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Resistance to Hypoosmotic Shock of Liposomes Containing Novel Pigments from an Antarctic Bacterium

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Although the antioxidant capacity of carotenoids and their role in regulating membrane fluidity have been well studied, their ability to confer resistance to hypoosmotic shock is poorly understood. In this work, we analyzed the effect of a mixture of carotenoid pigments obtained from an Antarctic microorganism belonging to the genus *Pedobacter* on liposomal resistance to hypoosmotic conditions. Intercalation of pigments into liposomal structures resulted in an improvement of membrane resistance by decreasing the percentage of calcein released in comparison to that by liposomes without pigments. Due to these properties, such pigments could be useful for biotechnological applications.

Keywords: Hypoosmotic shock, liposomes, carotenoids, calcein, Antarctica

Carotenoids are a class of terpenoids, representing one of the most widely distributed and structurally diverse classes of natural pigments. Their colors range from light yellow to deep red [2, 15]. Such pigments are one of the most important nonenzymatic antioxidant defense systems, neutralizing free radicals in cells to overcome oxidative damage [1, 17]. In addition to their well-established function as antioxidants, carotenoids might also play an important structural role in microorganisms [8]. It has been argued that carotenoids and other terpenoids appeared early in evolution, playing an essential role as membrane stabilizers. Indeed, there is no description on cellular membranes that lack terpenoids, suggesting their critical role since the origin of life [13]. As an example, cholesterol stabilizes eukaryotic membranes whereas bacterial membranes are reinforced by hopanoids and carotenoids [6, 14]. In many respects, polar carotenoids (dihydroxycarotenoids such as lutein, zeaxanthin, and violaxanthin) have similar effects on the structure and dynamics of the lipid bilayer to those of cholesterol [18, 19, 21, 20]. Moreover, reports have shown that carotenoids are involved in the regulation of membrane fluidity [8, 9, 22]. This regulation is a crucial strategy adopted by living cells to survive changes in environmental temperature, such as under the harsh conditions of Antarctica. Studies have also indicated that several psychrophilic bacteria from Antarctica contain carotenoid pigments [16, 10], and synthesis of these pigments increases in bacteria grown at 5°C compared to 25°C [3]. Thus, it is possible that the biosynthesis of carotenoids in microorganisms could be involved in regulating membrane fluidity, depending on the growth temperature.

A psychrotolerant aerobic red pigmented bacterium, belonging to the genus *Pedobacter* and designated yelcho2, was isolated from samples taken from Doumer Island, Antarctica during the Chilean Antarctic Scientific Expedition ECA45. This microorganism, as was previously described by Correa-Llantén *et al*. [5], produces different types of pigments that belong to the carotenoid group. These pigments have a range of molecular weights between 703-605 g/mol, with colors from yellow to red [5]. The antioxidant capacity of these pigments was shown to be strong when analyzed by different methods [5]. Additionally, the presence of intercalated yelcho2 pigments in liposomes improved their resistance to UVB-induced lipid peroxidation [5]. Although the antioxidant capacity of carotenoids and their role in the regulation of membrane fluidity have been well studied, their ability to confer resistance to osmotic shock, in particular, to different concentrations of

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NaCl, is poorly understood. Therefore, in this work, we analyzed the effect of the mixture of pigments obtained from the yelcho2 strain on liposomal resistance to hypoosmotic shock.

Growth of the microorganism and extraction of the pigment mixture were carried out as previously described [5]. In order to prepare liposomes, egg yolk phosphatidylcholine and asolectin (both from Sigma) were dissolved in chloroform:methanol (1:2). The organic solvent was removed by evaporation on a rotoevaporator. The thin lipid film thus obtained was dried under vacuum. Lipids were hydrated in a solution containing 420 mM NaCl and 80 mM calcein in 10 mM Tris–HCl buffer, pH 7.0. The suspension was subjected to ten cycles of freezing and thawing in order to obtain multilaminar vesicles and then it was extruded through a polycarbonate filter (pore size, 400 nm) ten times in order to obtain unilaminar vesicles. Excess calcein was removed by molecular-exclusion liquid chromatography using a Sepharose (GE Healthcare) column (1 cm ×15 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0)

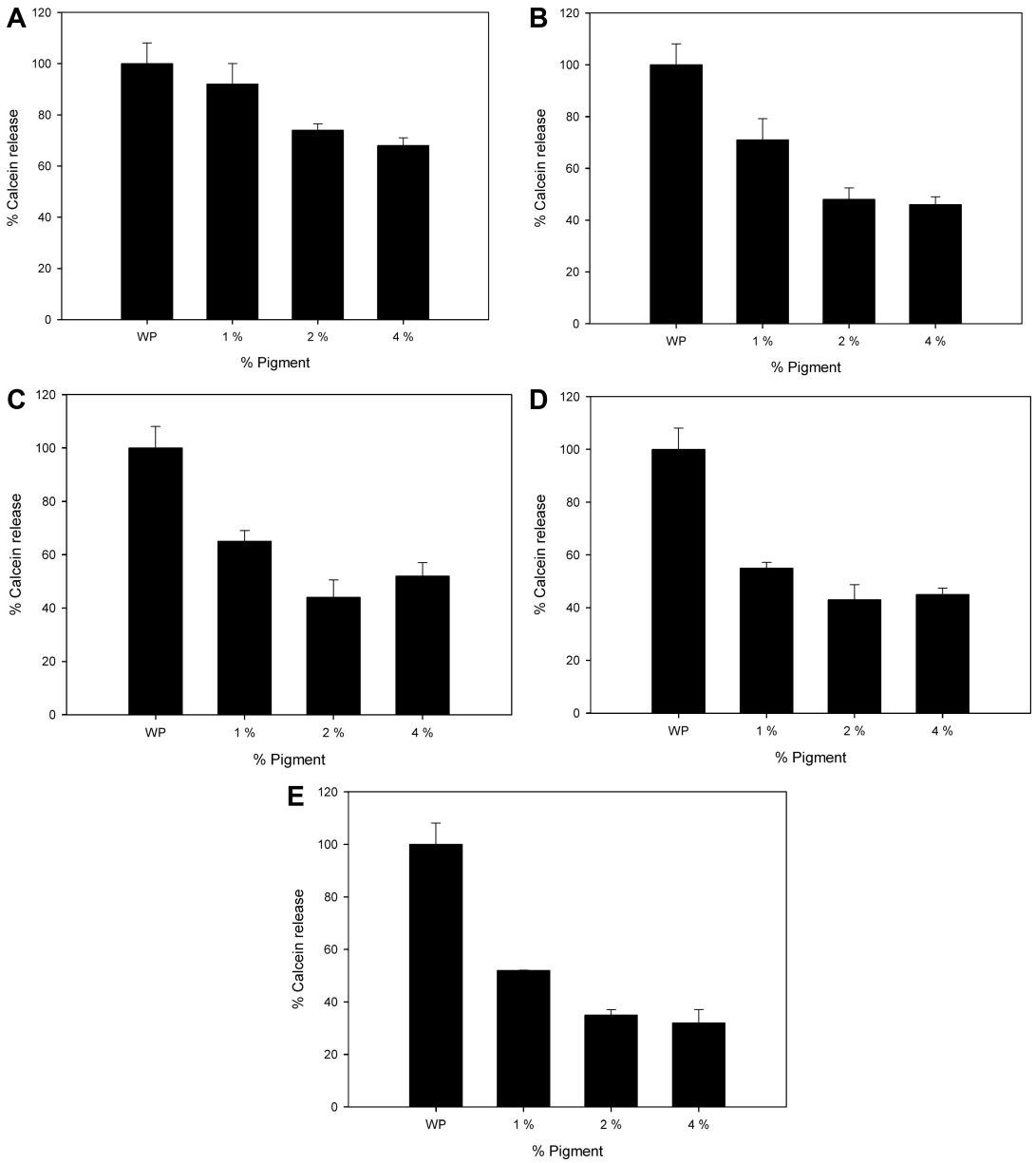

Fig. 1. Liposome resistance to hypoosmotic shock. Asolectin liposomes prepared with different pigment percentages (1, 2, 3 or 4%; WP: without pigments) were subjected to different NaCl osmolarities (0, 42, 84, 210 and 336 mM; A, B, C, D and E, respectively). Calcein release was expressed as a percentage.

containing 420 mM NaCl. All liposomes were maintained in darkness at 4ºC before experiments. Generated liposomes with and without pigments incorporated into their structure, and with encapsulated calcein, were exposed to different NaCl osmolarities. Release of calcein from liposomes was evaluated according to the method of Kuboi *et al*. [12]. Fluorescence intensity due to release of calcein was monitored with a fluorometer (Biotek series FLX 800 TBI) with excitation set at 490 nm and emission at 520 nm as previously described [12].

The behavior observed in Figs. 1 and 2 indicates that the presence of pigments intercalated into liposomes conferred resistance by reducing the degree of liposomal rupture under these conditions, because the percentage of calcein released was decreased in comparison to that by liposomes without pigments. Fig. 1 shows the results with asolectin liposomes with and without pigments in their structure. For all of the NaCl osmolarities tested, calcein release tended to decrease with increasing concentration of pigments in liposomes. Only in the case of the greatest hypoosmotic shock

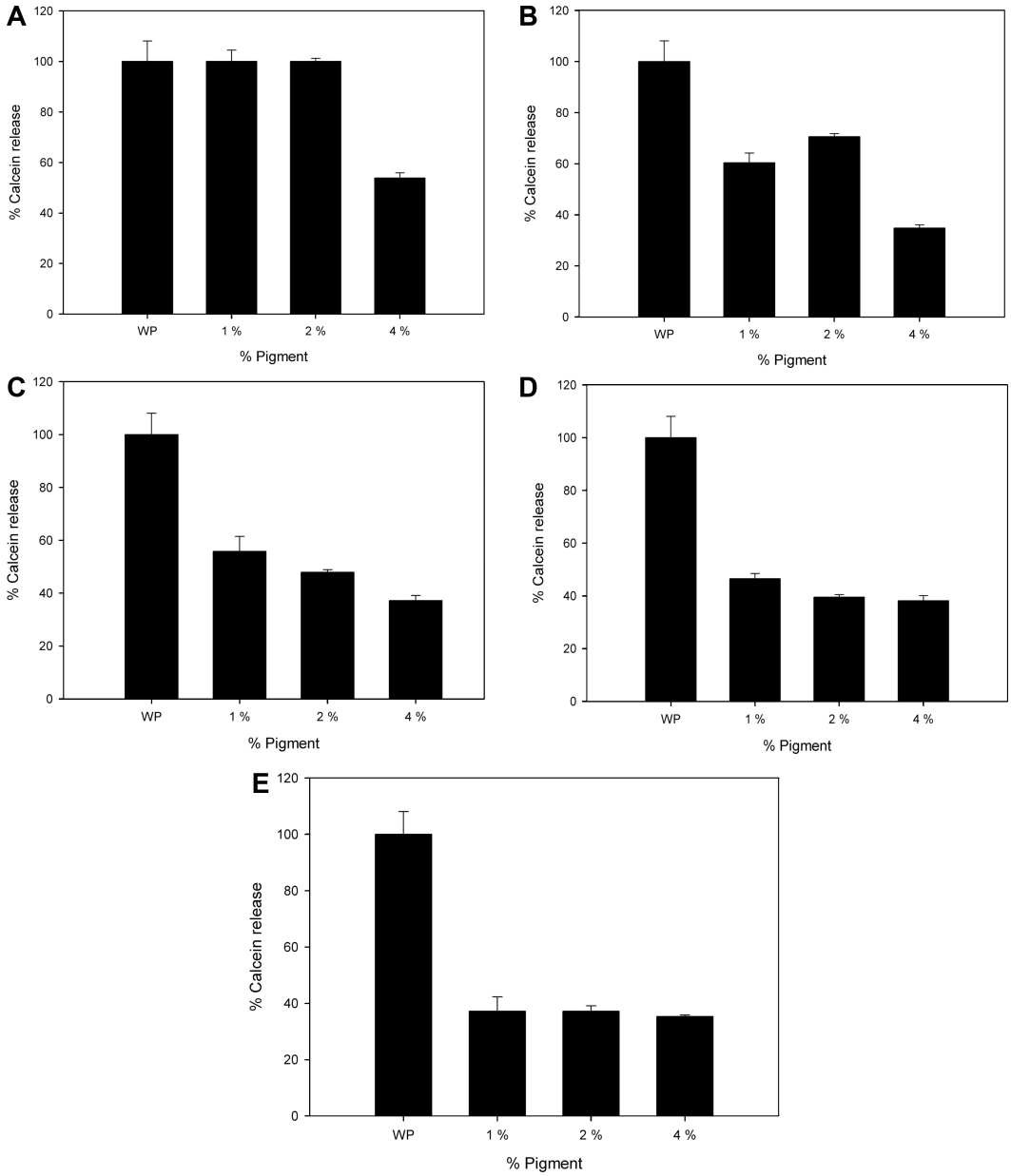

Fig. 2. Liposome resistance to hypoosmotic shock. Egg yolk phosphatidylcholine liposomes prepared with different pigment percentages (1, 2, 3 or 4%; WP: without pigments), were subjected to different NaCl osmolarities (0, 42, 84, 210 and 336 mM; A, B, C, D and E, respectively). Calcein release was expressed as a percentage.

(0 mM NaCl) did liposomes with the lowest pigment concentration (1% w/w) show a release of calcein similar to that without pigments. This result suggests that this pigment concentration was not sufficient to generate protection against and resistance to this degree of osmotic shock. On the other hand, the best protection was observed when 2 and 4% w/w pigments were incorporated into the liposomal structure. Fig. 2 shows the results with egg yolk phosphatidylcholine liposomes with and without pigments in their structure. Unlike the results obtained with asolectin liposomes, here the best protection was observed when 4% w/w pigments were incorporated into the liposomal structure.

These results suggest that the addition of the mixture of pigments from yelcho2 into the liposomes at the studied concentrations (1-4%) significantly affected the physical properties of the membranes, thus playing an important role in protecting against hypoosmotic shock. In addition, these pigments could also affect membrane fluidity, allowing metabolic functions to proceed even at low temperatures [3, 4, 7, 10, 11]. It is probable that the properties presented by the mixture of pigments from the yelcho2 strain are related to their biological function, enabling this microorganism to survive in the low temperature environment of Antarctica. This mixture of pigments could be very useful for biotechnological applications, as demonstrated by the resistance conferred to liposomes, which is a property of interest to the process of drug delivery.

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