

## Stabilization of liposomal membranes by carotenoids: Zeaxanthin, zeaxanthin glucoside and thermozeaxanthin

Masayuki Hara<sup>a,d,\*</sup>, Yumiko Yamano<sup>b</sup>, Yoshitsugu Sakai<sup>b</sup>, Eri Kodama<sup>d</sup>, Takayuki Hoshino<sup>c</sup>,  
Masayoshi Ito<sup>b</sup>, Jun Miyake<sup>d</sup>

<sup>a</sup> Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan

<sup>b</sup> Kobe Pharmaceutical University, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan

<sup>c</sup> Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8573, Japan

<sup>d</sup> Research Institute for Cell Engineering, AIST, 3-11-46 Nakoji, Amagasaki, Hyogo 661-0974, Japan

Received 22 September 2005; received in revised form 10 August 2006; accepted 12 October 2006

Available online 23 January 2007

### Abstract

We investigated the membrane-stabilizing effect of three synthesized polar carotenoids, thermozeaxanthin (TZ), zeaxanthin glucoside (ZG) and zeaxanthin (Z), using the fluorescent calcein-leakage measurement from the calcein-entrapped liposomes composed of dipalmitoylphosphatidylcholine (DPPC). The addition of TZ stabilized the liposomal membranes composed of DPPC at pH values ranging from 4.0 to 10.0. The addition of three carotenoids, TZ, ZG and Z, stabilized the membrane at acidic and neutral pH values ranging from 4.0 to 7.5. The values of leakage were lower at 30 °C and highest at 40 °C, and subsequently, gradually decreased at the higher temperature. The addition of TZ and Z stabilized the membranes, whereas, ZG destabilized the membranes at a temperature higher than 50 °C. In addition, the membrane-stabilizing effect of the carotenoids with the calcein-entrapped liposomes composed of lipids extracted from *Thermus thermophilus* were investigated. The addition of TZ resulted in stabilization of the membrane at all ranges of pH values. However, the addition of Z and ZG destabilized the membrane.

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**Keywords:** Thermozeaxanthin; Zeaxanthin; Liposome; Membrane; Stabilization; *Thermus thermophilus*

### 1. Introduction

Many researchers have shown an interest in the mechanism of the thermo-stability of bacterial cell membranes within thermophilic bacteria. It has been previously reported that the thermophilic bacteria *Thermus thermophilus* contain many glycolipids in which C<sub>15</sub> and C<sub>17</sub> branched fatty acids are contained, whereas, generally mesophilic bacteria contain a higher content of phospholipid in bacterial membranes [1,2]. The characteristic chemical composition of the lipid causes stability of bacterial membranes at temperatures around 70–80 °C. Another feature of thermophilic bacterial lipid composition is the high content of carotenoid compounds. Several bacterial strains

belonging to the genus *Thermus*, have a yellow or red colour as they contain carotenoids. It has been previously reported that the content of total phospholipid, i.e., glucolipid and carotenoid, increased when the growth temperature was increased from 50 to 75 °C [3]. Therefore, we believe that the presence of a sugar moiety in the glycolipids, the presence of a branched fatty acid chain, and a high content of carotenoid, can contribute to the thermal stability of the bacterial membrane in *T. thermophilus*.

Recently, several novel polar carotenoids have been found and have attracted much attention [4,5]. Thermozeaxanthins, carotenoid-glucoside esters existing in the cell membrane of the thermophilic bacterium *T. thermophilus*, were observed by Yokoyama et al. [4]. and were thought to stabilize the lipid membrane by inserting its hydrophobic core into the lipid bilayer [4]. Results from our experiments demonstrated that the TZ extracted from bacteria and the synthesized TZ, actually stabilized liposomal membranes composed of phospholipids

\* Corresponding author. Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan. Tel./fax: +81 72 254 9842.

E-mail address: [hara@b.s.osakafu-u.ac.jp](mailto:hara@b.s.osakafu-u.ac.jp) (M. Hara).

[6,7]. To further characterize the membrane-stabilizing effect of TZ, the calcein leakage was measured at various pH values using DPPC liposomes. However, the question as to whether TZ stabilized the membranes of thermophilic bacterial lipids remains. Therefore, we investigated the membrane-stabilizing effect on the liposomes composed of *T. thermophilus* HB27 lipids. The results obtained from these experiments are described in the present paper.

## 2. Materials and methods

### 2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma (St. Louis, MO, USA). 3,3'-bis [*N*, *N*-di(carboxymethyl)-aminomethyl] fluorescein (calcein) was purchased from Dojindo Laboratories, (Kumamoto, Japan). Sephacryl S-300 was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals used were of analytical grade.

### 2.2. Carotenoids

Thermozeaxanthin and zeaxanthin-mono- $\beta$ -D-glucopyranoside were synthesized according to a method previously reported [7]. The structure of the carotenoids Z, ZG and TZ is shown in Fig. 1.

### 2.3. Extraction of lipids from *T. thermophilus*

The thermozeaxanthin-deficient mutant strain of *T. thermophilus* HB27Cr2 was cultured in TM medium aerobically at 70 °C [8,9]. The bacterial cell body (48.8 g wet weight) was harvested from the 10 l culture by centrifugation (6000 rpm, 20 min), washed with 10 mM Tris-HCl (pH 7.5) and resuspended in 50 ml of the same buffer. Lysozyme was added to the suspension (4.2 mg/ml) and incubated at 37 °C for 30 min. Chloroform (100 ml) and methanol (200 ml) were mixed with the suspension and the lipids were extracted for 12 h at 4 °C. All following procedures for extraction of the lipids, except where otherwise stated, were carried out under a nitrogen atmosphere to prevent oxidation of the lipid. After centrifugation at 5000 rpm for 10 min at 4 °C, the supernatant was transferred to a flask, mixed with 95 ml methanol and 95 ml distilled water, and subsequently stirred

for 3 h. The chloroform layer (lower layer) was separated from the mixture using a separating funnel and was then evaporated. The lipid was stored as a crude lipid at -70 °C following measurement of the dry weight. The crude lipid was then washed with ice-cold acetone before preparation of the liposomes.

The crude lipid was dissolved in 2 ml chloroform and injected vigorously into a 200-ml ice-cold acetone solution containing 2 mM dithiothreitol (DTT), and was then incubated for 3 h at 4 °C. After centrifugation at 5000 rpm for 5 min, the resultant precipitate (lipid) was collected and dried using stream from nitrogen gas, and was dissolved in 200 ml of diethylether containing 2 mM DTT. After centrifugation for 5 min at 5000 rpm, the supernatant was collected and evaporated. A 140-mg lipid was obtained from the original 10 l bacterial culture. The lipid was re-dissolved in a chloroform solution to a concentration of 100 mg/ml and stored at 20 °C under a nitrogen atmosphere.

### 2.4. Preparation of calcein-entrapped liposomes

Calcein-entrapped large unilamellar vesicles (LUVs) were prepared by the extrusion method as described previously [6,10,11]. Briefly, 20 mg of DPPC was mixed with carotenoids (molar ratio 1.0%) in chloroform and dried to a lipid film by nitrogen flushing and rotary evaporation under a high vacuum. The dried lipid film was dispersed in 2 ml 100 mM calcein (pH 7.5) to form multilamellar large vesicles (MLVs) (lipid concentration 10 mg/ml). The MLV suspension was then frozen in liquid nitrogen and thawed in a water-bath at a temperature ranging from 25 to 50 °C (see below) for five cycles, followed by passing through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Costar, Cambridge, MA, USA.) at 50 °C on a high-pressure vesicle extruder, which was equipped with a temperature control (Lipex Biomembrane, Vancouver, BC, Canada). Unentrapped calcein was removed by gel filtration on a Sephacryl S-300 column (2×35 cm), which had been equilibrated with 50 mM Tris-HCl (pH 7.5) (buffer T). The yield obtained from the DPPC during the preparation of the liposomes using gel filtration was in the range of 15–85%. Calcein-free LUVs were prepared using the same procedures as described above except that buffer T was used to form MLVs instead of the 100 mM calcein solution.

The mean size (relative weight average) of the liposomes was analyzed by dynamic light scattering (DLS) as previously described [11]. A DLS analyzer equipped with an argon laser (488 nm) (DLS-6000 AS, Otsuka Electronics, Osaka, Japan) was used, with the scattering angle set at 90 °C and the temperature set at 25 °C. The liposomes remained in buffer T in the absence of free calcein for the DLS measurement. The values from the main peak were 90.0±33.8 nm for the control liposome (C, DPPC only), 102.4±38.2 nm for the liposome DPPC with zeaxanthin (Z), 127.8±29.8 nm for the liposome DPPC with zeaxanthin-mono- $\beta$ -D-glucopyranoside (ZG), and 93.9±31.1 nm for the liposome DPPC with thermozeaxanthin (TZ).

Preparation of the calcein-entrapped liposomes, consisting of *T. thermophilus* lipid (TTL), was carried out using the same

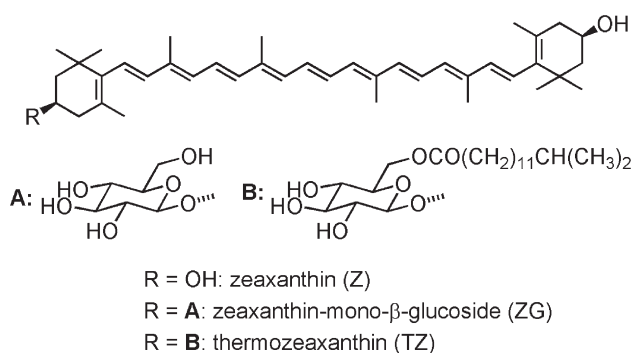


Fig. 1. Structure of the carotenoids: Z, ZG, and TZ used in the experiments.

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