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Self-assembled β-lactoglobulin–conjugated linoleic acid complex for colon cancer-targeted substance

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ABSTRACT

β-Lactoglobulin (β-LG) is a member of the lipocalin protein family and can bind a variety of hydrophobic molecules, such as fatty acids, in vitro. In this study, a potential colon-targeted antitumor drug was developed using bovine β-LG as a carrier loaded with *cis*-9, *trans*-11 conjugated linoleic acid (CLA). The intrinsic tryptophan fluorescence intensity of β-LG monitored by spectrofluorometer showed that 2.46 mol of CLA can be bound per mole of β-LG. Dynamic light scattering showed the formation of a β-LG-CLA self-assembled complex with particle size of 170 ± 0.08 nm. After treatment with gastrointestinal pH and digestive enzymes, β-LG-CLA complex showed very good stability in gastrointestinal conditions in vitro, measured by zeta potential analyzer and sodium dodecyl sulfate PAGE, respectively. In an intestinal model in vitro, the concentration of CLA in Caco-2 cells was detected by reverse-phase HPLC, and the level of CLA in cells after treatment with β-LG-CLA complex was significantly greater than after treatment with CLA, which means β-LG served as a capsular vehicle of CLA for intracellular transport. According to cell proliferation assay, β-LG-CLA complex can inhibit the viability of Caco-2 cells, and the inhibition rate is significantly greater than with the same concentration of CLA (100 μ*M*). The study revealed that bovine β-LG as a carrier binding with CLA can potentially be used for colon cancer therapy.

Key words: beta-lactoglobulin, conjugated linoleic acid, colon cancer, targeted substance

INTRODUCTION

Bovine β-LG, with an 18.4-kDa molecular mass for the monomer and 162 AA residues, is the major whey protein in cow's milk, and its properties have been studied extensively (Pervaiz and Brew, 1985). The secondary structure of β-LG has been reported: an 8-stranded antiparallel β-barrel forms a conical central calyx, with a 3-turn α-helix on the outer surface of the β-barrel and a ninth β-strand flanking the first strand (Papiz et al., 1986; Brownlow et al., 1997). The central cavity of β-LG provides a ligand-binding site for hydrophobic molecules, and this special property makes β-LG a core member of the lipocalin family, which shows a variety of biological functions related to the binding and transport of metabolites (Flower, 1994). It has been reported that β-LG has an observable affinity for retinol (Futterman and Heller, 1972) and many fatty acids, such as palmitic acid (Wu et al., 1999; Ragona et al., 2000), n-3 polyunsaturated fatty acids (Zimet and Livney, 2009), conjugated linoleic acid (**CLA**), and myristic acid (Considine et al., 2007), and in cow's milk, the main ligands that have been found to bind to β-LG are fatty acids (Pérez and Calvo, 1995). Therefore, it is likely possible to make a self-assembled complex by bovine β-LG binding *cis*-9, *trans*-11 CLA (***c*9,*t*11-CLA**).

Conjugated linoleic acid was originally described as an anticarcinogen isolated from grilled ground beef (Ha et al., 1987). As positional and geometric isomers of linoleic acid that have conjugated double bonds, several isomers of CLA have been identified. It has been found that ruminant dairy products are good sources of CLA isomers, especially *c*9,*t*11-CLA (Kay et al., 2004). Many publications have reported CLA isomers altering carcinogenesis, with the main focus on 2 major forms: *c*9,*t*11-CLA and *trans*-10, *cis*-12 CLA (***t*10,*c*12-CLA**). However, the growth inhibitory effects of CLA isomers varied with the model used, such as mammary, colon, and stomach cancer in vitro and in vivo, and different CLA isomers act through different mechanisms of inhibiting tumor growth (Kelley et al., 2007). There are very few studies of *c*9,*t*11-CLA in inhibiting cell growth of colon, colorectal cell lines, and more studies are needed to determine a preferable concentration of CLA during antitumor treatment.

Colorectal cancer is the third most common cause of cancer-related death worldwide (Parkin et al., 2005). Finding an effective therapeutic approach for colorectal

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cancer is very important to inhibit the rate of recurrence. A colon-specific drug-delivery system has the potential to cure bowel disease, including colorectal cancer (Zhao et al., 2008). Because the physiological conditions along the gastrointestinal tract include various enzymes and drastic changes in pH, which make the drugs likely to be absorbed or degraded before reaching colon, a special dosage form is required for the oral colon-specific drug-delivery system to the colon. It has been reported that β-LG can resist digestive enzyme hydrolysis in the gastrointestinal tract (Reddy et al., 1998) and remain intact while reaching the upper portion of the small intestine. In addition, β-LG is stable in low-pH conditions and could play a protective role for bound ligands in the acidic conditions of the stomach (Papiz et al., 1986). Thus, to some extent, β-LG has the ability to be a coating for CLA delivery to the colon.

In the present research, we synthesized a self-assembled β-LG-CLA complex beyond the hydrophobic ligand binding property of bovine β-LG, observed its stability in gastrointestinal conditions, detected the absorption level of CLA in the colon model after treatment with β-LG-CLA complex, and measured the inhibition effect of β-LG-CLA complex on colon cancer to evaluate whether this self-assembled complex has the potential to be a colon-specific drug for antitumor application in colon carcinoma.

MATERIALS AND METHODS

Materials

Bovine β-LG A was purchased from Sigma Chemical Company (St. Louis, MO), and the purity was >99%. The *c*9,*t*11-CLA was purchased from Cayman (Ann Arbor, MI), and the purity was >98%. Human colon tumor cancer Coca-2 cells were obtained from Cell Bank, Chinese Academy of Science (Shanghai, China). The 24-well and 96-well plates and a 6-well transwell plate were purchased from Corning Costar Corporation (Cambridge, MA). Pepsin and trypsin; Dulbecco's modified Eagle's medium (**DMEM**) and fetal bovine serum for cell culture; and acetonitrile, methanol, ethyl acetate, and acetic acid for reverse-phase HPLC (**RP-HPLC**) were all purchased from Sigma Chemical Company.

Preparation of β-LG-CLA Complex

The β-LG-CLA was synthesized beyond the hydrophobic ligand binding property of bovine β-LG (Wu et al., 1999; Ragona et al., 2000; Kontopidis et al., 2004) with a titration experiment for *c*9,*t*11-CLA binding to bovine β-LG. Intrinsic fluorescence of the tryptophan residues of bovine β-LG was measured before and after the addition of different amounts of CLA, which was predissolved in ethanol (10 mg/100 μL), to 1 μ*M* bovine β-LG solution. The CLA added ranged from 2 to 12.4 μL with 2 incremental aliquots, and bovine β-LG was dissolved in 2.5 mL of 100 m*M* Tris-HCl buffer solution with a pH of 7.0. After vigorous stirring, the β-LG-CLA mixtures (molar ratio range from 1:1 to 1:3) were incubated for 10 min at room temperature before the measurements. The binding parameters were studied by measuring the binding-induced quenching of the intrinsic Trp 19 fluorescence of the protein, using a fluorescence spectrofluorometer (Hitachi, Tokyo, Japan), at excitation and emission wavelengths of 279 and 332 nm, respectively (Christiaens et al., 2002). Measurements were performed in triplicate. The number of CLA molecules involved in binding per β-LG molecule was calculated by fluorescence intensity, expressed as the percentage of the initial fluorescence of CLA-free β-LG versus that with the added CLA concentration. The raw data were analyzed according to the model described by Christiaens et al. (2002). After the suitable binding molar ratio between β-LG and CLA was obtained, the β-LG-CLA complex was prepared by the titration experiment mentioned earlier, and the particle size at pH 7.0 was measured by dynamic light scattering using a Delsa Nano C particle size and zeta potential analyzer (Beckman Coulter, Brea, CA). Then, β-LG-CLA complex was synthesized and stored with 0.1% Tween 40 to keep a micelle state for the following research.

Stability of β-LG-CLA in the Gastrointestinal System

The stability characteristics of β-LG-CLA were considered by electrophoretic mobility under a wide range of physical pH conditions, and the degree of digestion with various digestive enzymes in vitro was also studied. To investigate the stability of β-LG-CLA under stomach and intestinal pH-value conditions, the complex solutions contained were titrated at pH 1.0, 1.2, 1.4, 1.8, 2.4, 2.8, and 3.0 to imitate the acidic environment of the stomach and pH 6.0, 6.2, 6.4, 6.8, 7.0, 7.2, and 7.4 to imitate the intestinal environment. A zeta potential analyzer (Delsa Nano C, Beckman Coulter) was used under a 3-V/cm electric field at 25°C to detect the electrophoretic mobility of the complex.

To investigate the stability of β-LG-CLA in the presence of digestive enzymes, pepsin and trypsin were used as digestive enzymes. The in vitro digestion experiments were performed at 37°C with an enzyme–substrate ratio of 1:100 (wt/wt) using 0.5 mg/mL enzyme and 5 mg/mL protein solutions. The pH for pepsin digestion was titrated at 1.2 using 1 mol/L HCl, and for trypsin