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Binding of retinyl acetate to whey proteins or phosphocasein micelles: Impact of pressure-processing on protein structural changes and ligand embedding

Claire Blayo [a], Duala Puentes-Rivas [a,1], Laëtitia Picart-Palmade [a], Dominique Chevalier-Lucia [a], Reinhard Lange [b,2], Eliane Dumay [a,*]

[a] Université Montpellier 2, UMR 1208, Ingénierie des Agropolymères et Technologies Emergentes, Équipe de Biochimie et Technologie Alimentaires, CC023, 2 Place Eugène Bataillon, 34095 Montpellier Cedex 5, France
[b] Université Montpellier 2, INSERM U710, UMR INSERM, UM2, EPHE, 2 Place Eugène Bataillon, 34095 Montpellier Cedex 5, France

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ABSTRACT

A whey protein isolate (WPI) and native phosphocaseins (PC) at pH 6.5–6.6 were processed with retinyl acetate (RAC) using pressure-assisted technological tools to improve RAC embedding through processing-induced protein structural changes. To this end, protein-RAC dispersions were submitted to ultra-high pressure homogenisation (UHPH) at 300 MPa and an initial fluid temperature (T_{in}) of 14 °C or 24 °C, or isostatic high-pressure at 300 MPa and 14 °C or 34 °C for 15 min. A short-time thermal treatment (STTT, 73 °C for 4 s) able to generate WPI aggregates was assessed for comparison. Processing effects were investigated in terms of protein particle sizes and molecular weights (M_w). M_w calculated using protein size determination obtained from light scattering measurements were in agreement with the known values. The amounts of RAC retained in WPI particles (unfolded and/or aggregated proteins) or in PC assemblies were quantitated after protein precipitation by ammonium sulphate. A 2.3–3.7 nmol RAC was carried per mg of pressure-denatured whey proteins, significantly less than after STTT (6.3 nmol RAC per mg of heat-denatured whey proteins) indicating that RAC embedding varied according to the technological tool, pressure or temperature. A 3.8–5.4 nmol RAC was carried per mg of PC assemblies through pressure-induced dissociation/reassociation of PC micelles. Combined pressure and mild temperature increased RAC embedding in PC assemblies.

1. Introduction

Natural hydrophobic biocompounds such as vitamins, polyphenols or polyunsaturated ω−3/ω−6 fatty-acids are highlighted for their benefits in promoting health and/or in reducing risks of diseases. The development of oral delivery systems in the nano-submicron range, able to embed and protect such bioactive compounds during processing and storage, then the further digestion steps in the body, without decreasing molecule bioaccessibility and biological efficiency has been the focus of recent attention (Acosta, 2009; Cohen Benshitrit, Shani Levi, Levi Tal, Shimoni, & Lesmes, 2012; Gonnet, Lethuaut, & Boury, 2010). Delivery systems intended for oral absorption have to be tailored using food-grade ingredients at an accessible cost. To that purpose, dairy proteins amongst the protein concentrates and isolates produced at an industrial scale for their techno-functional and nutritional properties are good candidates (Livney, 2010).

Amongst whey proteins, β-lactoglobulin (β-Lg) whose structural and biochemical characteristics have been extensively studied (Brownlow et al., 1997; Farrell et al., 2004) is known to bind hydrophobic molecules (retinoids and other vitamins, fatty acids and aroma) in its central cavity (or calyx) delimitated by β-barrels and/or at the β-Lg surface cleft (Jameson, Adams, & Creamer, 2002; Kontopidis, Holt, & Sawyer, 2002; Sawyer, Brownlow, Polikarpov, & Wu, 1998; Wang, Allen, & Swaisgood, 1997). It is expected that the binding to β-Lg protects unstable ligands from degradation caused by environmental factors such as temperature, air oxidation, UV-light, acid pH and gastric digestion (Diarrassouba et al., 2013; Hattori, Watabe, & Takahashi, 1995; Li, Ma, & Ngadi, 2013; Liang & Subirade, 2012; Liang, Zhang, Zhou, & Subirade, 2013; Stojadinovic et al., 2013). Lately, renewed interest has focused on single- or multi-ligand binding involving spectroscopic methods and molecular modelling techniques, in view of developing β-Lg-based carriers (Gholami & Bordbar, 2014; Zhang, Liu, Subirade, Zhou, & Liang, 2014). However few studies deal with the effects of technological treatments (other than chemical modifications) on the β-Lg–ligand binding efficiency, and different outcome is obtained depending on

* Corresponding author. Tel.: +33 467 143 351; fax: +33 467 143 352.
E-mail address: Eliane.Dumay@univ-montp2.fr (E. Dumay).
[1] Present address: INTEVEP, Centro de Investigacion y apoyo Tecnologico Filial de PDVSA, Santa Rosa, Caracas 1070A, Venezuela.
[2] Present address: Université Montpellier 2, UMR 1208, Ingénierie des Agropolymères et Technologies Emergentes, Équipe de Biochimie et Technologie Alimentaires, CC023, 2 Place Eugène Bataillon, 34095 Montpellier Cedex 5, France.

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processing parameters and environmental conditions. Prior heating of β-Lg aqueous dispersions (1–10 g L^{-1}; pH 6.5–7.5) at ≥75 °C for 3–20 min, decreased (or not significantly changed; $p = 0.05$) the number of binding sites of retinol to heated β-Lg and surprisingly, decreased the corresponding dissociation constant, in relation to the extent of β-Lg unfolding and the exposure of unspecific binding sites, the collapse of the β-Lg hydrophobic pocket and the protein aggregated state (Keppler, Sönnichsen, Lorenzen, & Schwarz, 2014; Laligant, Dumay, Casas Valencia, Cuq, & Cheftel, 1991; Mousavi, Bordbar, & Haertlé, 2008). Preheating of β-Lg at pH 2.3 and 80 °C for 1 h increased the aroma-binding ability of the protein in the molten globule state (Tavel, Andriot, Moreau, & Guichard, 2008). Combined pressure–temperature processing (600 MPa, 50 °C for 32 min) did not improve the binding ability of purified β-Lg for various ligands at pH 7.0, as assessed after pressure release (Yang, Powers, Clark, Dunker, & Swanson, 2003). More recently, we have followed the retinol binding process to β-Lg at neutral pH under isostatic high pressure by in situ fluorescence spectroscopy (Blayo, Marchal, Lange, & Dumay, 2014). Moderate pressure levels up to 150 MPa enhanced the retinol binding. In contrast, higher pressure levels up to 350–400 MPa induced an irreversible drop-out of the ligand retinol in accordance with previous studies (Dufour, Hoa, & Haertlé, 1994; Tanaka, Tsurui, Kobayashi, & Kunugi, 1996).

β-Lg aggregation induced by pH-cycling (Giroux, Houde, & Britten, 2010) or physical treatments such as dynamic high-pressure (Grácia-Juliá et al., 2008) have been proposed to developed protein aggregate-ligand co-assemblies able to cage hydrophobic compounds. Recently, Relkin and Shukat (2012) submitted aqueous dispersions of whey protein concentrate and α-tocopherol at pH 6.5, to a thermo-mechanical treatment (successive heating at 65 °C for 5 min, mixing at 10,000 rpm for 5 min then twelve-pass homogenisation at 120 MPa) to induce protein aggregation. No change in vitamin retention after 8-week storage at 4 °C of the processed protein-α-tocopherol dispersion, suggested the ability of HP-homogenisation to prepare whey protein aggregates for encapsulation. Mensi et al. (2013) added retinol or β-carotene (at a molar ligand/β-Lg ratio of 1/4) to aqueous dispersion of purified β-Lg previously processed by isostatic high-pressure (600 MPa and 20 °C for 10 min) at pH 2 or 7. Entrapment of retinoids into β-Lg aggregates was attempted through protein precipitation by a centrifugation step. Although a good entrapment was obtained (≥89%), β-Lg aggregates were not efficient to cage retinol during further storage at 4 °C, but a better stabilisation was obtained for β-carotene. In contrast, a good nano-entrapment of the polyphenol epigallocatechin-gallate (EGCG) and its significant protection against oxidation was obtained with a pre-heated (75–85 °C for 20 min) β-Lg isolate at pH 6.9 (Shpigelman, Israeli, & Livney, 2010).

Phosphocasein (PC) micelles, the other main protein components in bovine milk, are natural self-assemblies of individual caseins (α_{S1}, α_{S2}, β and κ at a molar ratio close to ~4:1:4:1.6) interconnected through hydrophobic areas and phosphocalcium bridges anchored to phosphoserine residues (Farrell et al., 2004). PC micelles could be interpreted as areas of high protein density interlocked together into supra-molecular structure to form highly hydrated micelle assemblies (~3.7–4.5 g of water per g of protein) with a rheomorphic character (Farrell, Malin, Brown, & Qi, 2006; Horne, 2002; McMahon & Oommen, 2008). Phosphocaseins offer natural nano-encapsulation potential. The ability of individual bovine phosphocaseins or casein micelle assemblies (re-assembled or native-like phosphocasein micelles) to bind hydrophobic biocompounds such as curcumin and polyphenols (Bohin et al., 2014; Bourassa, N'soukpoé-Kossi, & Tajmir-Riahi, 2013; Sahu, Kasoju, & Bora, 2008), vitamin D_2 (Semo, Kesselman, Danino, & Livney, 2007), ω−3 polyunsaturated fatty acids (Zimet, Rosenberg, & Livney, 2011) or drugs (Elzoghby, Helmy, Samy, & Elgindy, 2013; Shapira, Assaraf, & Livney, 2010) has recently focused research attention in view of developing oral delivery systems. In previous studies, we observed that native PC micelles at pH 6.6 and atmospheric pressure bind curcumin (Benzaria, Maresca, Taieb, & Dumay, 2013) and retinol (Blayo et al., 2014) with relatively weak binding constants ($K_b \approx 10^4$ M^{-1} and $\approx 10^3$ M^{-1} for curcumin and retinol, respectively) compared to the retinol–β-Lg complex ($K_b \approx 10^7$–10^8 M^{-1}, Blayo et al., 2014; Sawyer et al., 1998; Wang et al., 1997), suggesting moderate affinity of ligands to the hydrophobic domains in PC micelles, and/or non-specific binding processes. Relatively weak binding constants were also found for retinol ($K_b \approx 10^5$ M^{-1}) (Bourassa et al., 2013) or EGCG ($K_b \approx 10^4$ M^{-1}) (Bohin et al., 2014) linked to individual bovine α_S- or β-caseins.

However, few studies have investigated the binding ability of PC micelles subjected to technological processing. Benzaria et al. (2013) observed that processing PC micelles by dynamic high-pressure at 300 MPa prior to curcumin addition, increased the PC-curcumin binding constant by 1.5–2-fold compared to untreated PC micelles, which was attributed to the pressure-induced decrease in PC micelle size and the subsequent increase in particle surface area available for ligand adsorption. An increase in curcumin binding to milk proteins as assessed by fluorescence spectrometry was also observed after heating (80 °C for 10 min) or high-pressure processing (>200 MPa; isostatic pressure) of skim milk prior to curcumin addition comparing with untreated samples (Yazdi & Corredig, 2012; Yazdi et al., 2013). A combined pressure–temperature treatment (600 MPa at 50 °C) increased vitamin D_2 loading in re-assembled casein micelles (Menéndez-Aguirre et al., 2014). Processing PC micelles by dynamic high-pressure up to 300 MPa in the presence of α-tocopherol acetate significantly improved the amount of bound ligand to PC micelles by 1.5- to 3-fold depending on the combined processing temperature, as compared to non-treated PC micelles (Chevalier-Lucia, Blayo, Gràcia-Julià, Picart-Palmade, & Dumay, 2011).

The aim of the present study was to facilitate and improve retinyl acetate entrapment into whey protein aggregates or phosphocasein micelles through mild processing avoiding excessive heating, in view of process implementation. Retinyl acetate was chosen as a model hydrophobic biocompound for technological applications, since it is more resistant to oxidative degradation than retinol. Mild technological processing was chosen on the basis of our previous studies: isostatic high-pressure at 300 MPa and mild temperature for 15 min or dynamic high-pressure (or ultra-high-pressure homogenisation, UHPH) at 300 MPa and initial fluid temperature T_{in} of 14 °C or 24 °C. Short-time thermal treatment at 73 °C for 4 s, able to generate WPI aggregates was assessed for comparison. Retinyl acetate entrapment was quantitated after protein precipitation using ammonium sulphate. In parallel, the changes in protein particle sizes and molecular weights induced by processing, were assessed by photon correlation spectroscopy to follow whey protein aggregation or PC micelle dissociation/re-association phenomena.

2. Material and methods

2.1. Reagents and purified proteins

All chemicals were of analytical grade. Retinyl acetate (R7882, $M_w = 328.5$ Da) came from Sigma (St. Louis, MO). Ammonium sulphate (1.01217.1000) was from Merck (Darmstadt, Germany). Sodium mono- and di-phosphate (480087; 480137), tri-potassium citrate (471027), tri-sodium citrate (479487), $CaCl_2$ (43338), KCl (471177), K_2CO_3 (470807), KH_2PO_4 (361507), KOH (472173), K_2SO_4 (474167), $MgCl_2$ (414606), NaCl (479687), n-hexane (446907) and absolute ethanol were from Carlo Erba (Milano, Italy). Purified bovine β-Lg (L2506) non-crystallized and lyophilised (A and B variants) came from Sigma-Aldrich (St. Louis, MO, USA). Purified β-Lg concentration was determined using a weight extinction coefficient ($\varepsilon_{1‰}$) of 0.96 L g^{-1} cm^{-1}. The whey protein isolate (WPI) was obtained from Lactalis (Prolacta 90, lot 38, Retiers, France). WPI has been industrially prepared in mild conditions using microfiltration of milk followed by ultrafiltration then spray drying of the ultrafiltration-retentate. Native phosphocasein