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Annual Report

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Title: Production of β-carotene and other carotenoids by the alga *Dunaliella* and their use as a source of vitamin A and pigments in aquatic organisms

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Introduction

The eukaryotic unicellular alga, *Dunaliella bardawil*, is the most β-carotene enriched organism known, containing more than 10% β-carotene on a dry weight basis [1-3]. This unique property and the availability of commercial scale supplies of dry *Dunaliella* powder have attracted attention to the alga as a natural source of β-carotene [4-6].

The β-carotene of *D. bardawil*, and of many fruits and vegetables, is composed mostly of two isomeric forms, all-trans and 9-cis β-carotene, with a minor amount of a few other β-carotene monocis and dicis stereoisomers [7]. It was recently demonstrated that the β-carotene-rich *D. bardawil* can fully replace synthetic β-carotene or retinol as a source of vitamin A in chick and rat diets [8-9]. It was furthermore observed that when the β-carotene was supplied in larger amounts, both the chick and the rat livers accumulated at least a tenfold higher concentration of the algal β-carotene isomer mixture than did livers of animals fed on all-trans β-carotene [10]. The preferential accumulation of the natural isomer mixture of β-carotene was suggested to be due to the higher fat solubility of 9-cis β-carotene and to the tendency of all-trans β-carotene to crystalize at very low concentrations. However, it was not ruled out that this remarkable difference in the bioavailability of the two sources of β-carotene may have been due to a component of the algae which made its β-carotene accumulate to a much higher degree.

In the present first year study we grew *D. bardawil* in outdoor miniponds, harvested the algae by centrifugation and lyophilized the paste to a dry β-carotene-rich powder. In parallel, attempts were made to extract the β-carotene from the algae and partially purify the 9-cis stereoisomer. The bioavailability of the dry algae and of the different isomeric mixtures of β-carotene will be used to compare the natural β-carotene accumulation and conversion in aquatic organisms for vitamin A and pigmentation.

Research Protocol

Alga

Dunaliella bardawil was grown in media containing 2.0 M NaCl in outdoor culture under natural illumination as previously described [1]. Algae were collected by centrifugation and lyophilized. The dry algal powder contained around 4% β-carotene and an ash content of around 30% composed mostly of sodium chloride remaining from the growth medium.

β-carotene preparation

To purify the stereoisomers of β-carotene from *D. bardawil*, pigments were extracted and analyzed according to Ben-Amotz et al. [7]. and dry algae were extracted by repeated extractions with methanol. The pigments were phase separated into hexane with the addition of water and the hexane phase dried under vacuum and flushed with nitrogen. The pigments were redissolved in hexane (1:50, w/v) and placed for 24 h at -20°C for crystallization. Crystals of all-trans β-carotene were removed by filtration. The supernatant was concentrated by vacuum evaporation to a minimal volume in hexane, loaded on a heat activated silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, PA, USA) and washed with hexane. The hexane front fraction was collected, concentrated by flash evaporation and flushed with nitrogen. It contained a mixture of β-carotene stereoisomers with 9-cis β-carotene as the major isomer. The crystals of all-trans β-carotene and the 9-cis enriched mixture were used to make solutions of 30% β-carotene in soybean oil which were kept at -20°C until use. Synthetic β-carotene (30% in soy bean oil) was obtained from Hoffmann La Roche, Basel, Switzerland.

Pigment assays

The freeze-dried samples of the aquatic organisms were preliminarily assayed as described previously [10-12] with the following modifications: 5 ml ethanol were added to 0.5 g of dry sample, and the slurry was homogenized with 15 ml hexane. After mixing, the upper hexane was phase separated with the addition of 3 ml H_2O and centrifugation and was assayed spectrophotometrically at 326-370 nm for retinol and retinyl esters, and at 450-520 nm for total carotenoids. For fat-containing tissues, a saponification step was added to the procedure before extraction.

Isomeric Composition of β-Carotene of Additives

The procedure outlined above for the purification of β-carotene from *Dunaliella* yielded essentially pure crystals of all-trans β-carotene, and a mixture of β-carotene stereoisomers containing predominantly the 9-cis isomer. The latter fraction was further purified by a preparative HPLC column of Vydac with the same features as the analytical Vydac column to yield pure preparations of 9-cis β-carotene and the other β-carotene stereoisomers of *Dunaliella*.

Production Outline

In earlier studies, it was demonstrated that the bioavailability of β-carotene in mammals and poultry was much higher when the animals were fed a diet supplemented with a β-carotene-rich dry powder of *Dunaliella* than with synthetic all-trans β-carotene. The much higher liver β-carotene content in the natural β-carotene-fed animals was attributed to the presence in the algae of the 9-cis isomer of β-carotene, which is more fat-soluble and difficult to crystalize. To test the possibility that an algal component other than the β-carotene may have caused the marked increase in the bioavailability of β-carotene, the stereoisomers were purified as indicated above in preparative concentrations. It should be noted that the chemical synthesis of preparative quantities of β-carotene stereoisomers has not been achieved, irrespective of many trials, by the industrial chemical companies. The availability of preparative quantities of 9-cis β-carotene, 9,13 dicis β-carotene, 13,13 dicis β-carotene, etc., allows for the first time a search into the mode of absorption and accumulation of β-carotene stereoisomers. The HPLC preparative production of nutritional quantities of pure stereoisomers of β-carotene is lengthy and time-consuming. Nevertheless, by the end of 1990 the following products will be available:

a)	Lyophilized dry powder of *Dunaliella*	unlimited quantities
b)	Salt-free dry powder of *Dunaliella*	10 kg
c)	Purified all-trans β-carotene (*Dunaliella*)	50 g
d)	Stereosiomeric mixture of carotenes	50 g
e)	Purified 9-cis β-carotene	10 g
f)	Purified stereoisomers of β-carotene	1 g each

The above quantities of the β-carotene products will allow intensive dietary studies of nutrition on the selected aquatic organisms as outlined in the research proposal. The dietary studies will be initiated in 1991 and will run in parallel to the production of additional quantities of the β-carotene derivatives.

The two investigators, Dr. Ami Ben-Amotz and Dr. Jiamjit Boonsom, have already met a few times in Israel and in Thailand to discuss the mode of cooperation and to start the first phase of the joint project. At the first stage of the project, the Israeli and the Thai research groups worked independently with frequent correspondence and exchange of information on common related issues. The Israeli investigator studied the Thai aquatic organisms which will be tested for feeding on the natural carotenoids. The Thai investigator studied the chemistry of the carotenoids and their analytical evaluation on modern HPLC equipment. Since an HPLC system is not yet available in Thailand, this mode of cooperation is expected to allow the Thai investigator to acquire the basic knowhow in HPLC methodology for use in Thailand. A new system may be purchased later by the Thai institute. In view of the importance of natural food colors in the fishery market in the Far East, the analytical experience which will be gained by the Thai team will be widely used and implemented. The first and second stages of the joint project will be dedicated to studying the extraction of carotenoids from the fish organs, their purification and evaluation on the HPLC system. If successful, the analytical process will be introduced into the Thai market for analyzing carotenoids in different common fishes.

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