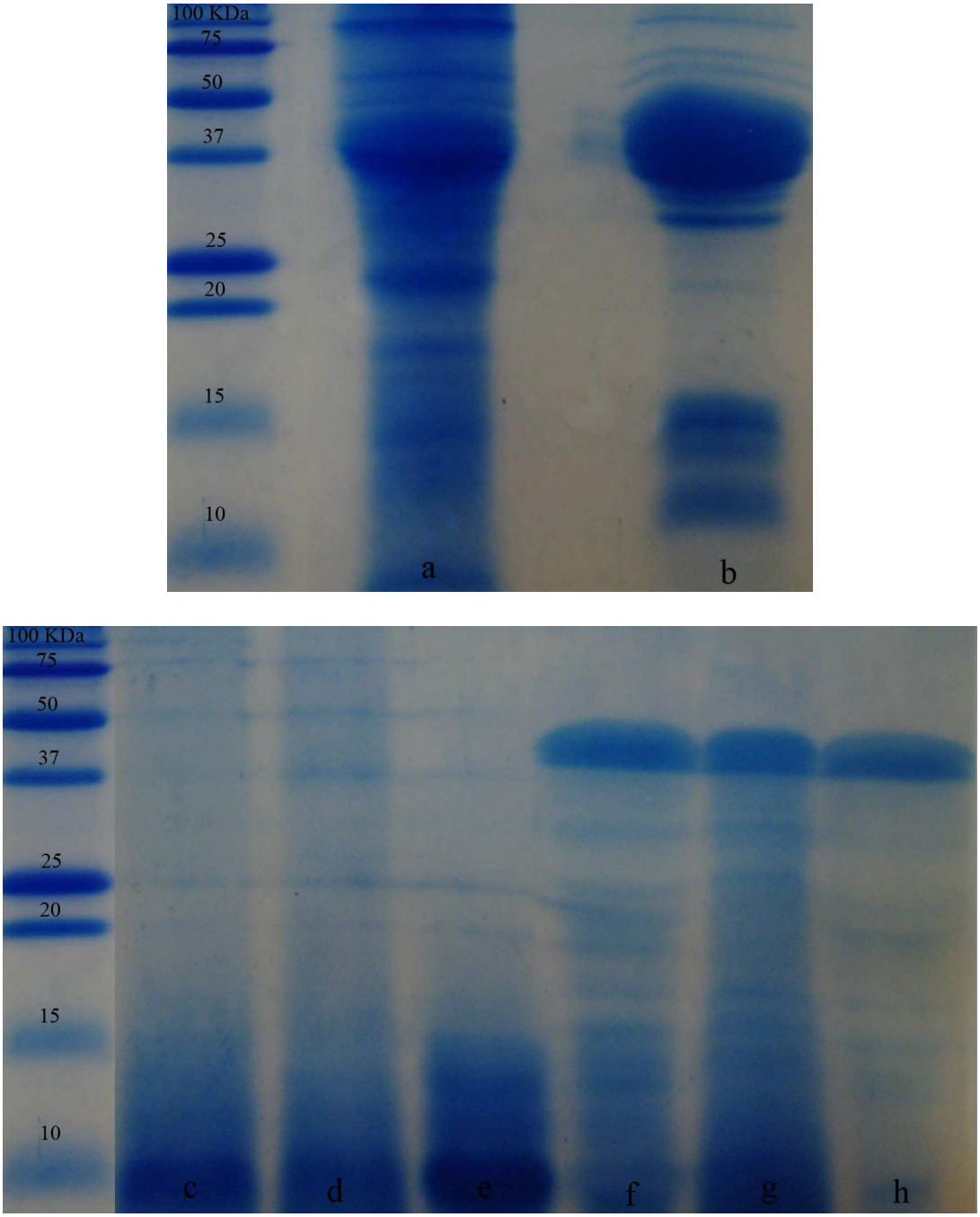


Figure 3-5 SDS-polyacrylamide gel electrophoresis
(a) barley glutelin, (b) barley hordein, and (c) SP_{BGH}, (d) SP_{BG}, (e) SP_{BH} after incubating BGH, BG and BH microcapsules in SGF with pepsin for 2 h, as well as (f) CP_{BGH}, (g) CP_{BG} and (h) CP_{BH} after incubating above microcapsules in SGF with pepsin for 2 h.

For a further structural understanding of these protein coating layers, their amino acid compositions were determined (Table 3-2). CP_{BGH}, CP_{BG} and CP_{BH} possessed obviously different amino acid compositions. Since glutamic acid (glutamine) and proline are two of the most abundant amino acids in both barley hordein and glutelin, the percentage of these two amino acids in CP_{BGH}, CP_{BG} and CP_{BH} were in proportion to their hordein and glutelin content. The CP_{BG} possessed 19.58% glutamine acid (glutamine) and 14.67% proline, similar to the amino acid composition of glutelin. CP_{BGH} and CP_{BH} possessed 34.75-39.45% glutamic acid (glutamine) and 29.15-31.19% proline, similar to the amino acid composition of hordein. This means that the protein layer coating on nanoparticles liberated from degradation of the BG matrix is probably mainly composed of subunits from barley glutelin, whereas, the protein layers coating on nanoparticles liberated from degradation of the BGH and BH matrix are probably mainly composed of subunits from barley hordein.

Table 3-2 Amino acid composition of the hordein, glutelin and protein coatings on nanoparticles liberated from BGH, BG and BH microcapsules (%)

Amino acid	Hordein	Glutelin	CP_{BGH}	CP_{BG}	CP_{BH}
asx	1.47	3.05	3.57	7.79	1.75
ser	5.44	13.11	4.35	7.00	3.23
glx	34.32	15.72	34.75	19.58	39.45
gly	2.68	7.88	4.44	8.68	1.65
his	0.89	1.93	0.78	1.08	0.30
arg	3.86	6.03	1.83	3.17	1.49
thr	2.30	4.31	2.65	4.12	1.24
ala	2.95	6.28	2.37	4.88	1.11
pro	21.13	11.87	29.15	14.67	31.19
cys	2.05	1.73	0.37	1.38	0.84
tyr	2.54	3.15	3.85	4.61	3.53
val	4.21	5.33	2.09	3.91	1.28
met	0.21	0.00	0.30	0.41	0.28
lys	0.84	3.94	1.09	1.95	0.39
ile	3.66	3.90	4.11	5.34	2.99
leu	6.37	7.66	3.79	6.21	2.41
phe	5.07	4.10	0.52	5.21	6.90

The order of amino acids listed in the table follows the elution order of the amino acids from the reversed-phase HPLC chromatographic column.

3.3.6 Nanoparticle formation mechanism discussion

In barley protein microcapsule preparation process, it is deduced that protein subunits in hordein or glutelin compete to adsorb the hydrophobic oil droplets during the pre-emulsion step. Upon high pressure treatment, these coated oil droplets aggregate to form larger granular particles, which are subsequently entrapped in a microcapsule matrix. In SGF, the bulk microcapsule matrices are rapidly degraded by pepsin. However, the protein layer directly contacting oil

droplets is resistant to pepsin digestion, leading to the liberation of nanoparticles incorporating β-carotene.

Two main reasons may explain at least part of this interesting property of degradation resistance. Firstly, proteins with high proline content are generally more resistant to degradation by digestive enzymes (Simpson, 2001). The proline content of CP_{BGH} and CP_{BH} was significantly higher than barley hordein, and also for CP_{BG} compared to glutelin. Secondly, the majority of pepsin-labile hydrophobic amino acid groups on protein chains were likely buried inside the matrix, leaving hydrophilic groups outside. CP_{BGH}, CP_{BG} and CP_{BH} all formed thin films with the hydrophobic amino acid residues in contact with the oil phase to stabilize the emulsions, so CP_{BGH}, CP_{BG} and CP_{BH} layers represented a less vulnerable substrate to pepsin digestion (Chen and Subirade, 2006; Morr and Ha, 1993). The slower bulk matrix degradation of BGH and BH microcapsules in SGF with pepsin compared to BG microcapsules can also be attributed to a higher proline content in hordein than that in glutelin. The aggregation of nanoparticles coated with CP_{BH} may be related to the “dough formation” property of hordein. Once librated from the BH microcapsule matrix, the CP_{BH} coating tended to aggregate, resulting in inter-particle bridges that finally led to caking and particle collapse. Extensive protein coating aggregation between adjacent emulsion droplets also could lead to coating rupture. This may explain the higher release of β-carotene from BH microcapsules after 3h of incubation in SGF with pepsin.

In SIF, the liberated nanoparticles remained well-dispersed within 30 min of incubation. Although some aggregation occurred afterwards, most of the particles

were in the range of 50-200 nm. It is expected that these nanoparticles would adhere to the intestinal mucosa owing to their submicron size which would prolong the particles' intestinal residence time. These nanoparticles are degraded in SIF by pancreatin which is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas (amylase, lipase and proteases). These enzymes could breakdown the protein-lipid and protein-protein interactions and therefore interrupt protein aggregation structures in the micro-particle and nano-particle matrices. Thus, nano-emulsions incorporating β-carotene were gradually formed during 6h of the test.

3.4 Conclusion

This research is the first to report that nano-encapsulations were formed as a result of enzymatic degradation of barley protein microcapsule bulk matrix in a simulated gastric tract. These nano-encapsulations delivered β-carotene to a simulated human intestinal tract intact, where they were degraded by pancreatic enzymes and steadily released the β-carotene. This *in vitro* system shows potential to facilitate lipophilic bioactive compound absorption in the human digestive tract, which needs to be proven in future *in vivo* experiments. The uniquely structured barley protein matrix microcapsules do not aggregate during storage or in harsh human gastric conditions. Additionally, they can be prepared by a simple and convenient process without the addition of organic solvents or surfactants. Compared to traditional submicron oil-in-water emulsions stabilized by surfactants and/or polymers (Simovic and Prestidge, 2007), these nanoparticle-coated emulsions may offer superior potential to serve as controlled release

systems for delivery of lipophilic bioactive compounds in the pharmaceutical and food industries.

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Chapter 4 General Discussions and Conclusion

4.1 A summary of research results

We successfully developed barley protein microcapsules incorporating lipophilic bioactive compounds (fish oil, β-carotene) by a pre-emulsifying process followed by a high pressure homogenization treatment. The optimal conditions for microencapsulation formation were determined to be 15% protein concentration and 1:1 oil/protein ratio. The formed microcapsules can be converted into white, free-flowing powders by a spray-drying process at the optimum inlet temperature of 150ºC. The formed microcapsules demonstrated a spherical shape and a smooth surface with a diameter ranging from 1 to 5μm as observed by the scanning electron microscope (SEM). Unlike hydrophilic proteins which usually form a thin layer membrane around oil droplets that stabilize the emulsion, barley protein can form solid coatings to entrap lipophilic compounds after passing a high pressure homogenizer, so that no solidification reagent or process was needed. The spray drying is therefore used as a drying method to turn wet-status microcapsules into dry powders. The microcapsules demonstrated multiple emulsions like inner structure with oil droplets well distributed/separated within the matrix. A high oil carrying capacity (encapsulation efficiency, 93-97%; loading efficiency, 46-49%) was observed for all well designed microcapsules. Lower moisture content was also noticed for spray dried barley protein microcapsules compared to spray dried whey protein microcapsules. The

microcapsule preparation process is simple, convenient, solvent friendly (no organic solvents or surfactants) and is able to undergo continuous production.

4.1.1 Results for protective ability

Oxidative stability of the encapsulated fish oil was studied in accelerated storage tests as well as in real food formulations (milk and yogurt) with the purpose of revealing the protective ability of barley protein matrix. For dry status microcapsules, all encapsulated fish oil demonstrated desirable oxidative stability. The relatively higher initial PV values observed for all encapsulation systems might be attributed to the oxidation of the surface/near surface oil of the matrix. Comparably, barley protein microcapsules provide better protective ability against fish oil oxidation than whey protein microcapsules, which might be due to their less surface/near surface oil and the lower moisture content. Among barley protein based microcapsules, the *D*-BG1H2 matrix demonstrated the best protective ability and generally, those containing high content of hordein all exhibited the lowest PV values suggesting that hordein may play an important role in preventing oil oxidization. For wet status microcapsules, all microcapsules verified extremely low oxidation levels of the encapsulated oil (PV < 30 meq peroxide/kg oil) compared to dry microcapsules after an 8 week treatment. This phenomenon suggests barley protein microcapsules (wet-status) might work as efficient carriers in liquid/semi-liquid food systems. Oxidative stability of encapsulated oil in real food formulation (milk and yogurt) was also studied. The *W*-BG1H2 and *W*-BWG systems confirmed desirable stability (PV < 20 meq peroxide/kg oil) especially for those in yogurt formulation in which the PV level

is below 5 meq peroxide/kg oil even after 5 weeks of storage. It has been recommended that PV levels shouldn't exceed 30 meq peroxide/kg oil in an edible food product (Naohiro and Shun, 2006). Thus, the above result proves the potential of barley protein microcapsules being used in liquid and semi-liquid food systems.

4.1.2 Results for controlled release

The release properties of barley protein microcapsules were investigated in the simulated gastric and intestinal fluids. For wet status microcapsules, nutrient was seldom released in simulated gastric fluid (SGF) with pepsin, while in simulated intestinal fluid (SIF) with pancreatin, nutrient was steadily and almost completely released during 6h. The desirable release profile for wet status microcapsules was found to be associated with barley protein matrix degradation profiles. After incubating barley protein microcapsules in SGF for 30 min, nano-encapsulations (20-30nm) were formed as a result of pepsin degradation of barley protein microcapsule bulk matrix. These nano-encapsulations delivered β-carotene to a simulated human intestinal tract intact, where they were degraded by pancreatic enzymes and steadily released the β-carotene. The *in vitro* data shows potential of using barley protein microcapsules to facilitate lipophilic bioactive compound absorption in the human digestive tract, which needs to be proven in future *in vivo* experiments. These uniquely structured barley protein matrix microcapsules do not aggregate during storage or in harsh human gastric conditions. Compared to traditional submicron oil-in-water emulsions stabilized by surfactants and/or polymers, the formed nanoparticle-coated emulsions from

micro-matrix degradation offer superior potential to serve as controlled release systems for delivery of lipophilic bioactive compounds in the pharmaceutical and food industries. Similar release profiles were found for dry status barley protein microcapsules, however no further research has been done so far, referring to their release mechanisms. Comparably, the dried microspheres seemed to show more retarded release than the wet-status microcapsules which might be attributed to the reinforcement of particle surface during the spray drying process.

4.2 Discussions and conclusions

Food proteins have already been widely used to prepare a wide range of matrices alone or in combination with other polymers in the form of hydrogel, micro- or nanoparticles, with the purpose to incorporate nutraceuticals to develop innovative functional food products. The most commonly used protein for encapsulating food ingredients is milk (or whey) proteins (Kagami et al., 2003; Rosenberg and Sheu, 1996). Other animal proteins such as gelatin, casein, and some plant proteins such as soy protein, zein and wheat proteins have also been developed as efficient coating materials (Ezpeleta et al., 1996; Latha et al., 2000; Lazko et al., 2004; Liu et al., 2005; Paynea et al., 2002; Swatscheka et al., 2002). Clear advantages of food protein matrices include high nutritional value, abundant renewable sources, and acceptability as naturally occurring food components degradable by digestive enzymes. Some limitations, however, still exist. For example, (a) the cross-linking reagent that is usually needed for coating solidification, may lead to toxic side effects, (b) the sustainable release might not be successfully achieved by hydrophilic protein matrix due to their high

permeability in aqueous environments and (c) high oxygen permeability was found in some cases for wall material comprised of only protein. As one type of hydrophobic protein, barley proteins, comparably, reveal promising potential of being used as protective vehicles for nutraceutical delivery. The demonstrated advantages include:

(1) No cross-linking reagents were needed in the microcapsule preparation process, since self-sustainable-solid-coating was able to form after high pressure emulsifying process.

(2) The high hydrophobic nature of barley protein matrix lead to its favorable protective ability against fish oil oxidation and the formed microcapsules were especially suitable to be used in liquid/semi-liquid food system.

(3) The hydrophobicity together with its unique structure also resulted in a limited nutraceutical release behavior in the simulated gastric fluid (SGF) but a sustainable controlled release in the simulated intestinal fluid (SIF).

(4) Nano-encapsulations (20-30nm) were observed as a result of enzymatic degradation of microcapsule bulk matrix in the simulated gastric tract. These nano-encapsulations delivered nutraceuticals to a simulated human intestinal tract intact, where they were degraded by pancreatic enzymes and steadily released the nutraceuticals.

Further research, however, is still needed for confirming the oxidative stability of various encapsulated nutraceuticals in a barley protein matrix.

Meanwhile, *in vivo* experiments are needed as well, to verify the potential of the barley protein matrix in facilitating lipophilic bioactive compound absorption in the human digestive tract. Furthermore, greater fundamental understanding of protein–protein and protein–nutraceutical interactions at the molecular level and their impact on the functionality of proteins is also required to ensure ideal design of these nutraceutical carriers for use in the food/pharmaceutical industry.

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