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Binding mechanisms between lycopene extracted from tomato peels and bovine β-lactoglobulin

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ABSTRACT

Fluorescence spectroscopy and computational methods were used to study the interaction mechanism between lycopene extracted from tomato peels and bovine β-lactoglobulin (β-LG). The chromatographic analysis of the extract allowed identification and quantification of nineteen carotenoids, with lycopene representing approximately 72% from the total carotenoids in the extract. The β-LG fluorescence has been regularly quenched upon increasing the lycopene concentration, suggesting the formation of the complex, whereas a dynamic quenching process was highlighted. The thermodynamic parameters suggested that the interactions between the protein and ligand are driven by van der Waals' force and hydrogen bonding. Molecular docking was employed to find out details on lycopene binding to the β-LG molecule equilibrated by means of molecular dynamics at different temperatures. The key interactions assuring β-LG – lycopene complex formation varied with the temperature. When the protein was heated at mild temperatures, the lycopene was found to bind on top of the calyx, whereas due to the conformational changes enabled by the thermal treatment at 90 °C, the most favorable ligand binding site involved amino acids responsible for stabilizing the dimmer.

1. Introduction

Tomato is one of the most widely cultivated vegetable crops and is globally used for human nutrition [1]. Significant amounts of tomatoes, in terms of millions of tons, are processed every year to produce different food products such as ketchup and sauce, resulting in large amounts of peel, pulp, and seed as by-products, an amount almost equivalent to 40% of the raw material [1]. Vasapollo et al. [2] suggested that concentrations of lycopene in tomato vary from 30 to 200 mg/kg in fresh fruit, and from 430 to 2950 mg/kg on a dry basis, with lycopene representing more than 85% of the total carotenoids. The tomato peels contain about five times more lycopene than tomato pulp [3].

Lycopene ($C_{40}H_{56}$) a major carotenoid without provitamin A activity, has an open chain hydrocarbon with 11 conjugated double bonds. Lycopene is considered responsible for significant beneficial effects, such as for protecting cells against oxidative damage and thereby decreasing the risk of chronic diseases, inducing cell to cell communication [4] and modulating hormonal, immune systems and other metabolic pathways [5,6]. In food industry, lycopene has important applications as a natural red pigment mainly responsible for the characteristic deep-red color of tomato fruits and products. Lycopene is widely used as food additive because of the coloring and antioxidant effects [1,7–9]. However, the use of lycopene and other carotenoids in food and nutraceutical applications is limited due to their poor water solubility and stability at processing at different parameters in terms of pH, temperature, salts, light, oxygen etc. McClements et al. [10] suggested the potential role of encapsulation for improving the aqueous solubility, physicochemical stability, and bioavailability of these compounds.

Proteins have a potential role in encapsulation, protection and delivery of bioactive components in functional foods because of their ability to form protein-ligand complexes, possibly protecting the bound components against oxidation and degradation and also providing means of stimulus-induced release [11,12]. One of the most studied ligand-binding food proteins is β-lactoglobulin (β-LG), the principal whey protein. β-LG 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 [13]. β-LG has the ability to bind various hydrophobic compounds and drugs such as fatty acids, lipids, aromatic compounds, vitamins and polyamines [14,16]. From a structural point of view, β-LG possesses 16 free amino groups that can act as binding site for potential covalent ligands [17]. However, three

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potential binding sites were reported, with the primary ligand-binding site in the internal cavity of the β-barrel. Some other potential sites are at the surface near Trp^{19}-Arg^{124}, the hydrophobic pocket in the groove between the α-helix and the β-barrel, a site near the aperture of the β-barrel, and a site at the monomer-monomer interface of the protein dimer [12,18,19]. 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 [20]. 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 [21]. At the quaternary structure level, the protein is mostly present in monomeric or dimeric form, this equilibrium being significantly influenced by the environmental conditions.

The main objective of this study was to advance the knowledge regarding the binding of lycopene extracted from tomato peels to whey protein, from the perspective of micro- and nanoencapsulation. The investigations assumed the use of fluorescence spectroscopy quenching experiments and molecular docking and molecular dynamics simulation methods. The obtained results describe in detail the protein-ligand binding particularities, facilitating the selection of the method and encapsulation conditions.

2. Materials and methods

2.1. Materials

β-LG (purity > 90%, genetic variants A and B) from bovine milk was purchased from Sigma (Sigma–Aldrich Co., St. Louis, MO). Unless otherwise stated, all other reagents were of analytical grade.

Fresh ripe tomatoes were purchased from a local market and stored at 4 °C for a maximum of 2 days before use. After removal of damaged parts and washing, whole tomato fruits were immersed in boiling water for 1–2 min. Then they were cooled under tap water and hand peeled. The peels were dried in air on tissue paper at room temperature (20–22 °C) in the dark for 4–5 h. Then they were wrapped in aluminum foil and stored at 4 °C for not more than 24 h before use [22].

2.2. Lycopene extraction

Five grams of dried tomato peels 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 lycopene extract was concentrated at 40 °C to dryness, dissolved in 10 mL ethanol (70%) and filtered through 0.45 μm membranes. The lycopene was quantified using a colorimetric method. Optical density of the extract was measured spectrophotometricaly at 503 nm against a hexane blank. Concentration of lycopene in extract was calculated using the extinction coefficient of 3150 M^{-1} cm^{-1} [23].

2.3. HPLC quantification of carotenoids from the extract

In order to identify and quantify the carotenoid pigments from tomato peels extract, a chromatographic analysis was performed. The system used was an HPLC from Thermo Finnigan Surveyor (Finnigan Surveyor LC, Thermo Scientific, SUA), controlled by Xcalibur software system. The carotenoids from each sample were analyzed at 450 nm on a Lichrosorb RP-18 (5 μm) Hibar RT 125–4 column. The elution solvents were 90% acetonitrile (A) and 100% ethyl acetate (B). The injection volume was 20 μL, and the flow rate was maintained at 0.800 mL/min.

The elution profile used was: 0–16 min, 15% B; 16–54 min, 15–62% B, 54–56 min, 62% B; 56–60 min, 62–15% B; 60–70 min, 15% B. The quantification of carotenoids was done using a lycopene calibration curve. The calibration curve for the lycopene standard was prepared using six different concentrations (0.04–0.2 mg/mL), by dissolving in ethyl acetate before the analysis. The linear regression factor of the calibration curve for this standard was 0.991.

2.4. Heat treatment

Plastic tubes (1 cm diameter) were filled with 0.150 mL of β-LG solution. The samples were heated at different temperatures ranging from 25° to 90°C for 15 min, using a thermostatic water bath (Digibath-2 BAD 4, Raypa Trade, Barcelona, Spain). The samples were then cooled in ice water to avoid any further thermal denaturation of the protein.

2.5. Quenching experiments with lycopene extract

The un-treated and heat-treated protein samples (0.100 mL of 1 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 1:10 diluted lycopene extract (LE) in ethanol. 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. 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 Stern-Volmer constants, binding constants and number of binding sites were calculated as previously reported [24].

2.6. Molecular modeling investigation

The crystal structure of the bovine β-LG (4DQ3.pdb; [25]) was taken from the RCSB Protein Data Bank. The β-LG model was preliminary refined by removing all other compounds accompanying the protein, optimized *in vacuum*, solvated by using explicit water molecules and further optimized in the water-like environment. In order to simulate the effect of the thermal treatment applied to the β-LG in the laboratory scale experiment, the solvated model was further heated at 25 °C, 60 °C and 90 °C using a Berendsen thermostat and finally equilibrated through the procedure reported by Aprodu et al. [26], such as to reduce any temperature and energy oscillations of the systems. GROMACS 5.1.1. package [27] was used for performing molecular dynamics simulations in parallelization conditions on the High Performance Computer System (HPC) from *Dunarea de Jos* University using Intel E5 2680 v3, 12-cores, 2.5 GHz.

The β-LG models equilibrated at different temperatures served as receptors for the lycopene molecule in the docking procedure, based on the use of molecules shape complementarity principles. The docking solutions generated through PatchDock algorithm [28] were further optimized by adjusting the relative orientation of the molecules within complexes through FireDock web server [29].

For each simulated temperature, analysis of the best docking results, decided based on the binding energy values, was performed using PDBsum [30], PDBePISA [31], DoGSiteScorer web server [32] and LigPlot+ [33] tools to gather details on structure and interaction particularities within complexes.

2.7. Data analysis

Data were analyzed using the Stern Volmer equation (Eq. (1)):

$$\frac{F_0}{F} = 1 + K_{SV}[LE] \quad (1)$$

where F_0 and F are the fluorescence intensities of β-LG in the absence