Beta-Lactoglobulin (β-Lg) - Polysaccharide Complexes as Nanovehicles for Hydrophobic Nutraceuticals

Y. D. Livney* & N. Ron

Faculty of Biotechnology & Food Engineering, Technion, IIT- Haifa, Israel
livney@technion.ac.il

Introduction

The enrichment of staple foods with nutraceutical substances is a promising strategy for promoting health of wide populations. However, the desirable reduction of fat in a healthy diet creates a difficulty in sufficiently providing essential hydrophobic nutraceuticals (HN) like fat-soluble vitamins, antioxidants and poly-unsaturated fatty acids, many of which are also sensitive to degradation during processing, shelf life and digestion. This necessitated the development of novel technologies for solubilizing, stabilizing and protecting such HN in aqueous food systems. An ideal vehicle for the task would be a natural, GRAS ("generally recognized as safe") and inexpensive nano-sized food component, which is capable of solubilizing and protecting HN in aqueous media while retaining sensory quality, and promoting absorption of the HN in the digestive tract. β-lactoglobulin (β-Lg) is as an excellent vehicle for such tasks. It is the major whey protein of ruminant species: Cow milk contains 2-3g/l β-Lg (Kontopidis et al., 2004). β-Lg is a globular protein of 18.4 kDa Mw with 162 amino acid residues. It is rich in essential amino acids (Hattori et al., 2000). β-Lg folds up into an 8-stranded, antiparallel β-barrel with a 3-turn α-helix on the outer surface and a ninth β-strand flanking the first strand (Brownlow et al., 1997). Under physiological conditions β-Lg exists as a dimer. β-Lg belongs to the lipocalin protein family, most of whose members exhibit some ligand binding. However, the physiological function of β-Lg remains elusive. The location of the major binding site of β-Lg lies inside the β-barrel of the protein. This location is the binding site of retinoids and vitamin D (Kontopidis et al., 2004). β-Lg is quite resistant to gastric digestion (Wang et al, 1997).

Attractive biopolymer interactions mainly occur between positively charged proteins (pH<pI) and anionic polysaccharides or negatively charged proteins (pH>pI) and cationic polysaccharides. These interactions result in complex formation, and depending on pH, ionic strength and molar ratio of the two biopolymers, may lead to either soluble complexes or to coacervation, i.e. associative phase separation. Soluble complexes may be obtained when opposite charges carried by the two macroions within a complex are not equal in number. The resulting net charge allows the complex solubilization thanks to the high entropy of the low molecular weight counterions, as well as repulsion between the similarly charged complexes. However, when the opposite charges carried by the two biopolymers neutralize each other, the complexes become insoluble (Schmitt et al., 1998; de Kruif et al., 2004; Livney, 2007).

The aim of this research was to introduce and develop a novel process for nanoencapsulation of hydrophobic nutraceuticals within β-Lg based nanoparticles, having a secondary protection by a polysaccharide, which is electrostatically complexed with the protein, for enrichment of clear acid beverages (and other drinks or foods), and to study the protection conferred to the ligand.

Materials and Methods

Materials

Bovine β-Lg isolate was obtained from Davisco food international, dialyzed against de-ionized water and freeze-dried. Vitamin D_2 was purchased from Sigma. An experimental sample of low

molecular weight (Mw≈20,000Da) low methoxyl pectin (LMP) with a degree of esterification (DE) of 5-10% was kindly provided by CP Kelco.

Methods

Solution preparation

β-Lg (0.2% wt) and pectin (1%wt) solutions were freshly prepared in filtered doubly distilled water and stirred for at least 6 hr for complete hydration. 276 μL of 5mg/mL vitamin D_2 in absolute ethanol were added per 100mL protein solution, where vitamin encapsulation was studied.

β-Lg -pectin complexation

A series of solutions containing a constant protein concentration (0.05%wt) but varying pectin contents (0-0.15%wt) was prepared by adding different amounts of 0.2%wt stock polysaccharide solution and buffer to the protein solution. These solutions were titrated using HCl and NaOH to the desired pH, than stirred for 1 h and stored overnight at room temperature prior to analysis. Four criteria were used in order to evaluate the complexes and find the conditions (pH and ratio) which give small and stable nanoparticles: Sedimentation- Qualitative observation following overnight equilibration; Turbidity- Measured by Ultrospec 3000 spectrophotometer at 600nm: Samples were stirred before measurement; Zeta potential (ζ)- Measured using a Brookhaven Zeta-PALS. (Best stability when $|\zeta| \geq 30$mV). Particle size- measured by Dynamic Light Scattering (DLS) using a Malvern Zeta-Sizer nano ZS.

Encapsulation of Vitamin D_2, as a model HN encapsulant, in β-Lg – Pectin Complexes

Vitamin D_2, dissolved in ethanol, was added while stirring to β-Lg solution at pH ≈6.8 and stirred further for half an hour. Pectin solution was added to the β-Lg-vitamin solution at the desired quantity, than the pH was adjusted while stirring to the desired value, and stirred for another hour. Vitamin content was analyzed by solvent extraction followed by reversed phase HPLC according to the method described earlier (Semo et al., 2007).

Results and Discussion

β-Lg- pectin complexes

To deliver HN in acid drinks, like fruit drinks, the complexes need to be stable at a low pH. Complexes of β-Lg with pectin form below the pI of the protein (~5.15), where it is positively charged. We studied different ratios of LMP and β-Lg, in order to find a range in which the system would have the desirable properties of stability and transparency. The results of the ratio scan at pH 4.0 are presented in Figures 1-2.

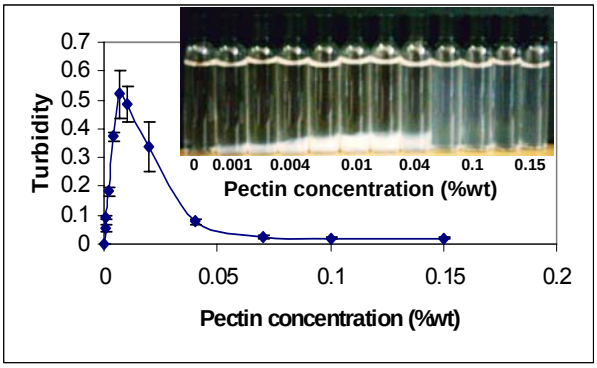

Figure 1. Influence of pectin concentration on turbidity of 0.05 %wt β-Lg solution at pH 4. Insert: Visual sedimentation test results.

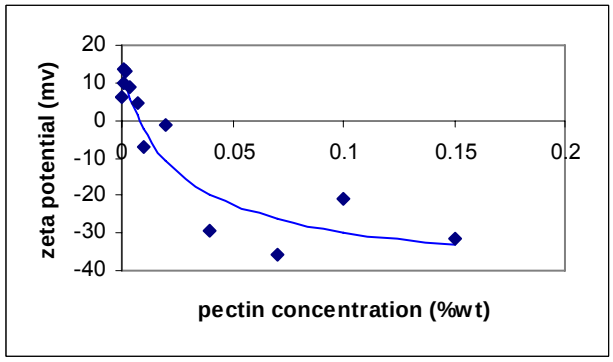

Figure 2. Influence of pectin concentration on zeta potential of 0.05%wt β-Lg solution at pH 4.

These observations indicated that the range having the desired properties of transparency and stability ($\zeta \geq |-30|$mV) was above 0.07% pectin (at least to the highest level tested, 0.15%wt). We next studied the effect of pH in the range 3.5-4.5, within the above concentration range, on particle size and turbidity.

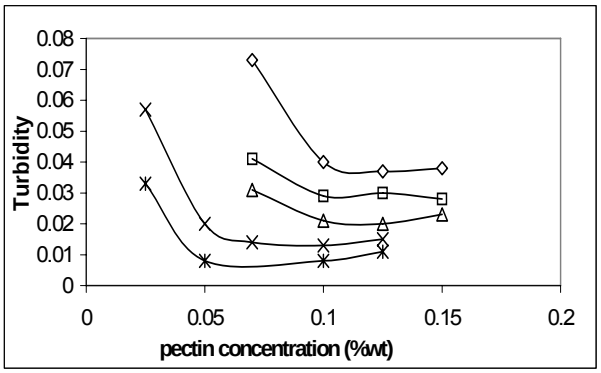

Figure 3. Turbidity vs. pectin concentration at different pH values. β-Lg concentration was 0.05%wt in all samples. ◊ pH=3.5; □ pH=3.75; Δ pH=4.0; x pH= 4.25; ∗ pH=4.5

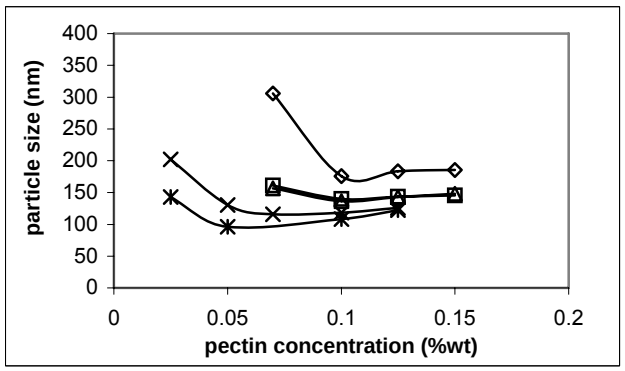

Figure 4. Particle size vs. pectin concentration at different pH values. β-Lg concentration is 0.05%wt in all samples. ◊ pH=3.5; □ pH=3.75; Δ pH=4.0; x pH= 4.25; ∗ pH=4.5

Two major trends are apparent from Figures 3 and 4: (1) Particle size decreased and solutions become clearer with increasing pH at the studied range; (2) Pectin:β-Lg ratio at minimum particle size decreased with increasing pH. These trends apparently result from the fact that as the pH rises towards the pI of β-Lg, the positive charge of the protein decreases and the negative charge of the pectin increases. Consequently, the complexes become more negative resulting in larger repulsion between them and dissociation into smaller and more stable complexes. Based on Figures 3 and 4 the minimum particle size was obtained at pH 4.5, and 0.05% pectin. When particle size was minimal, its distribution was also the narrowest (not shown). This trend was observed at all the pH values studied. Hence, at this minimum point the particles were most homogenous, and probably most stable, as aggregation would tend to broaden size distribution..

Next, we incorporated a model HN in the complexes, by binding it to β-Lg prior to pectin addition. We have chosen vitamin D_2 as a known HN ligand with a strong binding constant to β-Lg: Ka=2.04×10^8 (Wang et al., 1997).

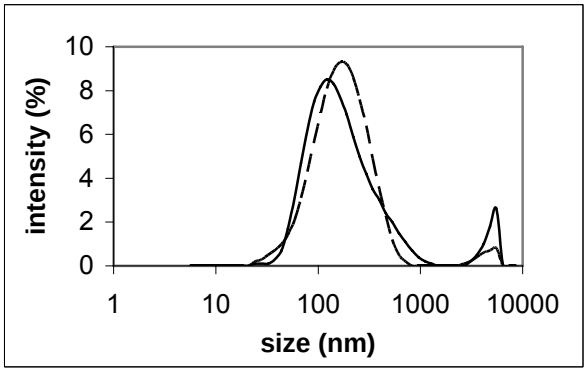

Figure 5. Size distribution of β-Lg pectin complexes with (solid line) and without (dashed line) encapsulated vitamin D.

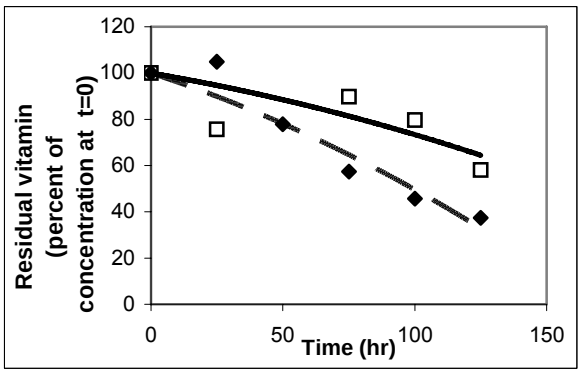

Figure 6. β-Lg/complexes protection against Vitamin degradation: β-Lg (♦); complexes (□). The data is presented relative to the result obtained by extraction at time zero: β-Lg - 9.52μg/ml, complex 1.29μg/ml.

Fig. 5 shows β-Lg-pectin nanoparticles at pH 4.0 with and without vitamin D_2. Average sizes were 146 nm and 151 nm respectively. Differences between these size distributions were not significant. To evaluate the encapsulation efficiency we used an ultra centrifuge (15,000xg) to separate the complexes (pellet) from the serum (supernatant), and compared the concentration of vitamin D_2 in the pellet to that in the serum, and to the concentration of vitamin D_2 in the system before separation. Vitamin D_2 concentration in the pellet was found to be 77.26 μg/ml compared to only 1.4μg/ml in the supernatant, suggesting that the vitamin concentration in the nanoparticles was 55 times higher than that in the serum and 5.7 times higher than the initial concentration of the vitamin in the system (13.5 μg/ml) suggesting a good encapsulation efficiency.

The protective effect of β-Lg-pectin complexes on the vitamin D_2 bound within them was tested on β-Lg and β-Lg-pectin complexes. Samples were stored at 30°C in closed vials containing 10ml of solution, and 10ml of air. The results, plotted in figure 6, show a somewhat slower degradation (apparently caused by oxygen and light) of the vitamin in the β-Lg-pectin complexes compared to encapsulation in β-Lg only.

Conclusions

We have encapsulated a model HN in β-LG and developed a new "nanocoating" technology based on forming electrostatically stable nanoparticles, of about 100 nm in size, comprised of β-LG (to which the HN is bound) and an anionic polysaccharide (e.g. pectin). The systems formed were almost completely transparent, which may be suitable for enrichment of most fruit juices. Complexes provided a higher protection to the vitamin against degradation than β-Lg alone.

Acknowledgements

This research was supported by The Danone Institute, Israel, and by The Mallat Family Fund.

References

Brownlow, S., J. H. M. Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. North, and L. Sawyer. (1997) *Bovine β-lactoglobulin at 1.8 .ANG. resolution - still an enigmatic lipocalin.* Structure (London) 5(4):481-495.

de Kruif, C. G., F. Weinbreck, and R. de Vries. (2004) *Complex coacervation of proteins and anionic polysaccharides.* Current Opinion in Colloid & Interface Science 9(5):340-349.

Hattori, M., K. Numamoto, K. Kobayashi, and K. Takahashi. (2000) *Functional changes in beta-lactoglobulin by conjugation with cationic saccharides.* J Agric Food Chem 48(6):2050-2056.

Kontopidis, G., C. Holt, and L. Sawyer. (2004) *Invited Review: beta-lactoglobulin: Binding properties, structure, and function.* J. Dairy Sci. 87(4):785-796.

Livney, Y. D. (2007) *Complexes and conjugates of biopolymers for delivery of bioactive ingredients via food.* In: Delivery and controlled release of bioactives in foods and nutraceuticals. N. Garti, ed. Woodhead Publishing Ltd., Cambridge, England.

Schmitt, C., C. Sanchez, S. sobry-Banon, and J. Hardy. (1998) *Structure and technofunctional properties of protein-polysaccharide complexes: a review.* Critical Reviews in Food Science and Nutrition 38(8):689-753.

Semo, E., E. Kesselman, D. Danino, and Y. D. Livney. (2007) *Casein micelle as a natural nano-capsular vehicle for nutraceuticals.* Food Hydrocolloids 21(5-6):936-942.

Wang, Q. W., J. C. Allen, and H. E. Swaisgood. (1997) *Binding of vitamin D and cholesterol to beta-lactoglobulin.* J. Dairy Sci. 80(6):1054-1059.