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Thermal stability of the complex formed between carotenoids from sea buckthorn (*Hippophae rhamnoides* L.) and bovine β-lactoglobulin

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ABSTRACT

Sea buckthorn has gained importance as a versatile nutraceutical, due to its high nutritive value in terms of carotenoids content. β-Lactoglobulin (β-LG) is a natural carrier for various bioactive compounds. In this study, the effect of thermal treatment in the temperature range of 25 to 100 °C for 15 min on the complex formed by β-LG and carotenoids from sea buckthorn was reported, based on fluorescence spectroscopy, molecular docking and molecular dynamics simulation results. Also, the berries extracts were analyzed for their carotenoids content. The chromatographic profile of the sea buckthorn extracts revealed the presence of zeaxanthin and β-carotene, as major compounds. The Stern-Volmer constants and binding parameters between β-LG and β-carotene were estimated based on quenching experiments. When thermally treating the β-LG–carotenoids mixtures, an increase in intrinsic and extrinsic fluorescence intensity up to 90 °C was observed, together with blue-shifts in maximum emission in the lower temperature range and red-shifts at higher temperature. Based on fluorescence spectroscopy results, the unfolding of the protein molecules at high temperature was suggested. Detailed information obtained at atomic level revealed that events taking place in the complex heated at high temperature caused important changes in the β-carotene binding site, therefore leading to a more thermodynamically stable assembly. This study can be used to understand the changes occurring at molecular level that could help food operators to design new ingredients and functional foods, and to optimize the processing methods in order to obtain healthier food products.

1. Introduction

β-Lactoglobulin (β-LG), the major whey protein in bovine milk, is a small globular protein with 162 amino acid residues, having a molecular mass of 18,400 Da. The protein is classified as a member of the lipocalin-protein family because of its high affinity to small hydrophobic ligands [28]. Thus, β-LG can bind various hydrophobic compounds and drugs such as fatty acids, lipids, aromatic compounds, vitamins and polyamines [29,31,34]. Burova et al. [8] suggested that β-LG has two intramolecular disulfide bonds (Cys^{66}-Cys^{160}, Cys^{106}-Cys^{119}) and one free thiol group (Cys^{121}), which is buried between the β-barrel and the major α-helix. The protein contains 16 free amino groups that can act as binding site for potential covalent ligands as well [37]. The tertiary structure is dominated by the β-barrel and consists of nine anti-parallel β-sheets and a major α-helix at the C-terminal end of the polypeptide chain [53]. The β-barrel is formed by two β-sheets, where strands A to D form one sheet, and strands E to H form the other (with some participation from strand A, facilitated by a 90° bend at Ser [21]). The loop EF that connects strands E and F at the open end of the β-barrel acts as a gate ([50], chap. 6, [28]). At the quaternary structure level, the protein is mostly present in monomeric or dimeric form, this equilibrium being significantly influenced by the environmental conditions.

β-LG is frequently used as ingredient in food industry, because of the good techno-functional properties, high nutritional value, solubility over a wide pH range and GRAS (generally recognized as safe) status. Due to the abovementioned structural particularities, β-LG is considered as a natural carrier for various bioactive compounds to improve their bioavailability. The binding ability depends on the pH value. At pH higher than 7.0, the EF loop is open, allowing ligands to enter into the hydrophobic core [28]. Harvey et al. [19] suggested that β-LG contains three binding sites for different hydrophobic molecules: the first one is in the central cavity, known as *calyx*, the second is the surface cleft which lies between the α-helix and the surface of the barrel, and the third one is located at the monomer-monomer interface.

Carotenoids are tetraterpenoid pigments found in plants, bacteria, and fungi that are among the most widely distributed colored compounds in nature [42]. They are lipophilic biomolecules and are classified as carotenes if their sole constituents are hydrocarbons or xanthophylls, and if they also contain one or more oxygen atoms [5]. β-Carotenes (BC) are naturally occurring and the most abundant lipophilic carotenoid precursors of vitamin A. BC is widely used as a colorant

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in foods and beverages. However, the utilization of BC as a functional food in the food industry is currently limited because of its poor water solubility, high chemical instability, and low *in vivo* bioavailability. Encapsulation of BC can be used to improve the aqueous solubility, physicochemical stability, and bioavailability [33].

Provitamin A activity is the ability of carotenoids to form vitamin A (retinol and retinal) by the action of carotene dioxygenase [52]. Although provitamin A activity is the major function of carotenoids, potent antioxidant activity of carotenoids through singlet oxygen quenching and deactivation of free radicals plays important roles in the prevention of certain types of cancer, cardiovascular diseases, and macular degeneration [38]. Apart from this, carotenoids play important roles in cellular and organelle function [40].

Sea buckthorn berries are among the most nutritious of all fruits and have important medicinal properties [23]. The composition of sea buckthorn has been extensively studied, revealing that numerous positive effects are associated to the high nutritive value in terms of carotenoids, vitamins, organic acids, flavonoids, macro- and micronutrient elements. Sea buckthorn has gained much importance as a versatile nutraceutical crop with diverse uses, from controlling soil erosion to being a source of horse fodder, nutritious foods, drugs, and skin-care products ingredient [15]. Due to its natural antioxidant activity, different parts of the sea buckthorn can be used for the treatment of diseases, such as flu, cardiovascular diseases, mucosal injuries and skin disorders. Regarding the content in carotenoids, Anderson et al. [3] identified mainly the following compounds: zeaxanthin, β-carotene, β-cryptoxanthin, lutein, lycopene and γ-carotene.

The aim of the present study was to deepen the understanding of thermal treatment effects on the β-LG-sea buckthorn extract complex (further called as β-LG-CSB), in relation with protein structural changes as followed mainly by *in situ* fluorescence spectroscopy. The fluorescence spectroscopy methods involved the use of intrinsic and extrinsic intensity fluorescence, phase diagram, synchronous spectra, three-dimensional fluorescence spectroscopy and quenching experiments. The BC binding site on β-LG and the effect of complexation on the conformational stability and the secondary structure of β-LG are reported here, based on the quenching experiments, molecular docking and molecular dynamics simulation results.

2. Materials and Methods

2.1. Materials

β-LG (purity >90%, genetic variants A and B) from bovine milk, β-carotene, 1-anilino-8-naphtalenesulphonic acid (ANS), acrylamide and potassium iodide (KI) were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO). Sea buckthorn (*Hippophae rhamnoides* L.) berries were purchased from the local market (Galati, Romania) in the month of October of the year 2015, and immediately stored at −70 °C until use. Unless otherwise stated, all other reagents were of analytical grade.

2.2. Carotenoids Extraction

Five grams of sea buckthorn berries were extracted in 35 mL of ethanol:hexane solutions (4:3, v/v) containing 0.05 g magnesium carbonate on an orbital shaker for 1 h at room temperature. After extraction, the supernatant was separated and the residue was re-extracted with 70 mL ethanol:hexane solutions (4:3, v/v). The resulted residue was washed with 25 mL ethanol and afterwards with 12.5 mL hexane. The residue was washed again with 100 mL NaCl of 10% concentration and 150 mL of water. The carotenoids sea buckthorn (CSB) extract was concentrated at 40 °C to dryness, dissolved in 10 mL ethanol (70%) and filtered through 0.45 μm membranes. CSB were quantified using a colorimetric method. In brief, 1 mL of CSB ethanolic extract was added to 0.5 mL of 0.05 g/L NaCl, vortexed for 30 s, and centrifuged at 1500*g* for 10 min. The supernatant was diluted, and the absorbance at 460 nm was measured. The amount of CSB was calculated by plotting a calibration curve with β-carotene as standard (0–0.5 mg/mL).

2.3. Determination of Total Carotenoids Content

Total carotenoids content (TCC) in sea buckthorn extracts was analyzed using a colorimetric method. Briefly, 1 mL of aliquot of extract in ethanol was added to 0.5 mL of 0.05 g/L NaCl, vortexed for 30 s, and centrifuged at 1500*g* for 10 min. The supernatant was diluted, and the absorbance at 460 nm was measured. The amount of TCC was calculated by plotting a calibration curve with β-carotene as standard (0–0.5 mg/mL).

2.4. Quenching Experiments With β-Carotene

The (un)-heat treated protein samples (0.04 mL of 3 mg/mL β-LG in 0.01 M Tris-HCl buffer solution at pH 7.7) were diluted in 3 mL of appropriate buffer and titrated by successive addition of BC (1 mg of standard solution prepared in 2 mL of hexane). The excitation wavelength was set at 292 nm, while the emission spectra were collected from 310 nm to 400 nm with increments of 0.5 nm. Both the excitation and emission slit widths were set at 10 nm. The Stern-Volmer constants, binding constants and number of binding sites were calculated as previously reported [13].

2.5. Preparation of β-LG-CSB Complex

To obtain the protein solution, β-LG was weighed and dissolved in 0.01 M Tris-HCl buffer solution (pH 7.7) at a concentration of 3 mg/mL. Fresh CSB solution was prepared by dissolving the CSB in ethanol to give 4 μM concentrations. The β-LG-CSB complex was prepared by simple mixing of the two components. The CSB extract was added to the protein solution to reach a final protein/CSB molar ratio of 1:1. The resulting ethanol concentration never exceeded 5% (v/v), which had no appreciable effect on protein structure [31].

2.6. Heat Treatment

Plastic tubes (1 cm diameter) were filled with 0.04 mL of β-LG-CSB complex. The samples were heated at different temperatures ranging from 25 to 100 °C for 15 min, using a thermostatic water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain). Then, the tubes were cooled in ice water to avoid any further thermal denaturation.

2.7. Fluorescence Spectroscopy

2.7.1. Intrinsic Fluorescence

All fluorescence spectra were performed on a LS-55 luminescence spectrometer (Perkin Elmer Life Sciences, Shelton, CT, USA), equipped with the software Perkin Elmer FL Winlab. The excitation wavelength was set at 274 nm, 280 nm and 292 nm, while the emission spectra were collected from 310 nm to 420 nm, with increments of 0.5 nm. Both the excitation and emission slit widths were set at 10 nm.

2.7.2. Extrinsic Fluorescence

In order to evaluate the binding of hydrophobic ANS, the thermally (un)-treated β-LG-CSB complex was incubated for 15 min in the dark with 10 μL of 8 mM ANS solutions. Then, the samples were excited at 365 nm and emission was collected between 400 and 600 nm. The excitation and emission slits were both 10 nm, and the scan speed was 500 nm/min.

2.7.3. Phase Diagram

Measurements of the fluorescence intensity were performed at excitation wavelength of 292 nm, whereas the intensity was collected at