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# Distribution of Carotenoids, Vitamin A in Vital Organs of Rats Fed with *Dunaliella bardawil* Whole Cells and Synthetic β-Carotene

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## ABSTRACT

Experimental rats of Wistar strain were fed with carotenoid-rich whole cells of *Dunaliella bardawil* and synthetic β-carotene for 14 days. The accumulation of β-carotene, lutein and vitamin A were determined in different vital organs. β-carotene and lutein significantly accumulated in the organs of rats fed with *D. bardawil* biomass. In rats fed with 2.5 and 5.0 g/kg b.w. *D. bardawil* biomass, maximum accumulation of β-carotene was observed in the liver (1.8 mg/g) while rats fed with synthetic β-carotene (50 mg/kg b.w) accumulated 1.85 µg/g β-carotene in the liver. In serum, 18.7 and 20.0 µg/l β-carotene was found in rats fed with 2.5 and 5.0 g/kg b.w. *D. bardawil* biomass, respectively. Lutein accumulation was exclusively found only in the groups treated with *Dunaliella* biomass. The vitamin A accumulation was maximum in the serum of rats fed with *D. bardawil* biomass (6.4 and 16.4 µg/dl with 2.5 and 5.0 g/kg b.w. *D. bardawil* treatment, respectively). In the same group liver retinol was found to be 2.9 and 3.52 µg/g respectively. The present study provides evidence for the accumulation of β-carotene, lutein and vitamin A in vital organs of rats when the diet is supplemented with carotenoid-rich *D. bardawil* biomass. This has an implication in the antioxidant status of the treated animals.

**Keywords:** algae, HPLC, *in vivo*, liver, lutein

## INTRODUCTION

Carotenoids are terpenoids synthesized by the isoprenoid pathway (Gray 1987). The biosynthetic pathway of carotenoids in plants originates from isoprenoids through phytoene (3-double bond colorless hydrocarbon), phytofluene (5-double bond hydrocarbon) and a few intermediate carotenoids (δ-carotene, neurosporene, lycopene) to form β-carotene. In higher plants, carotenoids are synthesized and stored in chloroplasts, whereas in animal tissues it is exclusively obtained through the diet, and then gets accumulated and metabolized. The hydroxylation of β-carotene leads to the formation of xanthophylls such as lutein (Delgado *et al*. 2000). A molecule of β-carotene is converted to two molecules of retinal by a central cleavage which is catalyzed by β-carotene-15, 15, dioxygenase, which is mainly present in the liver, intestine and also to some extent in other vital organs like the brain, kidney and lung (During *et al*. 1996). The retinal formed is further metabolized to retinoic acid or retinol (Nagao 2004).

In recent years algae have been gaining importance in diet supplements, as they possess nutraceutical properties. *Dunaliella* is a eukaryotic photosynthetic micro-algae belonging to the Chlorophyceae, which can produce β-carotene in large quantities under high solar radiation stress (Ben-Amotz *et al*. 1983). The alga lacks a rigid cell wall, which makes it easily digestible and accessible by most animal species (Ben-Amotz *et al*. 1989). Earlier studies have shown that *Dunaliella* has no toxic effects in rats (Mokady *et al*. 1989; Kuroiwa *et al*. 2006). Recently we have reported the antioxidant and hepatoprotective activity of *D. salina* in *in vivo* models (Chidambara Murthy *et al*. 2005a, 2005b). Therefore it is essential to understand the accumulation pattern of these carotenoids in vital organs. Ben-Amotz *et al*. (2005) reported the selective distribution of vitamin A and β-carotene isomers in rat tissues. The vitamin A content was highest in liver, and adrenals compared to other organs viz., spleen, kidney, heart and plasma when the diet was supplemented with *Dunaliella* biomass. However the β-carotene concentration was high in liver and spleen. Further, there are no reports on experimental studies dealing with supplementation of carotenoid-rich *Dunaliella* biomass and accumulation of β-carotene, lutein and vitamin A in a biological system.

The objective of the present study was to determine the distribution and accumulation of β-carotene, lutein and vitamin A in different vital organs of rats fed with carotenoid-rich biomass of *D. bardawil* and compared with the synthetic β-carotene.

## MATERIALS AND METHODS

### Source of *D. bardawil* and culture conditions

An authenticated indigenous strain of *D. bardawil* was isolated from the Sambar lake of Rajasthan, India. The culture was maintained in AS-100 medium (Vonshak 1986). Chemical composition of the medium (AS-100) is presented in **Table 1**. The Tris buffer (1 g/L) used in AS 100 medium was replaced with $NaHCO_3$ (4.0 g/L). The 14-day-old culture was subjected to carotenogenesis under sunlight in outdoor ponds subjected to a light intensity of 30-35 Klux at a temperature of 22-28°C. After 4 days, cells accumulated carotenoids were harvested using online centrifuge at 8000 rpm (M/s Sharples, UK). The wet biomass was freeze dried (Model-10XB, Lyophylization Systems Inc. USA), for 7 hours by spreading the sample with a thickness of 2-5 cm. The dried powder was stored at –80°C, used for feeding the experimental animals.

### Estimation of carotenoid content from *Dunaliella*

The carotenoids from the biomass were extracted using ethyl acetate and the carotenoid content was estimated by HPLC. Estimation was carried out using a Bondapak C18 column (5 µm x 250 mm) with methanol:acetonitrile:chloroform (47:47:6) as the mo-

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**Table 1** Chemical composition of AS-100 media.

| Chemicals | | (g/L) |
|---|---|---|
| $MgSO_4$ | | 2.44 |
| $CaCl_2$ | | 0.3 |
| $KH_2PO_4$ | | 0.05 |
| KCl | | 0.6 |
| $NaNO_3$ | | 1.0 |
| NaCl | | 50.0 |
| Tris buffer | | 1.0 |
| Trace metal solution | | |
| $H_3BO_3$ | 3.426 g/L | |
| $CoCl_2.6H_2O$ | 1.215 mg/L | |
| $MnCl_2.4H_2O$ | 0.432 mg/L | 10 ml of stock |
| $ZnCl_2$ | 31.5 mg/L | |
| Conc.$H_2SO_4$ | 1.0 mL | |
| $(NH_4)_6.Mo_7O_2.4H_2O$ | 31.19 mg/L | |
| Chelated iron solution | | |
| (10 g of $Na_2EDTA$ dissolved in hot water + 0.81 g of $FeCl_3.6H_2O$ in 500 mL of 0.1 N HCl, mix and make up to 1 L). | | 3 ml of stock |

bile phase at a flow rate of 1 ml/min (Vanitha *et al*. 2007). Peak identification was achieved by comparing with the retention time of standards (Sigma, USA) and confirmed by spiking the standards with individual samples. Further confirmation was carried out using LC- mass spectra.

## Experimental design

Albino rats of Wistar strain (120-150 g body weight) bred in the Animal House of the Central Food Technological Research Institute were used for the study. Animals were grouped into following groups each consisting 6 rats (n=6, 3 males and 3 females, maintained separately). Group-1 served as the control (receiving normal diet without *D. bardawil*); group-2: *D. bardawil* biomass at a dose of 2.5 g/kg body weight; group-3: *D. bardawil* biomass at a dose of 5 g/kg body weight (groups 2 and 3 are equivalent to 50 and 100 mg of β-carotene/kg, respectively); group-4 were treated with synthetic β-carotene at 50 mg/kg body weight.

These animals were housed in a room with a barrier system, and maintained under the following conditions: temperature of 24 ± 1°C, relative humidity 55 ± 5%, and a 12 h light/dark cycle. The animals were housed in polypropylene cages (3 rats/cage) on soft sawdust bedding. Throughout the experiment, rats were given commercial basal diet and water *ad libitum*. All the experiments were carried out under the regulation of the Institute Animal Ethical Committee.

## Test compound and administration dose levels

The *D. bardawil* biomass as dried powder was ground with a minimal amount of water (vehicle) for suspension consistency, and rats were fed once daily with *D. bardawil* biomass for 14 days by forced feeding at different doses. Similarly water suspension of synthetic β-carotene was prepared and fed to the rats. The control group was administered with only the vehicle.

## Biochemical screening

Clinical signs and general appearances were checked daily and body weights were measured once a week. Before the day of necropsy, the animals were deprived of food overnight and sacrificed by anaesthetizing the animals with ether. Blood was collected from the animals and the serum obtained was analyzed. The vital organs were weighed and transferred to ice-cold containers.

### *Extraction of carotenoids and vitamin A from organs*

The extraction of pigments and vitamin A was carried out according to Schmitz *et al*. (1991). Briefly, tissue was ground with ethanol: water with BHT, then saponified using 5 ml of 10% NaOH in ethanol for 30 min at 60°C, 10 ml of water was added and extracted with *n*-hexane. Carotenes and vitamin were extracted from the liver, spleen, lungs, adrenals, kidney and serum. Analysis was carried out by HPLC at 450 nm for carotenoids and at 320 nm for vitamin A.

## Statistical analyses

Mean and standard deviation values were calculated for all the parameters studied. Results were statistically analyzed by analysis of variance (ANOVA) using MS-Excel. The carotenoids and vitamin content in *D. bardawil* treated group was compared with synthetic β-carotene treated groups. Statistical significance was considered at $p < 0.01$ and $p < 0.05$.

# RESULTS

## Estimation of carotenoid content from *D. bardawil*

HPLC estimation revealed the presence of various carotenoids, the major one being β-carotene (~70%), lutein (~20%), α-carotene (~3%), lycopene (~2%) and traces of chlorophyll (**Table 2**).

## Effect of algal feeding on body and organ weight

Administration of *D. bardawil* biomass did not show much variation in body weight when compared to controls with a normal diet (**Table 3**). No clinical signs of any toxicity or notable changes in the behavior of the animals were observed among rats fed with either algal or synthetic carotenoid. Furthermore, no mortality occurred during the experimental period. There was no significant ($p < 0.05$) variation in the relative weight of vital organs (**Table 4**).

## β-carotene content in different organs and serum

No detectable level of β-carotene was observed in vital organs of rats fed with a normal diet devoid of carotenoid supplement. Among the analyzed organs, maximum accumulation of β-carotene was observed in the liver (51 μg/g and 1.8 mg/g at 2.5 and 5.0 g/kg *D. bardawil* biomass, respectively) and next being kidney (15 and 20 μg/g tissue at 2.5 and 5.0 g/kg *D. bardawil* biomass, respectively) in the *D. bardawil*-treated group. When this was compared with the rats fed with synthetic β-carotene, the accumulated β-carotene was 1.85 μg/g in liver. In serum, high content (18.7 and 20.0 μg/dl) of β-carotene was found in rats fed with 2.5 and 5.0 g/kg b.w. *D. bardawil* biomass, respectively when compared to the synthetic β-carotene treated group (12.2 μg/dl; **Table 5**).

**Table 2** Carotenoid content of *D. bardawil* biomass.

| Carotenoid | Content (%) |
|---|---|
| β-carotene | 70-75 |
| Lutein | 15-20 |
| Lycopene | 1.5-2 |
| α-carotene | 2.5-3 |
| Neoxanthin | 1-1.5 |
| Phytoene | 1-1.2 |

The carotenoid content was analyzed by HPLC with the conditions mentioned in materials and methods. The values are average analysis of triplicate samples.

**Table 3** Body weight of the experimental rats fed with carotenoid-rich whole cell biomass of *D. bardawil* and synthetic β-carotene compared with the control devoid of a carotenoid source.

| Group§ | Body weight (g) | |
|---|---|---|
| | Initial | Final |
| Control | 128 ± 3 | 175 ± 6 |
| *Dunaliella* (2.5 g/kg b.w.) | 128 ± 5 | 155 ± 4 |
| *Dunaliella* (5 g/kg b.w.) | 133 ± 13 | 167 ± 10 |
| Synthetic β-carotene (50 mg/kg b.w.) | 128 ± 14 | 166 ± 7 |

§ Average of 6 animals per group.
The animals were fed with the above diet for 14 days and the initial and final body weight was recorded weekly. Values are expressed as mean ± SD.

**Table 4** Relative weights of different organs§ of the experimental rats fed with carotenoid-rich whole cell biomass of *D. bardawil* and synthetic β-carotene compared with control devoid of a carotenoid source.

| Group | Liver | Kidney | Heart | Lung | Spleen | Testis | Brain |
|---|---|---|---|---|---|---|---|
| Control | 3.80 ± 0.30 | 0.80 ± 0.07 | 0.34 ± 0.07 | 0.52 ± 0.10 | 0.37 ± 0.08 | 1.35 ± 0.14 | 0.80 ± 014 |
| *Dunaliella* (2.5 g/kg b.w.) | 3.20 ± 0.10 | 0.77 ± 0.07 | 0.35 ± 0.10 | 0.47 ± 0.07 | 0.37 ± 0.04 | 1.42 ± 0.20 | 0.94 ± 0.12 |
| *Dunaliella* (5 g/kg b.w.) | 3.40 ± 0.40 | 0.78 ± 0.13 | 0.33 ± 0.10 | 0.55 ± 0.09 | 0.29 ± 0.15 | 1.50 ± 0.20 | 1.00 ± 0.09 |
| Synthetic β-carotene (50 mg/kg b.w.) | 3.70 ± 0.90 | 0.72 ± 0.07 | 0.34 ± 0.04 | 0.54 ± 0.10 | 0.25 ± 0.10 | 1.35 ± 0.05 | 0.90 ± 0.10 |

§ Average of organ weight from 6 animals per group
The animals were fed with the above diet for 14 days and the weights of different organs were recorded. Values are expressed as mean ± SD.

**Table 5** β-carotene content (μg/g tissue fresh weight) in rat tissues§ for control diets and diets supplemented with β-carotene–rich *Dunaliella* or synthetic β-carotene.

| Group | Liver | Kidney | Lung | Spleen | Adrenal | Serum (μg/dl) |
|---|---|---|---|---|---|---|
| Control | ND | ND | ND | ND | ND | 1.8 ± 0.4 |
| *Dunaliella* (2.5 g/kg b.w.) | 51.3 ± 13.2* | 15.56 ± 4.3* | ND | ND | ND | 18.7 ± 5.2** |
| *Dunaliella* (5 g/kg b.w.) | 1805.5 ± 97* | 20.89 ± 3.1* | 0.135 ± 0.08 | ND | ND | 20.0 ± 2.7** |
| Synthetic β-carotene (50 mg/kg b.w.) | 1.85 ± 0.2 | ND | ND | ND | ND | 12.2 ± 2.6 |

§ Average of organ weight from 6 animals per group
The animals were fed with the above diet for 14 days and the organs were removed and analyzed. Values are expressed as mean ± SD.
*, ** Statistically significant compared to synthetic β-carotene treated group at $p$ <0 .05 and $p$ <0.01, respectively.
ND: not detected.

**Table 6** Lutein content (μg/g tissue fresh weight) in rat tissues§ for control diets and diets supplemented with β-carotene–rich *Dunaliella* or synthetic β-carotene.

| Group | Liver | Kidney | Lung | Spleen | Adrenal | Serum (μg/dl) |
|---|---|---|---|---|---|---|
| Control | ND | ND | ND | ND | ND | ND |
| *Dunaliella* (2.5 g/kg b.w.) | 0.010 ± 0.001* | 0.01 ± 0.001* | 1.36 ± 0.38* | 0.014 ± 0. 005* | 1.05 ± 0.32* | 5.0 ± 1.24* |
| *Dunaliella* (5 g/kg b.w.) | 0.073 ± 0.010* | 0.04 ± 0.010* | 1.89 ± 0.42 * | 0.095 ± 0.020* | 1.87 ± 0.13* | 8.0 ± 1.50* |
| Synthetic β-carotene (50 mg/kg b.w.) | ND | ND | ND | ND | ND | ND |

§ Average of organ weight from 6 animals per group
The animals were fed with the above diet for 14 days and the organs were removed and analyzed. Values are expressed as mean ± SD.
*, ** Statistically significant compared to synthetic β-carotene treated group at $p$ <0.05 and $p$ <0.01, respectively.
ND: not detected.

**Table 7** Vitamin A content (μg/g tissue fresh weight) in rat tissues§ for control diets and diets supplemented with β-carotene–rich *Dunaliella* or synthetic β-carotene.

| Group | Liver | Kidney | Lung | Spleen | Adrenal | Serum (μg/dl) |
|---|---|---|---|---|---|---|
| Control | 0.02 ± 0.003 | 0.15 ± 0.02 | 0.80 ± 0.20 | 0.20 ± 0.050 | 0.17 ± 0.03 | 0.03 ± 0.01 |
| *Dunaliella* (2.5 g/kg b.w.) | 2.90 ± 0.050* | ND | 1.02 ± 0.60 | 0.03 ± 0.011** | 3.72 ± 0.80 | 6.40 ± 1.20 |
| *Dunaliella* (5 g/kg b.w.) | 3.52 ± 1.300* | 0.20 ± 0.09** | 1.50 ± 0.80 | 0.05 ± 0.020** | 3.12 ± 0.82 | 16.40 ± 2.60** |
| Synthetic β-carotene (50 mg/kg b.w.) | 1.39 ± 0.850 | 0.12 ± 0.06 | 2.12 ± 0.85 | ND | 3.18 ± 1.10 | 8.30 ± 2.30 |

§ Average of organ weight from 6 animals per group
The animals were fed with the above diet for 14 days and the organs were removed and analyzed. Values are expressed as mean ± SD.
*, ** Statistically significant compared to synthetic β-carotene treated group at $p$ <0.05 and $p$ <0.01, respectively.
ND: not detected.

## Lutein content in different organs and serum

Lutein was not detected in the vital organs in both the control and synthetic β-carotene treated group. Lutein accumulated in the vital organs of rats fed with the *D. bardawil* biomass (**Table 6**).

## Vitamin A content in different organs and serum

The vitamin A accumulation profiles in different organs are presented in **Table 7**. Maximum vitamin A was detected in the serum of rats fed with two doses of *D. bardawil* biomass (6.4 and 16.4 μg/dl, respectively) and the next being liver, in which vitamin A accumulated to 2.9 and 3.5 μg/g, at 2.5 and 5.0g/kg b.w., respectively (**Table 7**).

## DISCUSSION

β-carotene is a potent bioactive compound and processed in the body to yield vitamin A, which has a profound effect on development and on several diseases (Elliott 2005). The two major sites of β-carotene metabolism in humans are the intestine and the liver. In addition, the human liver was shown to have a four-times larger capacity for metabolizing β-carotene than the small intestine (During *et al*. 2001). In contrast to humans, rats convert most β-carotene in the intestine and accumulate it in the liver (During and Harrison 2004). Schmitz *et al*. (1991) reported that diet-containing carotenoids determine the carotenoid profile in vital organs. It was also observed that in humans hepatic tissues are the major sites for carotenoid accumulation and the control point for lipoprotein synthesis (Schmitz *et al*. 1991).

Underwood *et al*. (1979) reported that, since 90% of the body's reserves of vitamin A are stored in the liver, the measurement of hepatic and serum vitamin A content yields the most accurate information regarding the vitamin A status (Underwood *et al*. 1979). Britton *et al*. (1995) reported that even though more than 600 different carotenoids have been identified, only about 40 are found to occur in fruits and vegetables, of which only a few are absorbed, metabolized and stocked as liver retinol (Britton *et al*. 1995). Our observations suggest that *D. bardawil* biomass contains different carotenoids, which are absorbed, metabolized and stored in different organs (**Tables 5-7**).

The large intervention studies with synthetic β-carotene was stopped in between, because of failure to reduce the carcinogenecity and increased the mortality in a high risk population of heavy smokers and asbestos workers taking large doses of synthetic all *trans*-β-carotene (Albanes *et al.* 1995; Omenn *et al*. 1996). The negative effect of the synthetic all *trans*-β-carotene drew attention to other natural carotenoids coexisting with β-carotene mainly present in fruits, vegetables and algae. In *Dunaliella* sp. *cis*- and *trans*-β-carotene exists in equal concentrations (Shaish *et al*. 1990) in addition to other carotenoids (Vanitha *et al*. 2007). The importance of *cis* carotenoids is that they are the direct

precursor of *cis*-retinoic acid, which is a hormone in signalling processes (Nagao and Olson 1994; Lyn Patrick 2000).

Studies of supplementing *Dunaliella* biomass and accumulation of β-carotene have been carried out in different organs in rats (Werman *et al*. 1999), ferrets (Gugger *et al*. 1992), gerbils (Deming *et al*. 2002) mainly targeting the liver and serum. However no reports are available on the accumulation of lutein in these animals, which is another important carotenoid present in *D. bardawil*.

*Dunaliella* sp. are also rich in a spectrum of daily life nutrients, including vitamins, minerals, protein and amino acids, lipids, carbohydrates, chlorophyll and other important phytonutrients. The algae contain 10-11% carbohydrate, which include mono- and disaccharides, and lipids ranging from 6-8%. *Dunaliella* contains essential fatty acids including ω3 and ω6 linoleic acid and α-linoleic acid. Apart from this *Dunaliella* cells are rich in vitamins and other antioxidant minerals, particularly selenium and magnesium. It has been reported to contain the vitamins thiamine, pyridoxine, riboflavin, nicotinic acid and fairly high levels of biotin and α-tocopherol (Borowitzka and Borowitzka 1988).

Recently Ben-Amotz *et al*. (2005) reported the distribution of β-carotene stereoisomers in organs of rats fed with a *Dunaliella* diet. However their study did not discuss the accumulation of another important carotene, lutein found in *Dunaliella* sp. Lutein belongs to the xanthophyll family of carotenoids and is present in eye tissues. In plants lutein functions as an antioxidant and protects from photo-induced free radical damage (Demmig-Adams *et al*. 1996). In animals lutein is highly concentrated in the macula, a small area of the retina, which plays a major role in central vision and high visual acuity (Landrum and Bone 2001). It is possible that stable accumulation of lutein in different vital organs may be for the utilization at the time of requirement. Recent studies revealed that lutein supplementation increases lutein levels in the eye and improves the visual function in patients suffering from eye diseases (Rodrigues and Shao 2004).

Our report is in accordance with that of Schmitz *et al*. (1991) in which diet-containing carotenoids determined the carotenoid profile in vital organs. Hence lutein accumulation was observed in rats fed with *D. bardawil* biomass, suggesting that the diet supplemented has a profound effect on the type of carotene accumulated. The β-carotene, lutein absorption and accumulation was higher in rats treated with *D. bardawil* biomass than the synthetic all *trans*-β-carotene.

All these evidences suggests the important role of carotenoids in the diet and the present study provides evidence for the accumulation of β-carotene, lutein and vitamin A in vital organs of rats when the diet was supplemented with *D. bardawil* biomass. As more people take responsibility for their own health, the demand for natural foods and nutraceuticals like *Dunaliella* will undoubtedly increase, and it will become a good food supplement.

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