Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 65 (2007) 354–362
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Effect of preparation conditions on the nutrient release properties of alginate–whey protein granular microspheres

Lingyun Chen, Muriel Subirade *

Institut de recherche sur les nutraceutiques et les aliments fonctionnels (INAF/STELA), Université Laval, Sainte-Foy, Canada

Received 28 June 2006; accepted in revised form 5 October 2006
Available online 21 October 2006

Abstract

The effect of preparation conditions on nutrient release from alginate (AL)–whey protein isolate (WPI) granular microspheres obtained by an emulsification/internal cold gelation method was studied by varying WPI/AL ratio, microsphere diameter, total polymer concentration and riboflavin loading. Microsphere size distribution and nutrient encapsulation efficiency (EE) were examined. Riboflavin release profiles were investigated in simulated gastric and intestinal fluids. Values for EE above 80% were obtained for most microspheres, with the notable exceptions of high AL or pure AL. Variations in WPI/AL ratio, granule size and nutrient loading have major impact on nutrient release. Microspheres prepared with a WPI/AL ratio of 8:2, a riboflavin concentration of 1% in the initial aqueous phase and diameters near 94 μm retained the vitamin in SGF and released it in SIF. By careful process design, granular microspheres with potential as oral delivery vehicles for bioactive compounds may be developed.

Keywords: Microspheres; Alginate; Whey protein; Oral administration; Nutrient release

1. Introduction

The incorporation of bioactive compounds such as peptides and vitamins into food systems can provide a simple way to develop innovative functional foods that may have physiological benefits or reduce susceptibility to disease [1]. However, oral administration of these molecules is currently an inefficient means of delivery, due to low permeability and/or solubility within the gut and lack of stability under the conditions encountered in food processes or in the gastro-intestinal tract. Encapsulation of bioactive compounds increases both their shelf life and bioavailability after oral administration [2]. However, the development of materials that allow effective and selective delivery of bioactive agents to their site of action has proven a daunting task for scientists. Polymer-based delivery systems that trap molecules of interest within networks have been extensively developed for the biomedical and pharmaceutical sectors [3,4]. Despite successful elaboration of many synthetic polymers as delivery systems, these cannot be used in food applications, which require compounds generally recognized as safe (GRAS) for consumption in large quantities. Food proteins are widely used in food products because of their high nutritional value and functional properties, including the ability to form gels and emulsions [5–9]. These properties make proteins very good coating material for the encapsulation of bioactive compounds. A variety of processes have been developed to prepare protein-based microparticles, primarily extrusion [10], emulsifying-stabilization [11–13] and spray drying methods [14–16]. Although many of these protein-based microparticles exhibit the desired

* Corresponding author. Chaire de recherche du Canada sur les protéines, les bio-systèmes et les aliments fonctionnels, Institut de recherche sur les nutraceutiques et les aliments fonctionnels (INAF/STELA), Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Pavillon Paul Comtois, Sainte-Foy, Que., Canada G1K 7P4. Tel.: +1 418 656 2131x4278; fax: +1 418 656 3353.
E-mail address: Muriel.Subirade@aln.ulaval.ca (M. Subirade).

0939-6411/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2006.10.012

functionality profile, they require heating or organic chemical agents in at least one of the production steps, leading to some destruction of sensitive encapsulated compounds, as well as toxicity issues associated with organic residues.

Cold gelation provides an alternative gel matrix development method based on adding cations to a preheated protein suspension [17,18]. This method requires a heating step during which whey proteins are denatured and polymerized into soluble aggregates, followed by a cooling step and subsequent salt addition, which results in the formation of a network via Ca^{2+}-mediated interactions of soluble aggregates [19]. The formation of cold-set gels opens interesting opportunities for whey proteins as carriers of heat-sensitive bioactive compounds. Whey protein micro-beads (1–2 mm) have been prepared in our group by an extrusion method consisting of dropping a whey protein/oil emulsion into a calcium chloride solution [20]. Degradation assay by enzymatic hydrolysis reveals that these beads are gastro-resistant and form good matrixes for protecting fat-soluble bioactive molecules such as retinol, which have intestinal absorption sites. However, the large size of beads prepared by this method rules out their extensive application in foods, since only smaller particles ($\leqslant$100 μm) can be incorporated into most foods, including solid and semi-solid products, without changing their sensory qualities [21]. The nutraceutical industry is thus challenged to develop small protein-based particles for nutrient delivery.

In a recent study, we elaborated riboflavin-loaded alginate (AL)/whey protein isolate (WPI) granular microspheres 94 μm in diameter using an emulsification/internal cold gelation method based on the release of calcium from its carbonate by acidification [22]. Their special matrix/granular structure is visible microscopically (Fig. 1a), featuring WPI granules 3–10 μm in diameter homogeneously distributed within an AL spherical matrix. Yellow crystals of solid riboflavin are encapsulated in the small granules (Fig. 1b). Study of the vitamin release properties of these microspheres in simulated gastric and intestinal fluids (SGF and SIF) revealed that the AL matrix remained in its non-swollen state in SGF, creating a significant barrier to outward diffusion of the vitamin. In SIF, the matrix underwent swelling and erosion, followed by liberation of small riboflavin-containing WPI granules. Moreover, in the presence of pancreatin, the WPI granules were degraded resulting in complete riboflavin release. These observations allow us to expect gastric protection of microsphere-entrapped nutrients and their complete release in the small intestine, which could lead to the introduction of safe and highly functional oral delivery systems for bioactive compounds.

In the present study, the effects of formulation and phase dispersion conditions on microsphere release properties were investigated. AL–WPI granular microspheres with different WPI/AL ratio, particle size, total polymer concentration (C_{AL+WPI}) and nutrient loading were prepared and tested as vehicles of nutrient delivery in simulated gastric and intestinal fluids.

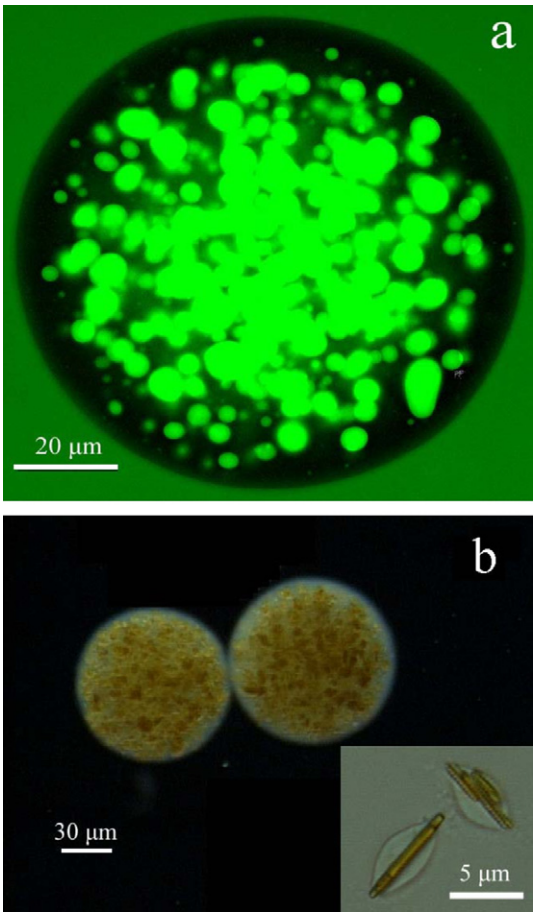

Fig. 1. Micrographs of AL–WPI granular microspheres: (a) labeled with phen green (b) loaded with riboflavin (WPI/AL ratio 8:2; particle size 94 μm and C_{AL+WPI} 5%). Insets show microsphere internal structure with loaded riboflavin inside granules about 3–10 μm in diameter (from Ref. [22]).

2. Experimental methods

2.1. Materials

Sodium alginate (Manucol DH) was purchased from Fluka AG, Buchs, Switzerland. Whey protein isolate (WPI) was obtained from Davisco Foods International Inc., Le Sueur, MN. WPI protein content was 93% (dry matter basis), as determined by the Kjeldahl method (nitrogen × 6.38) [23]. Soybean oil used to form the emulsions was purchased locally (Merit Selection brand). Calcium carbonate (40 nm) was kindly provided by NanoMaterials Technology Pte Ltd (Singapore). Pepsin 1:60,000 (from porcine stomach mucosa, crystallized and lyophilized), Span 80, Tween 80, riboflavin and the fluorescent dye fast green FCF were purchased from Sigma Chemical Co. (St. Louis, MO). Pancreatin 5X (from hog pancreas) was purchased from ICN Nutritional Biochemicals (Cleveland, OH). All other chemicals were reagent grade.

2.2. Microsphere preparation

AL–WPI granule microspheres were prepared by the emulsification/internal cold gelation technique [22]. WPI powder was rehydrated at 8% (w/v) in deionized water. WPI solution was adjusted to pH 8.0 with 1 N NaOH and heated at 80 °C for 30 min to denature proteins completely [24]. The denatured WPI solution was then cooled to room temperature. Sodium alginate powder was dispersed in distilled water and stirred overnight to form a 4.0% (w/v) solution. Denatured WPI solution and alginate solution were then combined to form solutions with various C_{AL+WPI} and WPI/alginate ratio as shown in Table 1. For preparing sample C1, 0.8 g alginate powder was dispersed in 20 ml 4% denatured WPI solution directly. Riboflavin was dissolved in the polymer solution at concentration $C_{riboflavin}$ (Table 1) to form a nutrient/polymer aqueous phase. Calcium carbonate (50 mM Ca^{2+} equivalent) was then added to 20 ml of this solution. The WPI–alginate–$CaCO_3$–riboflavin mixture was dispersed in 100 ml soybean oil by stirring at speeds ranging from 100 to 1100 rpm for 15 min to form W/O emulsions of droplet sizes in the 100–900 μm diameter range. During agitation, 40 μl glacial acetic acid was added to initiate droplet gelation. After 20 min, the oil/droplet suspension was added with gentle mixing to 150 ml of a 50 mM calcium chloride solution. After complete partitioning of microspheres to the aqueous phase, the oil was discarded and the samples were filtered on filter paper (Whatman) and washed with 1% (v/v) Tween 80 solution, lyophilized and weighed. To prepare 30–50 μm microspheres, Span 80 was added to the oil phase as a surfactant (1%). The oil/solution mixture was homogenized using an Ultra-Turrax T25 homogenizer for 3 min with an electrical input of 40 V (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) to prepare droplet of 30 μm. Pure WPI (8% w/v) and pure alginate (2% w/v) microspheres were also prepared by the emulsification/internal gelation technique as controls.

2.3. Particle size and size distribution analysis

Freshly prepared AL–WPI granule microspheres were dispersed in 0.2 μm filtered distilled water containing 1% (v/v) Tween 80. Size measurement was performed by static light scattering, using a Mastersizer 2000 (Malvern Instruments, Southborough, MA). All size measurements were performed at a 90° scattering angle at 25 °C with 180 s of recording. The mean hydrodynamic diameter was generated by cumulative analysis.

2.4. Microsphere morphology

Features such as shape and structure were observed for isolated washed microspheres using an Olympus BX50WI optical microscope (Olympus, Melville, NY, USA) fitted with epi-fluorescence and optical fluorescent filters (Chroma Technology Corp, Rockingham, USA) and a digital camera (model U-TV1 X, Olympus Optical, Tokyo, Japan). For fluorescence observation, microspheres were stained with phen green by dispersing dry samples into staining solution.

2.5. Determination of riboflavin loading

A 20–30 mg sample of dry AL–WPI granule microspheres was precisely weighed and dispersed in 25 ml

Table 1
Particle size, size polydispersity (*d*) and riboflavin loading of AL–WPI granular microspheres obtained under various preparation conditions

Sample	WPI/AL (w/w)	C_{AL+WPI} (%)	$C_{riboflavin}$ (%)	Stirring (rpm)	Size (μm)	*d*	LE (% ± SD)	EE (% ± SD)
A1	WPI	8.0	1.0	600	92.2 ± 1.4	0.55	9.1 ± 0.2	83.5 ± 0.3
A2	9:1	5.0	1.0	600	100.8 ± 1.5	0.53	13.4 ± 0.1	83.1 ± 0.3
A3	8:2	5.0	1.0	600	94.2 ± 2.0	0.54	14.6 ± 0.1	87.8 ± 0.2
A4	6:4	5.0	1.0	700	95.7 ± 2.4	0.75	13.8 ± 0.1	84.0 ± 0.2
A5	5:5	5.0	1.0	1000	92.1 ± 2.8	0.81	11.1 ± 0.1	68.0 ± 0.3
A6	AL	2.0	1.0	800	103.2 ± 2.5	0.74	18.0 ± 0.1	61.4 ± 0.3
B1	8:2	5.0	1.0	Ultra-Turrax[a]	32.2 ± 1.7	0.57	12.5 ± 0.1	78.5 ± 0.2
B2	8:2	5.0	1.0	1100[a]	58.5 ± 1.5	0.58	13.3 ± 0.1	84.1 ± 0.2
B3	8:2	5.0	1.0	600	94.2 ± 2.0	0.54	14.6 ± 0.1	87.8 ± 0.2
B4	8:2	5.0	1.0	300	439.5 ± 2.3	0.85	16.1 ± 0.2	93.7 ± 0.3
B5	8:2	5.0	1.0	100	885.5 ± 2.8	1.98	16.2 ± 0.1	94.6 ± 0.3
C1	8:2	8.0	1.0	800	98.6 ± 2.3	0.93	9.1 ± 0.1	79.1 ± 0.2
C2	8:2	5.0	1.0	600	94.2 ± 2.0	0.54	14.6 ± 0.1	87.8 ± 0.2
C3	8:2	2.0	1.0	600	91.7 ± 2.1	0.51	26.8 ± 0.1	86.5 ± 0.1
D1	8:2	5.0	1.0	600	94.2 ± 2.0	0.54	14.6 ± 0.1	87.8 ± 0.2
D2	8:2	5.0	0.5	600	95.4 ± 1.8	0.54	8.6 ± 0.1	96.8 ± 0.2
D3	8:2	5.0	0.2	600	91.3 ± 2.1	0.53	3.6 ± 0.1	98.5 ± 0.2

C, concentration in the aqueous phase during dispersion AL, pure alginate microsphere; WPI, pure whey protein microspheres; Ultra-Turrax, the emulsion was homogenized using an Ultra-Turrax T25 homogenizer for 3 min with an electrical input of 40 V; LE, riboflavin loading efficiency; EE, riboflavin encapsulation efficiency.

[a] Span 80 was added in the emulsion formation process.

of simulated intestinal fluid USP XXII at pH 7.4 with 1.0% pancreatin (w/v) at 37 °C under vigorous agitation for 6 h. The resulting mixture was centrifuged at 12,000 rpm for 20 min at 23 °C. Riboflavin concentration in the supernatant was determined from absorbance at 445 nm measured with a UV–visible spectrophotometer (model 8435, Hewlett-Packard, Palo Alto, CA) using a standard curve [25]. The riboflavin encapsulation efficiency (EE) and loading efficiency (LE) were calculated from

$$\mathrm{EE} = A/B \times 100,$$

$$\mathrm{LE} = A/C \times 100,$$

where A is the amount of riboflavin encapsulated in the microspheres, B is the total amount of riboflavin and C is the total weight of the microspheres.

2.6. In vitro release studies

Riboflavin release was determined by incubating an amount of dry microspheres encapsulating ~3 mg riboflavin in 30 ml of one of four release media with continuous agitation by magnetic bar (at ~100 rpm) at 37 °C. Release media: simulated gastric fluid (SGF) (HCl solution, pH 1.2) with or without 0.1% pepsin (w/v) and simulated intestinal fluid (SIF) (phosphate-buffered saline, pH 7.4) with or without 1.0% pancreatin (w/v). Samples (~1 ml) withdrawn at half-hour or one-hour intervals were centrifuged and the riboflavin content of the supernatant was determined by measuring absorbance at 445 nm. An equal volume of medium was added to the release mixture after each sampling to maintain a constant volume.

2.7. Microsphere degradation assay

Since riboflavin shows absorbance at 267 nm and microscopy observation revealed the same morphology, internal structure and behaviors in SGF and SIF for blank microspheres and riboflavin-loaded microspheres, microsphere degradation was investigated by re-suspending ~40 mg of dry microspheres without riboflavin in 30 ml of SGF or SIF with digestive enzymes. Samples (~2 ml) were withdrawn at half-hour or one-hour intervals and centrifuged and absorbance of the supernatant at 280 nm was measured to determine the digested WPI in the release media. An equal volume of medium was added to the reaction mixture to maintain a constant volume.

2.8. Statistical analysis

All experiments were performed at least in triplicate. Error bars on graphs represent standard errors obtained from the statistical model. Statistical comparisons were made using Student's t-test and analysis of variance (ANOVA). The level of significance used was $p < 0.05$.

3. Results

3.1. Microsphere preparation

AL–WPI granular microspheres at different WPI/alginate ratio (variable A), particle size (variable B), total polymer concentration (variable C) and riboflavin loading efficiency (variable D) were successfully prepared as demonstrated in Table 1 together with their preparation conditions. Encapsulation efficiencies of above 80% were obtained for most of the microspheres prepared in this work while loading efficiency ranged from 3.6% to 26.8%.

3.2. In vitro riboflavin release

3.2.1. Effect of WPI/AL ratio

Fig. 2 shows riboflavin release from microspheres with different WPI/AL ratios, particle size ranging from about 92 to 101 μm, $C_{\mathrm{AL+WPI}}$ fixed at 5% and LE ranging from about 11% to 15%. Release in SGF without pepsin was noticeably faster from pure AL and WPI microspheres ($p < 0.05$) than from AL–WPI granule microspheres, which all showed similar release profiles (Fig. 2a). In the presence of pepsin (Fig. 2b), riboflavin was released quickly from pure WPI microspheres, 90% after 30 min and completely within 1 h. Release from microspheres with a WPI/AL ratio of 9:1 was also relatively quick, 60% during the first 1 h and more than 80% after 6 h. At WPI/AL ratios $\leqslant$8:2, the microspheres regain the desired ability to retard riboflavin release and pepsin seemed to have little effect on their release properties. In SIF without pancreatin (Fig. 2c), the riboflavin release rate increased conspicuously with increasing alginate content until WPI/AL ratio reached 8:2 ($p < 0.05$). At least 75% was released within 90 min from alginate-rich microspheres. At WPI/AL ratios $\geqslant$8:2, release reached 60–75% only after 5 or 6 h. When pancreatin was included in the SIF medium (Fig. 2d), all microspheres showed rapid riboflavin release during the first hour with maximal release reached by the fourth hour.

3.2.2. Effect of particle size

Riboflavin release profiles of microspheres in the 30–900 μm diameter range, with a WPI/AL ratio of 8:2, $C_{\mathrm{AL+WPI}}$ of 5% and LE of about 12–16%, are shown in Fig. 3. Similar release rates were obtained in SGF with or without pepsin (Fig. 3a and b). No obvious differences were observed for microspheres 30–94 μm in diameter. However, those greater than 94 μm showed decreased release rates, particularly during the initial hour (10–20% release, $p < 0.05$), after which riboflavin release was steady and similar to that from the other microspheres. In SIF buffer without pancreatin (Fig. 3c), 94 μm microspheres gave the fastest release, followed by 400–900 μm microspheres. The slowest release was observed for 30–50 μm microspheres. When pancreatin was included in this medium (Fig. 4d), all microspheres showed increased initial riboflavin release rates. Release from microspheres

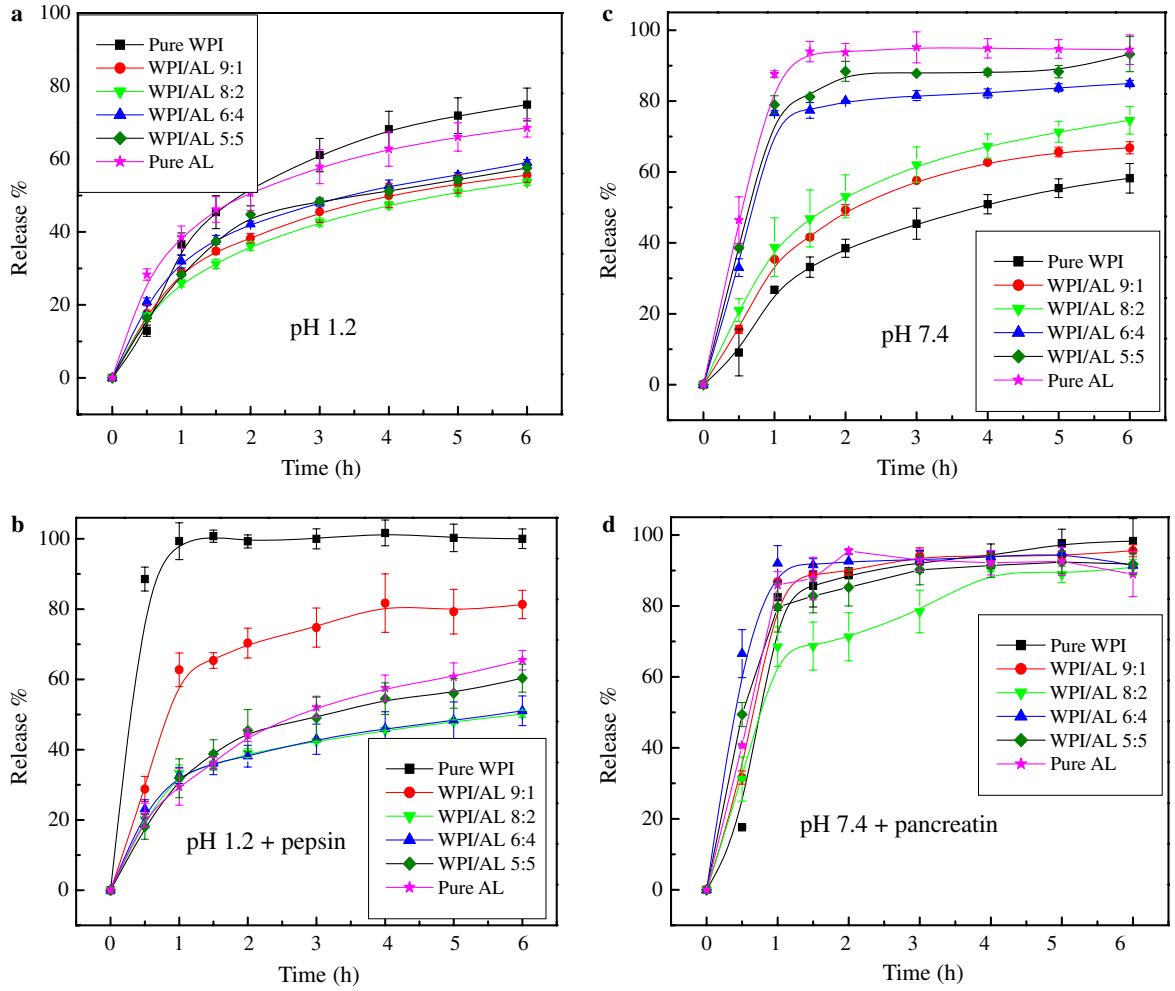

Fig. 2. Effect of WPI/AL ratio on riboflavin release from AL–WPI granular microspheres. (Particle size 94 μm, C_{AL+WPI} 5%, $C_{riboflavin}$ 1.0%).

⩽94 μm reached 80–90% by the sixth hour while leveling off at only 60% in the case of microspheres ⩾400 μm.

3.2.3. *Effect of polymer concentration and riboflavin loading*

Microspheres of diameters in the 90–100 μm range were produced with various C_{AL+WPI} and riboflavin loading at a WPI/AL ratio of 8:2. No dependence of riboflavin release on C_{AL+WPI} was observed (results not shown) but release rates in SGF (with or without pepsin) decreased with increasing riboflavin load ($p < 0.05$), as demonstrated in Fig. 4. Thirty-six percent of the riboflavin was released from microspheres prepared at an initial riboflavin concentration of 1.0% after 2 h of incubation, while those prepared at 0.2% lost their ability to retard nutrient release, liberating 80% after 2 h. In SIF without pancreatin, higher nutrient loading still resulted in slower release rate, but the difference was not as significant as in SGF. Preparation at 0.2% riboflavin brought 65% release within the first hour while 0.5% or 1.0% gave almost the same release rate (less than 40% after 1 h). In the presence of pancreatin, riboflavin was completely released from all these microspheres within 6 h.

3.3. *Degradation assay in simulated gastric and intestinal fluid*

No breakdown of alginate was visible microscopically during 6-h preliminary tests, while pure WPI microspheres and WPI granules inside AL–WPI microspheres were digested in the presence of pepsin or pancreatin. Fig. 5 shows degradation of the WPI from selected microspheres prepared without riboflavin. In SGF, pure WPI microspheres (A1) were degraded rapidly by pepsin and almost all of the WPI disappeared in 1 h. Quick degradation rate was also observed for WPI granules inside AL–WPI microspheres at a WPI/AL ratio of 9:1 (A2). However, increasing the alginate content to 20% delayed WPI degradation in the first hour of the test (A3) ($p < 0.05$) as did increasing particle size ($p < 0.05$). In SIF, similar degradation profiles were observed for microspheres prepared with different WPI/AL ratio or at sizes of 58 and 94 μm (B2 and A3), while microspheres of 440 μm (B4) showed a slower degradation rate ($p < 0.05$). Initial degradation rates were faster in SIF than in SGF ($p < 0.05$), except for pure WPI microspheres.

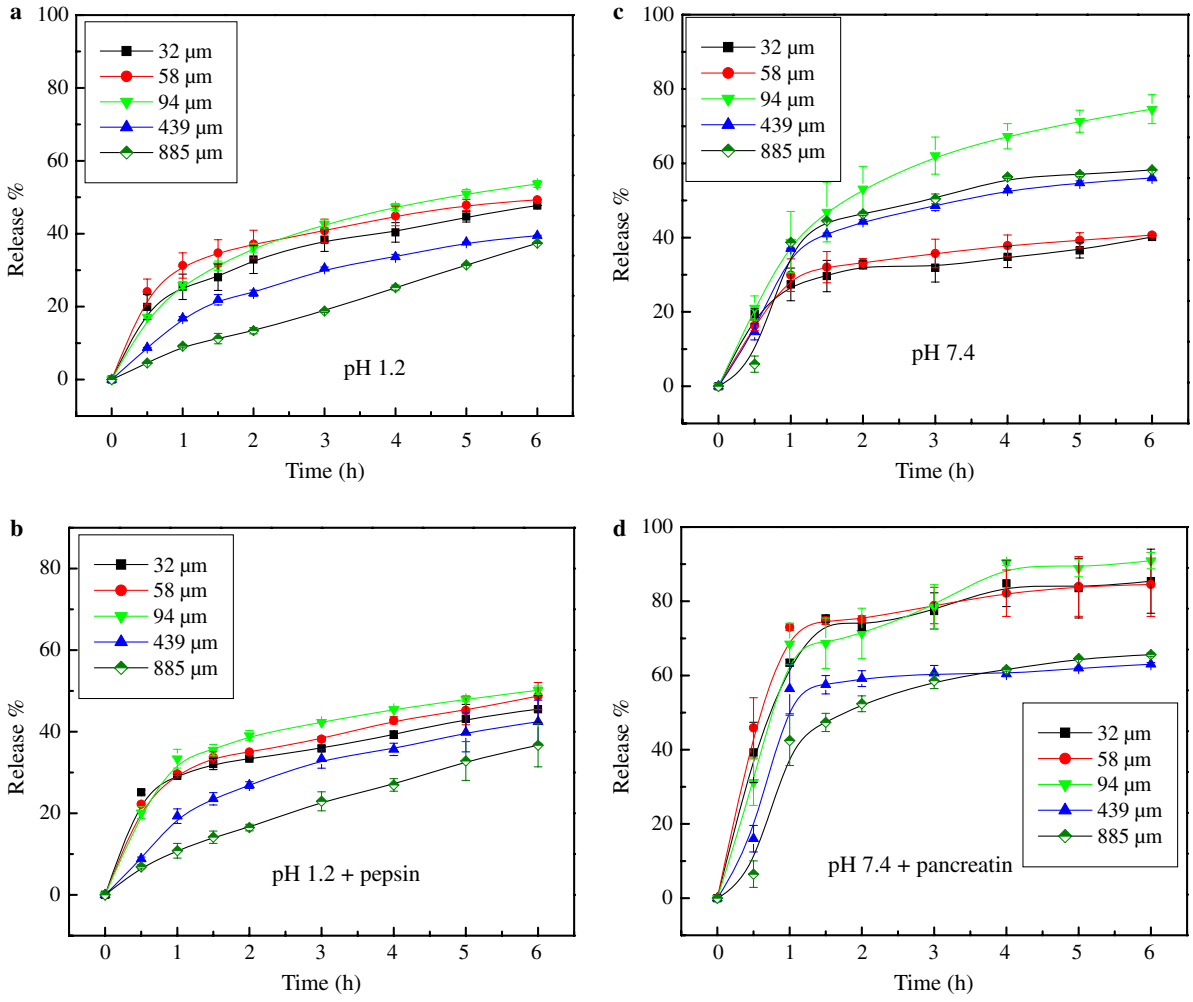

Fig. 3. Effect of particle size on riboflavin release from AL–WPI granular microspheres. (WPI/AL ratio 8:2, C_{AL+WPI} 5%, $C_{riboflavin}$ 1.0%).

4. Discussion

In emulsification/internal cold gelation, gelation occurs within the droplets. By controlling the conditions under which the aqueous phase is dispersed in the oil, the droplet size can be controlled from 32 to 886 μm in diameter. Strong dispersive force combined with surfactants (e.g. span 80) which are usually used to lower the interfacial tension between the water and oil phases and to stabilize emulsion droplets against coalescence are required to prepare microspheres below 50 μm in diameter. No physical disintegration was observed during particle washing, while shape distortion occurred for microspheres of 886 μm when the coverslip was placed on the samples for microscopic observation, suggesting that the emulsification/internal cold gelation method may not lead to strong bead formation at large sizes. Mastersizer analysis showed unimodal distributions ($d < 0.6$) for most of the microspheres. However, broader size distributions were observed with increases in alginate percentage or total C_{AL+WPI}, factors that both increase solution viscosity ($p < 0.05$) [22]. This also occurred with larger particles (440–886 μm, $p < 0.05$), because they were prepared by dispersion at lower energy input, apparently too low to produce droplets of uniform size. In the case of 886 μm microspheres, irregular shaped particles were also formed. It was noted that encapsulation of riboflavin had little influence on particle diameter, since particle size and size distribution remained unchanged as riboflavin concentration decreased.

High vitamin encapsulation efficiencies were obtained in spite of washing the microspheres (Table 1). When freshly prepared microspheres were separated directly from the oil phase by filtering on filter paper, rinsed rapidly with acetone and then lyophilized, EE values of $\geqslant 96.8$ were obtained in all cases (results not shown). However, after separating into calcium chloride solution, filtering and washing with Tween solution, EE values were somewhat lower. Since riboflavin is a low molecular weight water-soluble vitamin with no strong interaction with alginate or WPI, its diffusion in water is inevitable. It was noted that increasing the alginate content above 50% led to 30–35% riboflavin losses ($p < 0.05$) during the wash process, suggesting faster riboflavin diffusion from high-alginate microspheres. Riboflavin loss by washing also seemed to increase

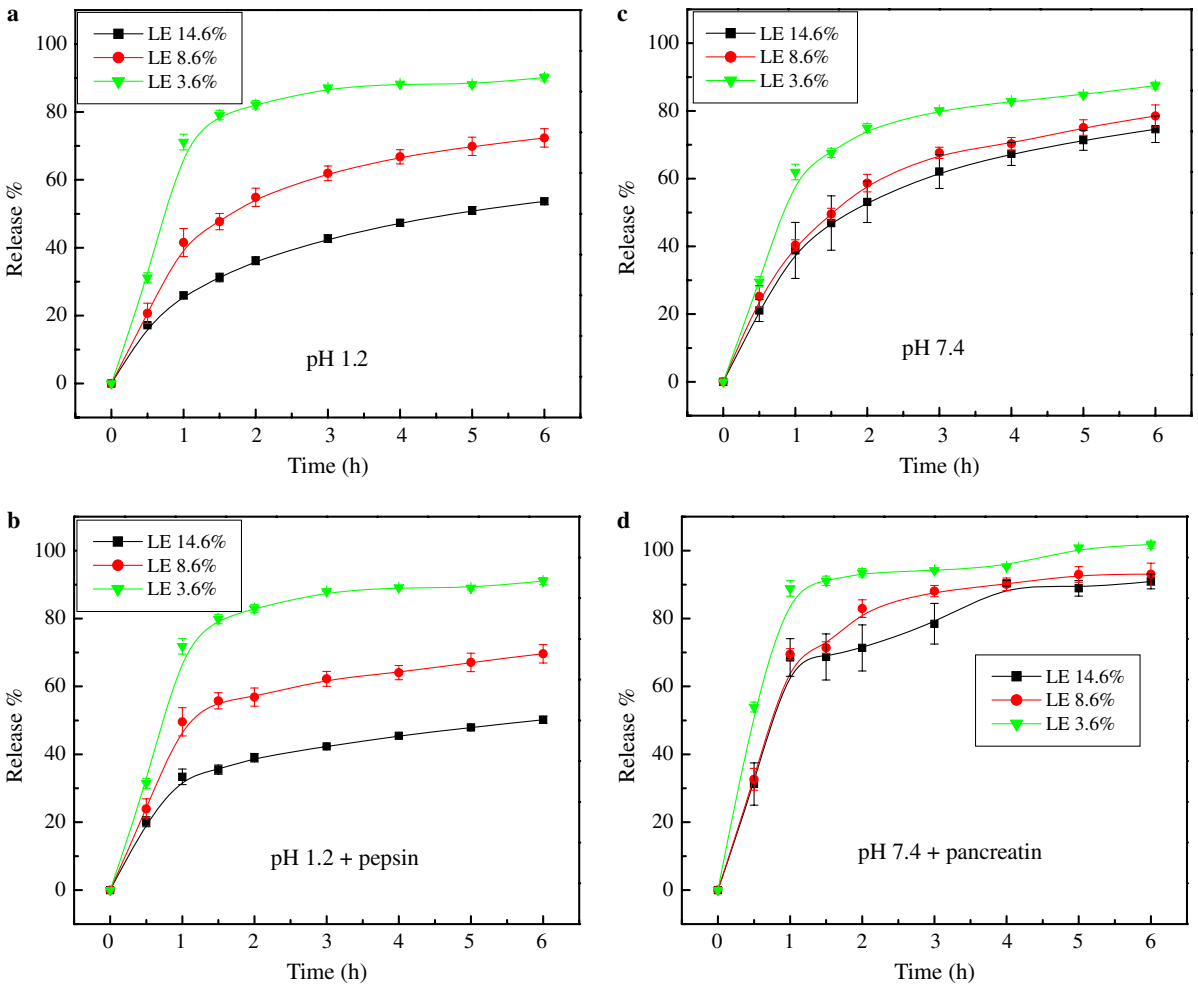

Fig. 4. Effect of riboflavin loading on riboflavin release from AL–WPI granular microspheres. (WPI/AL ratio 8:2, particle size 94 μm and C_{AL+WPI} 5%).

with decreasing particle size ($p < 0.05$), which may be expected, due to the increase in surface area/volume ratio [26] and decrease in the diffusion path length [27].

Degradation assay indicated that WPI was digested rapidly by pepsin. However increasing the alginate content in polymer bead forming solution or enlarging particle size delayed WPI granules degradation in the first hour of the test. These delays can be attributed to formation of thicker non-swollen AL matrix barriers (not degraded by digestive enzymes) to prevent pepsin degradation. When transferred to the SIF with pancreatin, quicker initial degradation rates for WPI granules from microspheres $\leqslant$94 μm were caused by rapid erosion of the AL matrix barrier [22] due to precipitation of Ca^{2+} with small cations in the weakly alkaline SIF buffer (pH 7.4), disrupting carboxyl cross-linkages between alginate chains. For microspheres $\geqslant$450 μm, AL matrix erosion took longer, thus preventing pancreatin degradation. Even after 6 h, some of these alginate matrixes were still observed in SIF medium.

Release properties of AL–WPI granular microspheres with different parameters were investigated. Although no dependence on polymer concentration was observed for riboflavin release from microspheres in SGF or SIF, WPI/AL ratio, particle size and nutrient loading did play important roles. Unlike pure AL or pure WPI microspheres, which showed noticeably faster riboflavin release due to direct diffusion from the simpler internal structure, certain granular AL–WPI microspheres were characterized by delayed riboflavin release. In SGF with pepsin, no degradation of pure alginate microspheres occurred, while pure WPI microspheres and WPI granules inside AL–WPI microspheres with a WPI/AL ratio of 9:1 underwent rapid degradation, leading to quick riboflavin release in the initial hours. However, at WPI/AL ratios $\leqslant$8:2, riboflavin release was delayed, the alginate being thick enough to provide a barrier against WPI degradation by pepsin. In SIF buffer, pure AL microspheres as well as the alginate matrix of microspheres with WPI/AL ratio $\leqslant$6:4 erode quickly [22]. This allows pancreatin to degrade the encapsulated WPI granules, which rapidly releases the riboflavin. Since the best nutrient release-delaying property was obtained for microspheres with a WPI/AL ratio of 8:2, this ratio was used for the study of the other three independent variables.

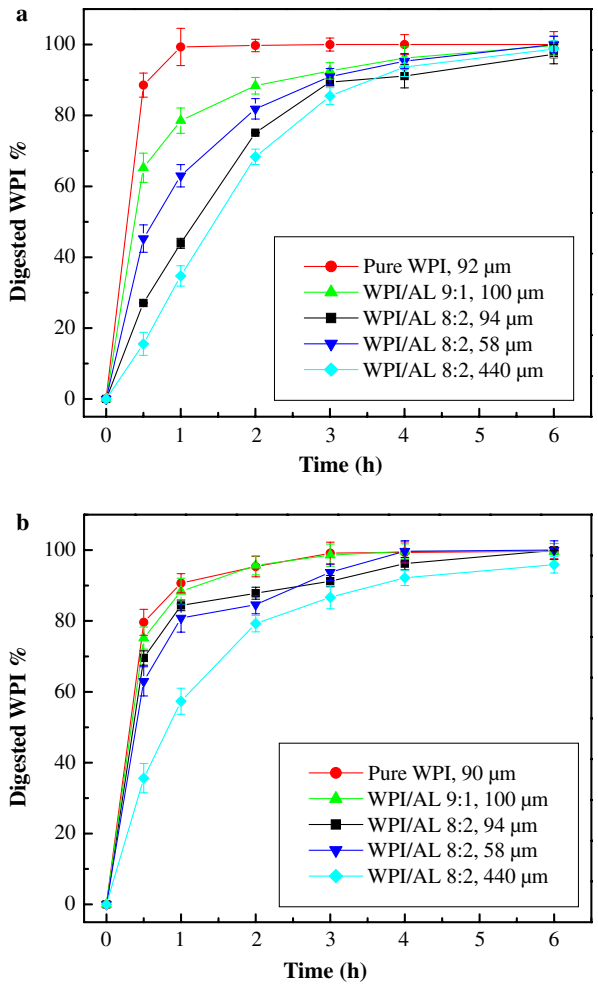

Fig. 5. Degradation of AL–WPI granular microspheres without riboflavin in SGF with pepsin (a) or in SIF with pancreatin (b), C_{AL+WPI} values were 5% for all the samples.

Microsphere size is a primary determinant of drug release rates. Larger particles generally release encapsulated compounds more slowly and over longer time periods due to smaller surface area/volume ratios and longer diffusion path lengths. Although release from granular microspheres 30–94 μm in diameter was similar in SGF with or without pepsin, increasing particle size above 94 μm decreased release, especially the initial rates. In SIF buffer without pancreatin, 94 μm microspheres gave the fastest release, followed by 400–900 μm microspheres. The slowest release was observed surprisingly for 30–50 μm microspheres. These results seemed contrary to the surface area/volume law. However, we have previously determined [22] that the alginate matrix of 94 μm microspheres underwent rapid swelling and erosion, releasing riboflavin-containing WPI granules. The quick riboflavin release rate in SIF buffer can be attributed to the increased surface area/volume ratio and decreased diffusion path length (due to the dispersion of the small granules from the 94 μm microspheres) and also to the disappearance of the alginate matrix barrier. While erosion of the alginate in microspheres $\geqslant$450 μm was slower, thus slowing riboflavin release as expected. For microspheres $\leqslant$50 μm, the obtained result could be due to their microstructure. Indeed, microscopy photographs demonstrated that the small granules within the alginate matrix appeared to aggregate with riboflavin crystals distributed among granules [22], unlike microstructure of 94 μm microsphere where riboflavin was located inside each granule (Ref. [22]). This aggregation may be attributed to strong intermolecular interactions among protein droplets forced together by strong interfacial tension. Although the thin alginate matrix eroded quickly, the aggregates did not, thus maintaining a significantly longer diffusion path for riboflavin release. When pancreatin was included in the SIF medium, both individual WPI granules and granule aggregates liberated from the microspheres after alginate matrix erosion were rapidly degraded, resulting in rapid riboflavin release in 1 h and almost complete riboflavin release after 6 h. However, size still mattered, since for microspheres $\geqslant$450 μm, riboflavin release was delayed to some extent (by the putative longer AL matrix erosion phase), with 40% of the vitamin still in the matrix after 6 h. Based on this work, microspheres 94 μm in diameter would allow release of small WPI granules into the intestinal medium, which should adhere to the intestinal wall to facilitate compound delivery. Microspheres of this size thus seem most suitable for oral delivery of nutrients. Moreover, they could be incorporated into a wide range of foods without changing the food sensory qualities.

Compound loading also had a noticeable effect on riboflavin release rate. Increasing the amount of vitamin loaded decreases its release rate in SGF with or without pepsin, likely because too many molecules are trying to diffuse, thus limiting their own permeation by hindering effects [28]. Microspheres with low loading efficiency (3.6%) did not exhibit delayed nutrient release, suggesting that higher loading of hydrophilic nutrients is suitable for AL–WPI granular microspheres.

The results discussed above suggest that microspheres prepared by the emulsification/internal cold gelation method at a WPI/AL ratio of 8:2, with 1% initial riboflavin concentration and having a particle size near 94 μm had the best matrix/granule structure and all conditions considered, the best compound release properties. They delay riboflavin release in SGF and liberate WPI granules in SIF, followed by complete release of the riboflavin. These microspheres therefore appear to be potentially useful as oral delivery vehicles for bioactive compounds in food and nutraceutical applications as well as in the pharmaceutical industry.

Acknowledgements

This work was supported by the Fonds Québécois de la Recherche sur la Nature et les Technologies Action Concertée FQRNT-AEE-MAPAQ-MIC. Lingyun Chen

is grateful to INAF, STELA, and FSAA for post-doctoral fellowships. Muriel Subirade thanks the Natural Sciences and Engineering Research Council of Canada Research Chairs Program for its financial support.

References

[1] R. Elliott, T.J. Ong, Science, medicine, and the future Nutritional genomics, Br. Med. J. 324 (2002) 1438–1442.
[2] P.M.M. Schrooyen, R. van der Meer, C.G. De Kruif, Microencapsulation: its application in nutrition, Proc. Nutr. Soc. 60 (2001) 475–479.
[3] N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, Eur. J. Pharm. Biopharm. 50 (1) (2000) 27–46.
[4] R. Langer, N.A. Peppas, Advances in biomaterials, drug delivery, and bionanotechnology, AIChE J. 49 (2003) 2990–3006.
[5] C.M. Bryant, D.J. McClement, Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey, Trends Food Sci. Tech. 9 (1998) 143–151.
[6] A.H. Clark, Gelation of globular proteins, in: S.E. Hill, D.A. Leward, J.R. Mitchell (Eds.), Functional Properties of Food Macromolecules, Aspen, Gaithersburg, MD, 1998, pp. 77–142.
[7] E. Dickinson, Colloidal aggregation: mechanism and implications, in: E. Dickinson, T. van Vlie (Eds.), Food Colloids, Biopolymers and Materials, Royal Society of Chemistry, Cambridge, 2003, pp. 68–83.
[8] P. Walstra, Studying food colloids: past, present and future, in: E. Dickinson, T. van Vlie (Eds.), Food Colloids, Biopolymers and Materials, Royal Society of Chemistry, Cambridge, 2003, pp. 391–400.
[9] L. Chen, G.E. Remondetto, M. Subirade, Food protein-based materials as nutraceutical delivery systems, Trends Food Sci. Tech. 17 (2006) 272–283.
[10] J. Franz, D. Pokorová, J. Hampl, M. Dittrich, Adjuvant efficacy of gelatin particles and microparticles, Int. J. Pharm. 168 (1998) 153–161.
[11] E. Esposito, R. Cortesi, C. Nastruzzi, Preparation parameters and thermal treatment on chemico-physical and biopharmaceutical properties, Biomaterials 17 (1996) 2009–2020.
[12] C. Bhattacharjee, K.P. Das, Characterization of microcapsules of β-lactoglobulin formed by chemical cross linking and heat setting, J. Dispersion Sci. Tech. 22 (1) (2001) 71–78.
[13] R. Dinarvand, S. Mahmoodi, E. Farboud, M. Salehi, F. Atyabi, Preparation of gelatin microspheres containing lactic acid – Effect of cross-linking on drug release, Acta Pharm. 55 (2005) 57–67.
[14] F. Pavanetto, I. Genta, P. Giunchedi, B. Conti, U. Conte, Spray-dried albumin microspheres for the intra-articular delivery of dexamethasone, J. Microencapsul. 11 (4) (1994) 445–454.
[15] M.L. Bruschi, M.L. Cardoso, M.B. Lucchesi, M.P. Gremiao, Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization, Int. J. Pharm. 264 (1–2) (2003) 45–55.
[16] T. Hino, M. Tanimoto, S. Shimabayashi, Change in secondary structure of silk fibroin during preparation of its microspheres by spray-drying and exposure to humid atmosphere, J. Colloid Interface Sci. 266 (1) (2003) 68–73.
[17] S. Barbut, E.A. Foegeding, Ca^{2+}-induced gelation of pre-heated whey protein isolate, J. Food Sci. 58 (4) (1993) 867–871.
[18] A. Maltais, G.E. Remondetto, R. Gonzales, M. Subirade, Formation of soy protein isolate cold-set gels: protein and salt effects, J. Food Sci. 70 (2005) 67–73.
[19] C.F. Roff, E.A. Foegeding, Dicationic-induced gelation of pre-denatured whey protein isolate, Food Hydrocoll. 10 (2) (1996) 193–198.
[20] L. Beaulieu, L. Savoie, P. Paquin, M. Subirade, Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol, Biomacromolecules 3 (2002) 2239–2248.
[21] M.A. Augustin, The role of microencapsulation in the development of functional dairy foods, Aust. J. Dairy Techn. 58 (2) (2003) 156–160.
[22] L. Chen, M. Subirade, Alginate–whey protein granular microspheres as oral delivery vehicles for bioactive compounds, Biomaterials 27 (2006) 4646–4654.
[23] NORM IDF; 20B: 1993. Kjeldahl (nitrogen) methods.
[24] G. Remondetto, M. Subirade, Molecular mechanisms of Fe^{2+}-induced β-lactoglobulin cold gelation, Biopolymers 69 (2003) 461–469.
[25] X.Z. Shu, K.J. Zhu, Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure, Int. J. Pharm. 233 (2002) 217–225.
[26] C. Berkland, M. King, A. Cox, K. Kim, D.W. Pack, Precise control of PLG microsphere size provides enhanced control of drug release rate, J. Control. Release 82 (2002) 137–147.
[27] W. K Lee, J.Y. Park, E.H. Yang, H. Suh, S.H. Kim, D.S. Chung, K. Choi, C.W. Yang, J.S. Park, Investigation of the factors influencing the release rates of cyclosporin A-loaded micro- and nanoparticles prepared by high-pressure homogenizer, J. Control. Release 84 (2002) 115–123.
[28] J. Djordjevic, B. Michniak, K.E. Uhrich, Amphiphilic star-like macromolecules as novel carriers for topical delivery of nonsteroidal anti-inflammatory drugs, AAPS PharmSci. 5 (2003), article 26.