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Study on the Anti-tumor Activity and Structural Changes of Bovine Lactoferrin-Oleic Acid and Linoleic Acid Complex

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Bovine lactoferrin (LF) was used to prepare complexes with oleic acid (OA) and linoleic acid (LnA), the anti-tumor activity of the lactoferrin-oleic acid complex (LF-OA) and lactoferrin-linoleic acid complex (LF-LnA) was measured on liver cancer, colon cancer and breast cancer cells using the methylene blue method. The structure changes of LF induced by binding of OA and LnA, including hydrophobic amino acids, hydrophobic regions, tertiary structure, secondary structure, was studied by intrinsic fluorescence, ANS-binding intrinsic fluorescence and circular dichroism spectrum, respectively. Both of LF-OA and LF-LnA exhibited profound anti-tumor activity on the three types of tumor cells. A significant red-shift of LF occurred after binding of OA and LnA, respectively. Meanwhile, the ANS-binding spectra of LF exhibited a blue-shift with an increasing fluorescence intensity. It can be indicated from the fluorescence spectra results that binding of OA and LA lead to the exposure of hydrophobic amino acids and hydrophobic regions. Furthermore, circular dichroism spectra suggested a partial loss of the tertiary structure, a decrease in the content of α-helix and β-turn with the increase of β-sheet and random coil structure. Here in this study, we successfully prepared the two anti-tumor LF-OA and LF-LnA complexes, which exhibited a profound prospect of cancer therapy.

1. Introduction

Epidemiologic research demonstrated that breast-feeding infants and young children exhibited a significant lower incidence in intestinal infection, respiratory infections and urinary tract infections (Schack-Nielsen and Michaelsen (2006)). Furthermore, breast-feeding protects both mother and child against tumor development, especially lymphoma (Martin et al. (2005); Shu et al. (1999)). The active fractions originating from breast-milk have been widely studied, the most popular fraction was the α-lactalbumin-oleic acid complex (α-LA-OA) (Hakansson et al. (1995)). It has been verified that α-LA-OA could selectively kill tumor cells both in the animal model and the clinical trials (Fischer et al. (2004); Mossberg et al. (2010); Gustafsson et al. (2004); Mossberg et al. (2007)). Due to the profound anti-tumor activity and application in cancer therapy, many researchers focused on prepared similar complexes using α-lactalbumin with different kinds of fatty acids (Svensson et al. (2003)) or other proteins with oleic acid (OA) (Vukojević et al. (2010)).
Lactoferrin (LF) is a globular, iron-binding protein that is widely represented in various secretory fluids, such as milk (Sánchez, Calvo and Brock (1992)). The structure of the iron-binding region of LF was similar to that of α-LA (Nuijens, van Berkel and Schanbacher (1996)). It was reported that both of the two proteins releases the bound ions at acid pH (Ohashi et al. (2003)) to yield a more open structure (Brisson, Britten and Pouliot (2007); Baker and Baker (2009)), which favors the binding of OA (Casbarra et al. (2004); Fast et al. (2005)). Therefore, in this study, we obtained complexes of LF with OA and linoleic acid (LnA). The anti-tumor activity was evaluated by methylene blue assay, meanwhile, the structural changes of LF was also measured by fluorescence spectroscopy and circular dichroism.
In this paper, a stabilized single frequency Brillouin fiber laser is experimentally demonstrated without using expensive instruments or complicated system. And a passive configuration using Mach-Zehnder

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interferometer is used to eliminate the polarization noise in the BOTDR. A BOTDR for distributed measurement over long range sensing fiber has been developed using the reference Brillouin laser.

2. Materials and Methods

2.1 Materials and Equipments

Bovine LF (95% purity), OA (C18:1:9 cis, ≥ 99.0% purity, cell culture tested), LnA (C18:2:9, 12 cis, ≥ 99.0% purity, cell culture tested) and 1-anilino-8-naphthalenesulfonate (ANS) were obtained from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) containing a high level of glucose, RPMI-1640, fetal calf serum, HEPES buffer solution, penicillin and streptomycin was purchased from Gibco (Life Technologies, USA). All the other chemicals used were of analytical grade.

Fluorescence spectrophotometer (RF-5301PC, Shimadzu, Kyoto, Japan), circular dichroism (JASCO J-810, Jasco, Japan), electrothermostat (DK-8B, Jinghong, Shanghai, China), Multiskan Spectrum Microplate Spectrophotometer (Infinite 200 Pro, TECAN, Switzerland).

2.2 Methods

2.2.1 Preparation of lactoferrin-oleic acid (LF-OA) and lactoferrin-linoleic acid (LF-LnA) complexes

LF was dissolved in phosphate buffer solution (PBS, 10 mM, pH 8.0) to a final concentration of 120 μM. OA or LnA was directly added into the protein solution at 50 molar equivalents (OA/LnA: LF). The mixtures were then incubated at 45°C in a water bath for 20 min after vortexing for 30 s. Excess fatty acid in the complexes was removed by centrifugation at 4°C followed by ultrafiltration using a 3000 kDa cut-off membrane (Sartorius). The LF solution was treated as above but without fatty acids acted as the control LF.

2.2.2 Cell viability assays

Cell viability was tested by methylene blue assay according to Felice et al.(2009). Briefly, cells were seeded in 96-well plates (Corning, USA) at a density of 1×104 cells/well and grown for 24 h. The medium was then removed, LF-OA and LF-LnA were added into a new medium without FBS to the final concentration of 1, 2, 4, 6, 8 μM and 2, 4, 6, 8, 10 μM, respectively. FBS was added into each well at a final concentration of 10% after 30 min. After 24 h incubation at 37°C, the medium was removed and cells were washed with PBS for 3 times. Then 50 μL methylene blue staining solution was added and incubated at 37°C for 1 h. After removing the media and washing with distilled water to clarification, 100 μL eluent was added and incubated for 20 min under room temperature. The absorbance was determined under 570 nm wavelength.

2.2.3 Intrinsic fluorescence spectra

A path length of 1 mm was used for all of the experiments. The emission spectra were collected between 300 and 500 nm with an excitation wavelength of 292 nm and a scan interval of 0.2 nm. The slit widths for excitation and emission were set at 3 nm. All of the experiments were repeated three times. The protein solutions were diluted with the corresponding buffer to a final concentration of 30 μM.

2.2.4 ANS-binding fluorescence spectra

Each protein sample was incubated with 50-fold ANS for 15 min at room temperature in the absence of light. The emission spectrum of ANS was recorded between 400 and 600 nm at a scan interval of 0.2 nm with an excitation wavelength of 390 nm. The emission fluorescence intensities of ANS were also measured to exclude the influence of unbound ANS. The slit widths for excitation and emission were set at 3 nm. The protein concentration was set at 30 μM for all of the measurements. A path length of 1 mm was used for all of the experiments. All of the experiments were repeated three times.

2.2.5 Circular dichroism

A path length of 1 mm was used for all of the experiments. The scan rate was set at 50 nm per min with an interval of 0.5 nm, and the response time was set as 8 s. Each spectrum was averaged from three scans. Baseline spectra were recorded for the buffer and subtracted from the sample. The protein concentration was set at 30 μM for all of the measurements. The secondary structure was calculated using the CD Pro software package (Jasco Corporation, Tokyo, Japan). All of the experiments were repeated three times.

2.2.6 Statistical analysis

Data are presented as mean values ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by the Student's t test and Duncan's test.

3. Results and Discussion

3.1 Antitumor activities of LF-OA and LF-LnA

The cell viability of HepG2, HT29 and MCF-7 cells after treatment with LF-OA and LF-LnA for 24 h was measured using the methylene blue assay (Fig. 1). The results revealed that increasing the concentration of LF-OA or LF-LnA led to a significant decrease ($p<0.01$) in the cell viability of the cells. According to the

concentration–cell viability curve, the half lethal dose (LD_{50}) values of LF-OA and LF-LnA were 4.88 and 9.14 μM towards HepG2 cells, 4.95 and 8.78 μM towards HT29 cells, 4.62 and 7.01 μM towards MCF-7 cells. When compared the activity with α-LA-OA (Fang et al. (2012)), the LD_{50} values of LF-OA and LF-LnA were only 10% of the value of α-LA-OA. Under all of the conditions tested in Fig. 1, the control LF solutions did not influence the cell viability (p>0.05). Furthermore, no anti-tumor activity was detected when treated cells with OA and LnA alone prepared by the method for the complexes.

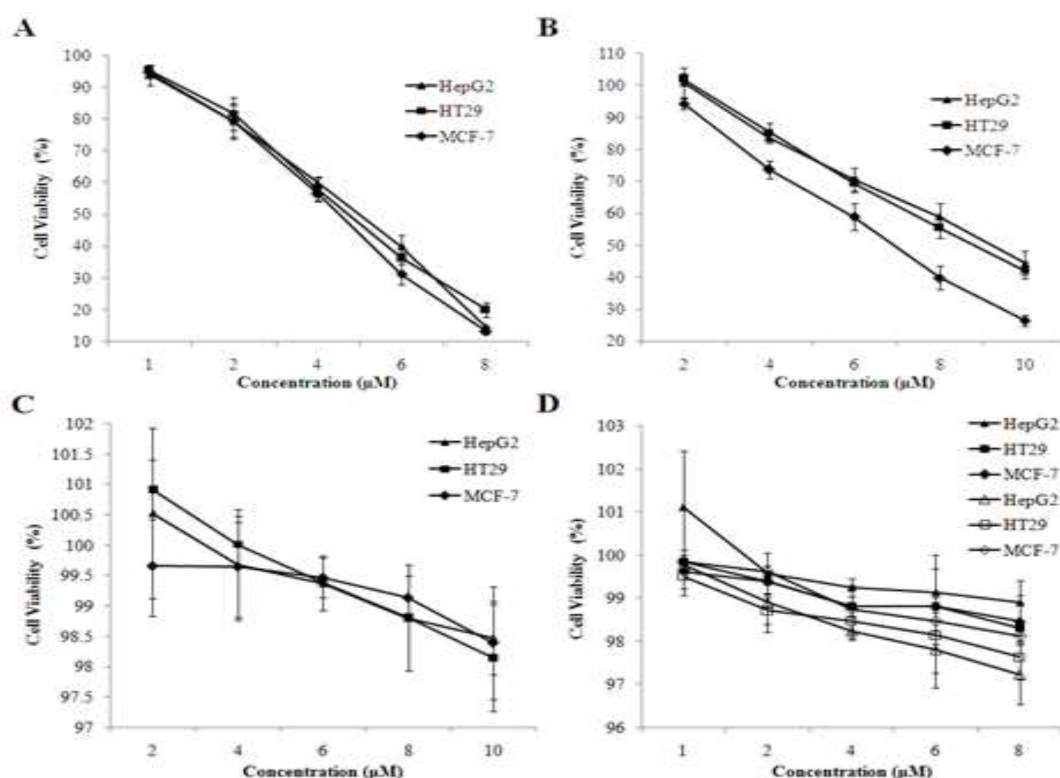

Figure 1: Cell viability after treatment with lactoferrin-oleic acid complex (A), lactoferrin-linoleic acid complex (B), lactoferrin (C) and fatty acids alone (D) prepared by the method for the complexes for 24 h on HepG2, HT29 and MCF-7 cells.

Note: In Fig.1D, the filled symbols corresponded to the control OA solutions while the blank symbols corresponded to the control LnA solutions.

3.2 Changes in the exposure of hydrophobic amino acid in LF induced by OA and LnA

Hydrophobic amino acids in the protein such as tryptophan (Trp) are very sensitive to a change of environment and the intrinsic fluorescence can be used to probe the flexibility of protein structure (Fang et al. (2012)). The intrinsic fluorescence emission spectra of LF, LF-OA and LF-LnA were measured to determine the changes of the exposure of hydrophobic amino acid induced by OA and LnA (Fig. 2). A significant red-shift was observed in the intrinsic fluorescence going from LF (332.87±0.50 nm, curve a) to LF-OA (339.10±0.71 nm, curve b) and LF-LnA (339.70±0.14 nm, curve c), indicating that the Trp residues were exposed to the solvent

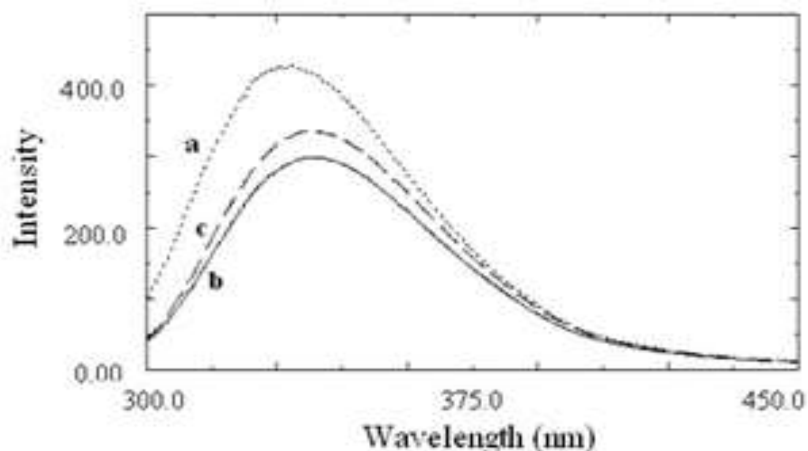

Figure 2: Intrinsic fluorescence emission spectra of LF (a), LF-OA (b) and LF-LnA (c)

3.3 Changes in the exposure of hydrophobic regions in LF induced by OA and LnA

ANS can bind to hydrophobic clusters in the protein and the changes in its fluorescence emission spectrum clearly indicate the folding intermediate state of the protein (Fang et al. (2012)). The λ_{max} of ANS decreased dramatically once binding to the proteins and was accompanied by an enhancement of the fluorescence intensity (Craig et al. (2009)). The ANS-binding spectra of LF with and without OA or LnA were measured by fluorescence spectroscopy (Fig. 3). According to the results in Table 1, it can be seen that both of the LF-OA and LF-LnA exhibited a significant blue-shift and an increased intensity compared with LF. These changes indicated that LF-OA and LF-LnA exposed more hydrophobic clusters than the control LF.

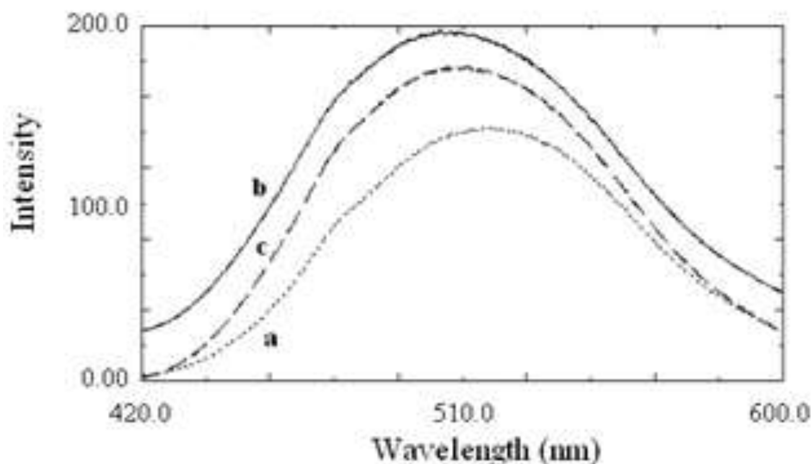

Figure 3: ANS-binding fluorescence emission spectra of LF (a), LF-OA (b) and LF-LnA (c)

Table 1: λmax and the fluorescence intensity of the ANS-binding spectra of LF, LF-OA and LF-LnA

	LF	LF-OA	LF-LnA
λ_{max} (nm)	511.7±1.8	504.8±1.4	507.6±0.6
Intensity	54.5±3.1	82.7±4.4	71.2±2.2

3.4 Changes in the secondary and tertiary structures of LF induced by OA and LnA

Near-UV (Fig. 4A) and far-UV (Fig. 4B) circular dichroism spectroscopy were used to compare the secondary and tertiary structures of LF following the binding of OA and LnA. Near-UV circular dichroism spectroscopy reflects the rigidity of the aromatic amino acid side chains in the protein, the increase in the ellipticity corresponded to the stability of protein tertiary structure. As shown in Fig. 4A, LF-OA and LF-LnA exhibited

decreased signal intensities compared with the LF control solution, indicating that the binding of OA and LnA led to the loss of the tertiary structure of LF.
The contents of the secondary structures were calculated based on their far-UV CD spectra (Fig. 4B) based on the Yang's reference (Table 2). The binding of OA and LnA to LF led to a decrease in the content of α-helix and β-turn, as well as an increase in the β-sheet and random coil structure.

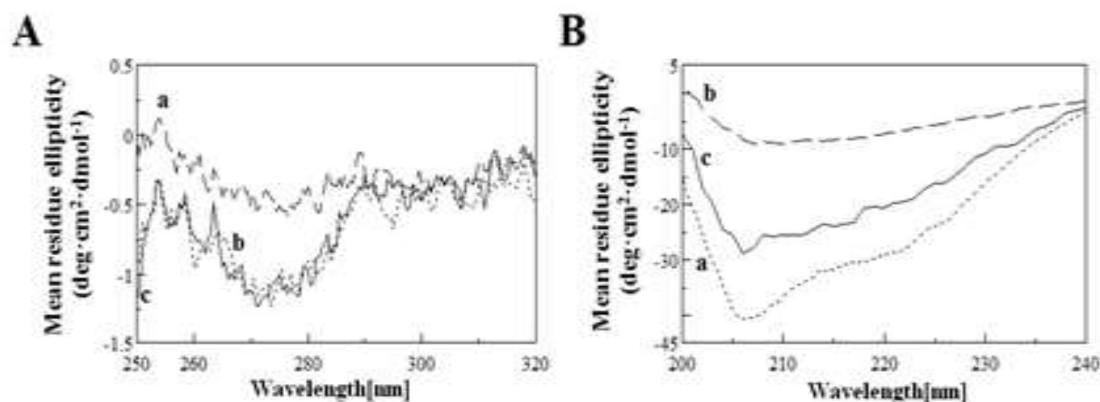

Figure 4: Near-UV (A) and far-UV (B) circular dichroism spectra of LF (a), LF-OA (b) and LF-LnA (c)

Table 2: Content of the secondary structure of LF, LF-OA and LF-LnA

Content/%	LF	LF-OA	LF-LnA
α-helix	19.0±0.6	12.5±0.5	13.7±0.4
β-sheet	30.5±0.9	35.2±1.8	34.0±1.5
β-turn	20.7±0.4	16.9±1.5	18.3±0.7
Random coil	29.8±0.1	35.5±0.5	34.0±0.3

4. Conclusion

This study revealed that LF could bind OA and LnA and exhibited a strong anti-tumor activity in HepG2, HT29 and MCF-7 cells, which was significant stronger than α-LA-OA. Structural changes of LF induced by binding OA and LnA were similar to that of α-LA-OA, including the exposure of Trp residues and hydrophobic regions, loss of tertiary structure and decreased content of α-helix and β-turn structures. Our study confirmed the presence of α-LA-OA-like complexes, which exhibited a profound prospect of cancer therapy.

Acknowledgements

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