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Nano-encapsulations liberated from barley protein microparticles for oral delivery of bioactive compounds

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ABSTRACT

Novel microparticles (3–5 μm) were created by pre-emulsifying barley proteins with a homogenizer followed a microfluidizer system. These microparticles exhibited a high oil carrying capacity (encapsulation efficiency, 93–97%; loading efficiency, 46–49%). Microparticle degradation and bioactive compound release behaviours were studied in the simulated gastro-intestinal (GI) tract. The data revealed that nano-encapsulations (20–30 nm) were formed as a result of enzymatic degradation of barley protein microparticle 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 microparticles may provide a new strategy for the nutraceutical and pharmaceutical industries to develop targeted delivery systems for lipophilic bioactive compounds.

1. Introduction

Oral administration is by far the most convenient way for the 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 capping agents and/or tailoring the particle surface charges to create separation through electrostatic repulsion (Elbadawy et al., 2010; Medina-Ramıírez 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 excel-

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lent emulsifying and film-forming properties of two major barley protein fractions: hordein and glutelin (Wang et al., 2010).

Our objective was to develop barley protein-based emulsion microparticles for the 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 paper describes the preparation, characterization, and evaluation of emulsion microparticles based on barley glutelin and hordein. Microparticle 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.

2. Materials and methods

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 U/mg) and pancreatin (from porcine pancreas) were purchased from Sigma–Aldrich Canada Ltd (Oakville, ON, Canada). All other chemicals were of reagent grade.

2.2. Microparticle preparation

Three types of emulsion microparticles 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 microparticles 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). Microparticles 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 microparticles were stored at 4 °C with 0.025% (w/v) sodium azide until used *in vitro* for release and degradation studies. A portion of the microparticles were spray-dried using a mini-spray dryer (Büchi 190 Mini Spray Dryer, Büchi Labortechnik, Flawil, Switzerland) at an air inlet temperature of 150 °C and an air outlet temperature of 55–65 °C for oil payload evaluation and particle morphology observation. The prepared samples were coded as BG, BH and BGH, corresponding to microparticles prepared from glutelin, hordein and their 1:1 (w/w) mixture, respectively.

2.3. Microparticle characterizations

The size of the microparticles 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 microparticle 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 microparticles was observed with a scanning electron microscope (SEM, S-2500, Hitachi, Tokyo, Japan) operating at 15 kV. The surfaces of the microparticles were sputtered with gold, observed and photographed. The powders were also fractured carefully after frozen in liquid nitrogen, and the interior morphology of the microparticles was studied and photographed using the SEM (Xu et al., 2007). The interior morphology of the wet microparticles was also observed using a transmission electron microscope (TEM, JEOL 2100 EX, Tokyo, Japan) at an accelerating voltage of 120 kV. Microparticles 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 °C for 48 h. The ultrathin section was stained in 2% uranyl acetate and 0.2% lead acetate and viewed and photographed (Leung et al., 2005).

2.4. Oil payload in the microparticle

Extraction of oil from barley protein microparticles was based on the method described by Beaulieu et al. (2002). The spray-dried microparticles (250 mg) were precisely weighed to the nearest 0.1 mg and added into 5 ml 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 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 1 h. After centrifugation (Beckman Coulter Avanti® J-E Centrifuge, CA, USA) at 8000 × *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: $EE\,(\%) = W_{\text{encapsulated oil}}/W_{\text{total oil}} \times 100$; where $W_{\text{encapsulated oil}}$ represents the weight of oil encapsulated in the microparticles and $W_{\text{total oil}}$ represents the oil added initially in the particle formation mixture. $LE\,(\%) = W_{\text{encapsulated oil}}/W_{\text{microparticles}} \times 100$; where $W_{\text{microparticles}}$ represents the weight of the microparticles encapsulating the oil inside.

2.5. In vitro release

Release profiles of the microparticles 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, microparticle 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 °C. Each tube contained ~250 mg (dry weight) microparticles and 25 ml release medium. The tubes were withdrawn at different time intervals. Digestive enzymes were inactivated by heating the release medium at 95 °C for 3 min. Quantitative analysis of the released β-carotene was based on the colorimetric method of Pan et al. (2007). The β-carotene content in the hexane was determined by measuring the absorbance at 450 nm with a UV–visible spectrophotometer (model V-530, Jasco, CA, USA). Blank SGF and SIF solutions were run as zero controls.

Beta-carotene release data were also fitted to the following equations using regression analysis.

$$\text{Zero-order equation} \frac{dM_t}{dt} = k \quad (1)$$

where k is the constant, t is time, and M_t is the amount of β-carotene released at time t.

$$\text{First-order equation} \frac{dM_t}{dt} = k(M_0 - M_t) \quad (2)$$

where k is the constant, t is time, M_0 and M_t are the amounts of β-carotene release at time 0 and t.

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 °C. The supernatants were filtered through Whatman No. 1 filter paper and the filtrates were then freeze-dried (FreeZone 6 Litre Console Freeze Dry System, Labconco Corporation, Kansas City, MO, USA) and weighed to the nearest 0.1 mg. Blank SGF and SIF with digestive enzymes were also run as controls. The percent protein matrix degradation was calculated by the following equations: degradation (%) = $W_{\text{degraded protein}}/W_{\text{microparticle protein}}$; where $W_{\text{degraded protein}}$ represents the weight of degraded soluble protein in the release medium, and $W_{\text{microparticle protein}}$ represents the total protein in the microparticles. Changes in microparticle 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. The size of the liberated nanoparticles was also determined using the Zetasizer NanoS instrument under the same conditions as indicated above.

2.7. Characterization of the protein layer stabilizing nano-encapsulations

After the *in vitro* degradation of BGH, BG and BH microparticles in SGF with pepsin (Section 2.6), the liberated nanoparticle precipitates were isolated using an ultra-centrifuge at 50,000 × *g* for 25 min at 4 °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 °C for 5 min. After cooling, 12 μl samples (5 mg/ml) were loaded on 5% stacking gel and 15% separating gel and then subjected to electrophoresis at a constant voltage of 75 V. 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. (1976) with slight modifications. Glass sample tubes (6 mm × 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 °C for 24 h, 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 254 nm. A reversed-phase AccQ.Tag 150 mm × 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 1.5 ml/min. Data acquisition was controlled by ChemStation software.

2.8. Statistical analysis

Each type of microparticle was prepared in three independent batches. The microparticle 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 microparticle, 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. Results and discussion

3.1. Microparticle preparation

The emulsifying-stabilization process is widely used to prepare globular protein (whey and soy protein) based microparticles. 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 microparticles 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 microparticles. 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). Plus, a high oil/protein ratio generally leads to a high carrying capacity of the final microencapsulation products. 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 microparticle formation. Further increasing the protein concentration and oil/protein ratio led to the formation of aggregated sticky substances, rather than well dispersed microparticles. Therefore, a protein concentration of 15% and oil/protein ratio of 1:1 were applied to prepare BGH, BG and BH microparticles 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 0.5 M NaOH at 23 °C. The dispersion pH was then reduced to 7 by adding 0.5 M 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 microparticles, 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.

3.2. Microparticle characterization

All three types of microparticles can be converted into white and free-flow powders by spray-drying. SEM photographs of the spray-dried BGH, BG and BH microparticles are shown in Fig. 1. These particles demonstrated diameters ranging from 3 to 5 μm with a spherical shape; however, their surface morphology differed. BGH and BG microparticles were dense, crack-free and possessed smooth surfaces (Fig. 1a and b) with many small pores homogeneously distributed inside (Fig. 1d). A porous structure was observed for BH microparticles (Fig. 1c). As revealed in our previous work, hordein forms a 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 microparticles indicate that oil droplets were well separated within the protein micron-matrix. Similar surface morphology of BGH microparticles and BG microparticles suggests that the coating wall surface was mainly composed of glutelin, forming a dense outside structure that prevented hordein ballooning. Fig. 1e shows the internal morphology of the BGH microparticle 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 1 μm. These granules then associated to form a microparticle. BG and BH microparticles (without spray-drying) showed a similar interior morphology compared to BGH microparticles (figures not shown). Such dense, cracks-free surface features and interior microparticle-coating-granule structures may allow BGH and BG microparticles to better withstand mechanical stresses and protect the incorporated ingredients against harsh environments (e.g. oxidation, low or high pH).

Table 1
Encapsulation efficiency (EE) and loading efficiency (LE) for barley protein microparticles.

Microparticles	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

3.3. Microparticle 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 1, three types of barley protein microparticles 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 microparticles. This is probably due to barley protein's excellent emulsifying properties (Wang et al., 2010) as well as its capacity to form solid microparticle-coating-granule structures during high pressure treatment which means that the oil droplets are retained inside the particle matrix during the particle formation and spray-drying processes. In spite of their porous structure, BH microparticles demonstrated similar EE and LE values to those of BGH and BG microparticles. This suggests that the BH matrix may bind oil droplets, which prevents their leakage during the spray-drying process.

The release properties of the three types of microparticles 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 microparticles without digestive enzymes in pH 2.0 and 7.4 buffers, indicating that the integrity of the microparticles was well maintained. Thus only β-carotene release profiles in SGF with pepsin and SIF with pancreatin were described in Fig. 2. In SGF with pepsin, β-carotene was slowly released from BGH microparticles and less than 5% β-carotene was detected in the release medium after 2 h of the test. This corresponds to the usual time for food and drugs to pass through the stomach to the small intestine (Fig. 2a). Even after 6 h of the test, only 11.3% β-carotene was released. Interestingly, in SIF with pancreatin, β-carotene was steadily released from the BGH microparticles at almost zero-order release kinetics ($r^2 = 0.97$) in the first 2 h. Over time the release curve levelled off gradually, until after 6 h when 91.6% of the β-carotene had been released. Similar β-carotene release profiles were observed for BG and BH microparticles in the simulated GI tract, except for BH microparticles in SGF with pepsin, where β-carotene release rates increased rapidly after 3 h and 60.5% of the β-carotene was released after 6 h (Fig. 2b and c). The release of β-carotene from BH and BG microparticles in SIF also followed the near zero-order release in the first 2 h ($r^2 \geq 0.97$).

All three types of microparticles 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, microparticles 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 microparticle suspensions for more than six months at 4 °C in different pH buffers (without digestive enzymes). This storage test demonstrates their excellent potential to encapsulate

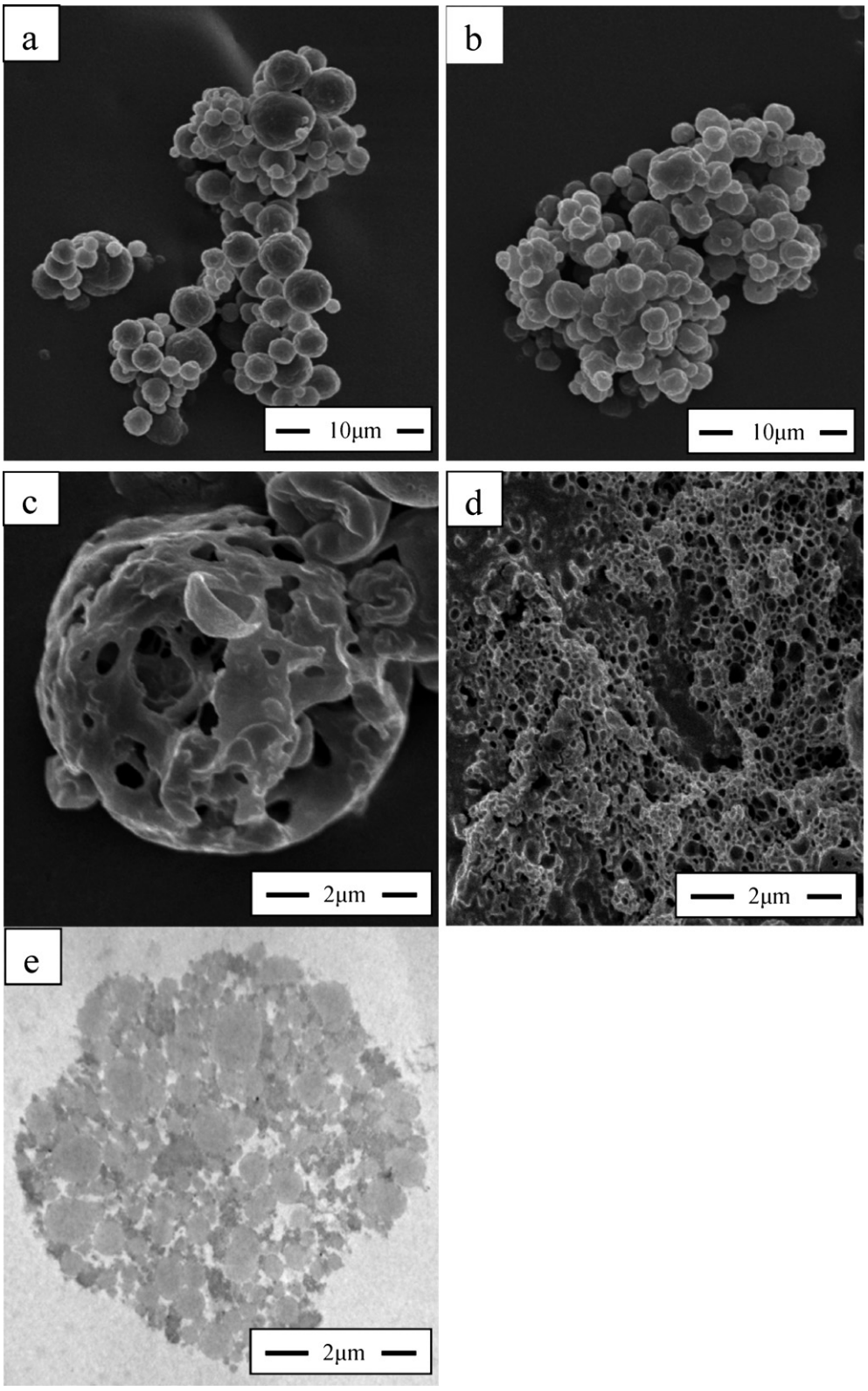

Fig. 1. Surface morphology of spray-dried microparticles prepared from (a) BGH, (b) BG, (c) BH, and (d) interior morphology of spray-dried BGH microparticles by SEM, as well as (e) interior morphology of BGH microparticles (without spray-drying) by TEM.

bioactive compounds for use in liquid and semi-liquid food and drug formulations. The stability of BH microparticles 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 microparticles to retard β-carotene release in SGF for 2 h 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 h would enhance their absorption in the small intestine.

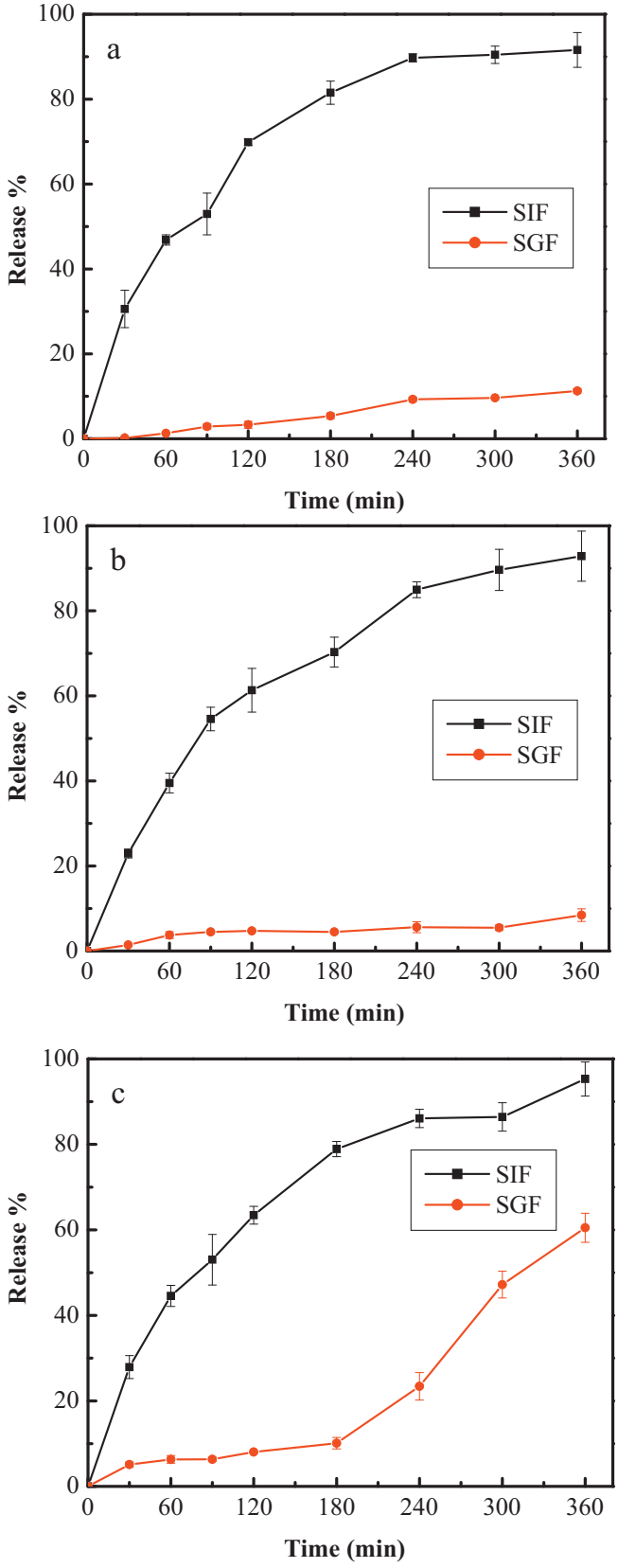

Fig. 2. Release profile of β-carotene in the simulated gastric (SGF) and intestinal (SIF) fluids with digestive enzymes from (a) BGH, (b) BG and (c) BH microparticles.

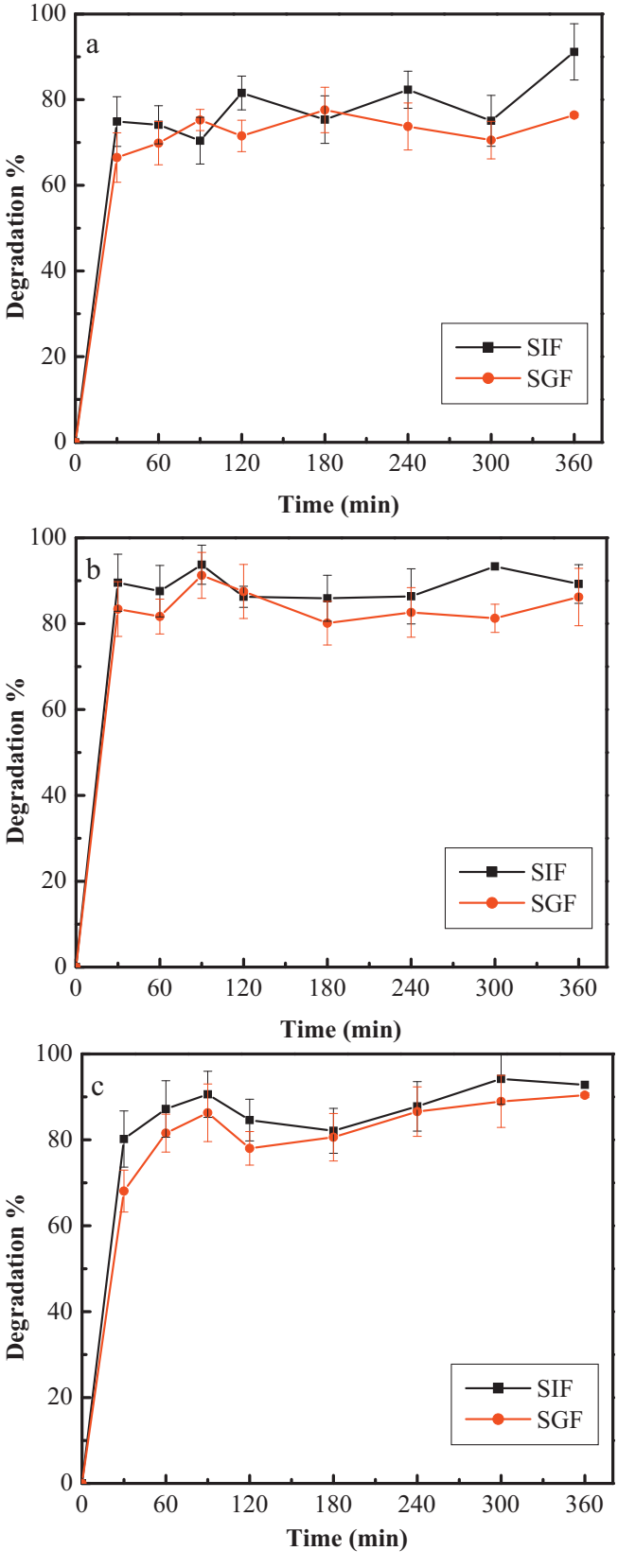

Fig. 3. Protein degradation profile of (a) BGH, (b) BG and (c) BH microparticles in the simulated gastric (SGF) and intestinal (SIF) fluids with digestive enzymes.

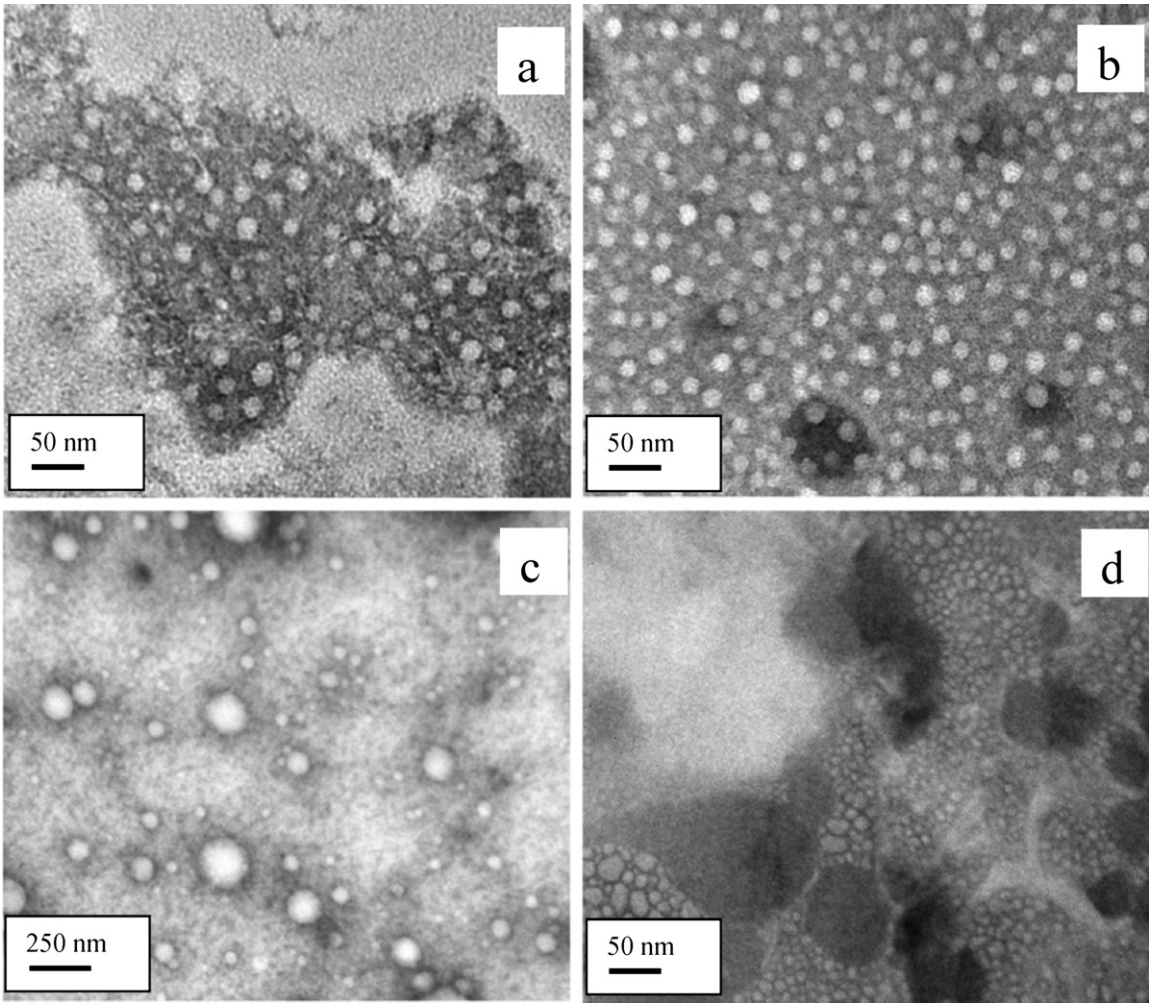

Fig. 4. Morphology changes of BGH microparticles in the SGF and SIF by TEM: nanoparticles observed after incubating microparticles in SGF with pepsin (a) for 30 min and (b) for 1 h, and nanoparticles incubated in SIF (c) without pancreatin for 2 h, and (d) with pancreatin for 6 h.

3.4. *In vitro degradation*

Protein degradation studies of BGH, BG and BH microparticles 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 Fig. 3. Surprisingly, all three types of microparticles 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 microparticles ($p > 0.05$). The rapid protein degradation in the simulated GI tract with enzymes indicates that all three types of barley protein microparticles are vulnerable for digestive enzyme attack, the same as microparticles 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 microparticles, seldom was β-carotene released in SGF within 2 h. Beta-carotene release rates in SIF were much slower than protein matrix degradation rates.

The degraded barley protein microparticles were then observed with a TEM after samples had been incubated in simulated GI tract with digestive enzymes. Fig. 4 shows the morphology changes of BGH microparticles in SGF and SIF. Nanoparticles with average sizes between 20 and 30 nm predominated as a result of microparticle bulk matrix degradation when incubated in SGF for 30 min (Fig. 4a). After 1 h of incubation, bulk matrices disappeared with monodispersed nanoparticles remaining in the release medium (Fig. 4b). The changes in the microparticles were also verified by Zetasizer analysis. A unimodal distribution with a peak of 115 ± 0.5 nm was obtained for BGH microparticles after incubating in SGF for 1 h, confirming the degradation of the bulk matrices and liberation of the nanoparticles. The smaller size observed from SEM can be attributed to shrink of the particles during drying process for microscopic observation. 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 2 h of incubation in pH 7.4 buffer; however, most of the particles exhibited a size of 50–250 nm (Fig. 4c). Zetasizer measurement showed a size of 140 ± 6 nm for these nanoparticles in pH 7.4 buffer. Interestingly, in SIF with pancreatin, both liberated nanoparticles and the original BGH microparticles were degraded within 6 h of incubation, leaving well dispersed nano-emulsions in the SIF medium. Fig. 4d shows emulsions released from nanoparticles. These released nano-emulsions were probably stabilized by the soluble protein hydrolysates in the release media, which can improve

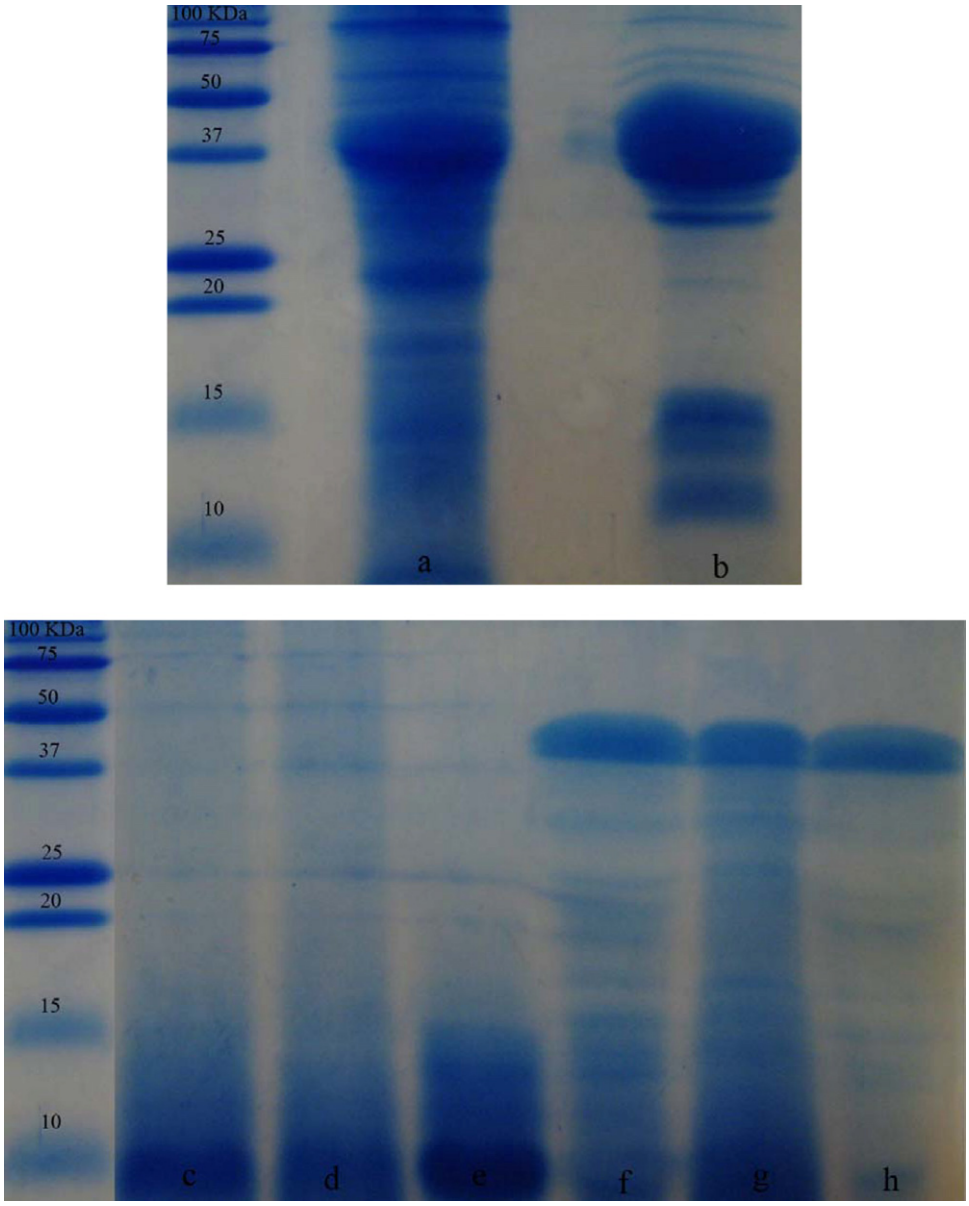

Fig. 5. SDS-polyacrylamide gel electrophoresis of the (a) barley glutelin, (b) barley hordein, and (c) SP_{BGH}, (d) SP_{BG}, (e) SP_{BH} after incubating BGH, BG and BH microparticles in SGF with pepsin for 2 h, as well as (f) CP_{BGH}, (g) CP_{BG} and (h) CP_{BH} after incubating above microparticles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

the absorption of the incorporated β-carotene in the small intestine (Rich et al., 2003). The same phenomenon was observed for BG microparticles, except a more rapid degradation occurred for BG microparticles 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 microparticles, however, these nanoparticles aggregated quickly once released, resulting in the formation of precipitates.

This phenomenon of obtaining nanoparticles from the degradation of a protein matrix has never been reported previously. This unique degradation behaviour of barley protein microparticles 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.

3.5. 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 microparticles 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 microparticles (SP_{BGH}, SP_{BG} and SP_{BH}), and the original barley hordein and glutelin (Fig. 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. 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

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

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.

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 are 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. 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 contaminating hordein (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 microparticles in SGF with pepsin for 2 h. Instead, new broad bands appeared at the bottom of lane (Fig. 5c–e), confirming that most of the proteins in BGH, BG and BH microparticles 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 (Fig. 5f–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.

For a further structural understanding of these protein coating layers, their amino acid compositions were determined (Table 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.

3.6. Nanoparticle formation mechanism discussion

In barley protein microparticle 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 microparticle matrix. In SGF, the bulk microparticle 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 microparticles in SGF with pepsin compared to BG microparticles can also be attributed to a higher proline content in hordein than 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 microparticle 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 release of β-carotene from BH microparticles after 3 h 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 released during the 6 h of the test.

4. Conclusion

This research is the first to report that nano-encapsulations were formed as a result of enzymatic degradation of barley protein microparticle 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. These uniquely structured barley protein matrix microparticles 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 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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