Hydrolysis Kinetics of Astaxanthin Esters and Stability of Astaxanthin of *Haematococcus pluvialis* during Saponification

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The reaction kinetics for the hydrolysis of astaxanthin esters and the degradation of astaxanthin during saponification of the pigment extract from the microalga *Haematococcus pluvialis* were investigated. Different concentrations of sodium hydroxide in methanol were used for the saponification under nitrogen in darkness at ambient temperature (22 °C) followed by the analysis of astaxanthins and other carotenoids using an HPLC method. The concentration of methanolic NaOH solution was important for promoting the hydrolysis of astaxanthin esters and minimizing the degradation of astaxanthin during saponification. With a higher concentration of methanolic NaOH solution, the reaction rate of hydrolysis was high, but the degradation of astaxanthin occurred significantly. The rate constants of the hydrolysis reaction (first order) of astaxanthin esters and the degradation reaction (zero-order) of astaxanthin were directly proportional to the concentration of sodium hydroxide in the saponified solution. Although the concentration of sodium hydroxide in the saponified solution was 0.018 M, complete hydrolysis of astaxanthin esters was achieved in 6 h for different concentrations (10−100 mg/L) of pigment extracts. Results also indicated that a higher temperature should be avoided to minimize the degradation of astaxanthin. In addition, during saponification, no loss of lutein, β-carotene, and canthaxanthin was found.

Keywords: *Astaxanthin; astaxanthin esters; hydrolysis; kinetics; carotenoids; stability; Haematococcus pluvialis*

INTRODUCTION

The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is produced by some microorganisms such as the green alga *Haematococcus pluvialis* and is synthesized from β-carotene through echinenone, canthaxanthin, and adonirubin (Grung et al., 1992; Fan et al., 1995; Yokoyama and Miki, 1995; Fraser et al., 1997; Harker and Hirschberg, 1997; Yuan et al., 1997). Due to its biological functions as a vitamin A precursor (Gobantes et al., 1998), higher antioxidative activity than those of β-carotene and vitamin E, and attractive pink color, astaxanthin can be used in medicine and as a food colorant (Johnson and An, 1991). Recent studies have demonstrated that astaxanthin has preventive effects against aflatoxin B_1 carcinogenicity (Gradelet et al., 1997) and possible immunomodulating activities (Okai and Higashi-Okai, 1996).

In *H. pluvialis*, astaxanthin is the major carotenoid and exists mainly as astaxanthin esters of various fatty acids (Johnson and An, 1991). The complexity of the pigment composition in the algal extract makes it difficult to separate all of these pigments and determine accurately the content of astaxanthin esters, because astaxanthin esters elute over a broad range of retention times (Sommer et al., 1991; Juhl et al., 1996). Therefore, the saponification of the algal extracts is recommended for reducing the number of chromatographic peaks and thus simplifies the quantification (Ittah et al., 1993; Minguez-Mosquera and Perez-Galvez, 1998).

The addition of alkaline solutions, for example, methanolic KOH or NaOH, which can break the pigment−fatty acid bond (Minguez-Mosquera and Perez-Galvez, 1998), is necessary for the hydrolysis reaction of astaxanthin esters. Weissenberg et al. (1997) established optimal conditions for mild saponification of xanthophyll esters with 5% methanolic potassium hydroxide solution overnight at ambient temperature. The addition of more concentrated alkaline reagents with shorter reaction times proved to be less effective (Weissenberg et al., 1997). Therefore, mild conditions using sodium methoxide as an acting reagent at ambient temperature were employed (Ittah et al., 1993).

It is imperative to establish the highest concentration of methanolic NaOH or KOH at which astaxanthin esters in the pigment extract can be readily hydrolyzed without significant degradation of the resulting labile astaxanthin. The major objective of the present work is to develop an optimal saponification method for the hydrolysis of astaxanthin esters from the pigment extracts of *H. pluvialis* and to study the hydrolytic reaction kinetics of astaxanthin esters and the degradation of astaxanthin.

EXPERIMENTAL PROCEDURES

Alga Culture and Pigment Extraction. *H. pluvialis* (*H. lacustris*, UTEX 16), obtained from the University of Texas Culture Collection, was grown in batch culture in a 3.7-L fermentor (Bioengineering, Wald, Switzerland) containing 2.5 L of MCM medium with 1 g/L sodium acetate at 25 °C (Yuan et al., 1996).

Algal cells were collected by centrifuging the culture fluid at 3000*g* for 10 min, the supernatant was discarded, and the cell pellet was rinsed with distilled water twice and then freeze-dried using a Heto FD3 freeze-dryer (Heto-holten, Allerod, Denmark). The dry cells (75 mg) were homogenized using a 15-mL tissue homogenizer (B. Braun, Melsungen,

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Germany), and the total pigments were extracted in the solvent mixture of dichloromethane and methanol (25:75, v/v). The mixture was then separated by centrifugation at 10000*g* for 5 min, and the supernatant containing pigments was collected. The extraction procedure was repeated at least three times until the cell debris was almost colorless. The total pigment extracts (50 mL) were centrifuged again at 10000*g* for 15 min and stored at −20 °C for subsequent saponification and HPLC separation. All above processes were conducted in darkness and under nitrogen.

Chemicals and Reagents. Sodium hydroxide, HPLC grade methanol, acetonitrile, and dichloromethane were obtained from BDH Laboratory Supplies (Poole, England). *trans*-Astaxanthin, lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* were obtained from Sigma Chemical Co. (St. Louis, MO). Canthaxanthin was kindly provided by Prof. Sammy Boussiba of Ben-Gurion University of the Negev (Israel).

High-Performance Liquid Chromatography (HPLC). HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The pigment extract and saponified extract solution were separated and analyzed (20 μL aliquots) by using a Beckman Ultrasphere C_{18} column (250 × 4.6 mm; 5 μm) at 25 °C. The mobile phase consisted of solvent A (dichloromethane/methanol/acetonitrile/water, 5.0:85.0:5.5:4.5, v/v) and solvent B (dichloromethane/methanol/acetonitrile/water, 22.0:28.0:45.5:4.5, v/v). For the simultaneous separation of free astaxanthin and astaxanthin esters, the following gradient procedure was used: 0% B for 8 min; a linear gradient from 0 to 100% B for 6 min; 100% B for 51 min. The flow rate was 1.0 mL/min. The tridimensional chromatogram was recorded from 250 to 700 nm. Peaks were measured at a wavelength of 480 nm to facilitate the detection of astaxanthins. Chromatographic peaks were identified by comparing retention times and spectra against known standards or by comparing their spectra with published data (Grung et al., 1992; Yokoyama and Miki, 1995; Harker and Hirschberg, 1997; Fraser et al., 1997; Yuan et al., 1997; Yuan and Chen, 1997, 1998).

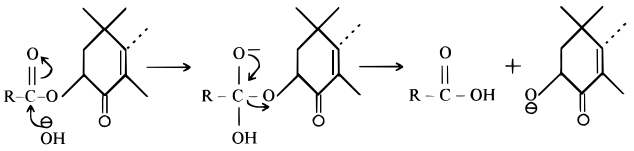
Saponification of Pigment Extracts and Stability of Carotenoids. Known volumes (e.g., 0.1 mL) of sodium hydroxide solutions in methanol at different concentrations, which were freshly prepared, were mixed with aliquots (e.g., 0.5 mL) of the pigment extract at the ratio of 1:5 (v/v) under a nitrogen atmosphere. The mixture (0.6 mL) was evaporated and concentrated to 0.5 mL under nitrogen. The hydrolysis reaction of astaxanthin esters was carried out in darkness under nitrogen at ambient temperature (22 °C). The reaction mixtures were sampled and analyzed by HPLC for monitoring the progress of hydrolysis during saponification until the end of the hydrolysis reaction. The contents of carotenoids were also determined simultaneously by the above HPLC method to measure the possible losses of carotenoids during saponification. Different concentrations of pigment extracts (the astaxanthin concentrations were between 10 and 100 mg/L) were used to test the effect of the initial concentration of astaxanthin esters on the rate constant of the hydrolysis reaction.

The degradation reaction of *trans*-astaxanthin was studied by adding 0.1 mL of different concentrations of NaOH solutions to 0.5 mL of *trans*-astaxanthin standard solution (27.1 mg/L) under a nitrogen atmosphere. The degradation reaction of astaxanthin was carried out in darkness under nitrogen at ambient temperature (22 °C). The degradation process was monitored by analyzing the changes in astaxanthin content in the reaction mixtures using the above HPLC method.

Kinetic Study of the Hydrolysis of Astaxanthin Esters. The kinetic study of the hydrolysis of astaxanthin esters was carried out on the basis of the following reaction:

$$\text{astaxanthin} - \text{FA} + \text{NaOH} \rightarrow \text{astaxanthin} + \text{Na} - \text{FA}$$

While NaOH is added, the hydroxyl anion attacks the carbonyl group of the astaxanthin ester; this is then followed by a second step in which the astaxanthin alcohol would be a leaving group.



Although astaxanthin was joined to more than one fatty acid, the reaction that takes place is essentially the same (Minguez-Mosquera and Perez-Galvez, 1998). Because adequate NaOH was added, the concentration of NaOH remained almost constant throughout. The reaction was supposed to follow first-order kinetics.

Suppose that at the beginning of the hydrolysis reaction, the concentrations of astaxanthin esters and free astaxanthin were C_{AE}^0 and C_{Ast}^0, respectively, and that at time *t* the amount of astaxanthin esters which had been hydrolyzed per unit volume was *x*, then the rate of hydrolysis of astaxanthin esters might be expressed by the following equation (Laidler, 1987):

$$dx/dt = k\,(C_{AE}^{\ 0} - x) \tag{1}$$

Separation of the variables *x* and *t* in eq 1 and integration give

$$\ln[C_{AE}^{\ 0}/(C_{AE}^{\ 0} - x)] = kt \tag{2}$$

Equation 2 can be rewritten as

$$\ln\left(1 - \frac{x}{C_{AE}^{\ 0}}\right) = -kt \tag{3}$$

In eq 3, *x*, the concentration of astaxanthin esters hydrolyzed may be evaluated from the difference between the concentration of free astaxanthin at time *t* obtained in each HPLC analysis and the concentration of free astaxanthin at the beginning of the hydrolysis reaction, that is

$$x = C_{Ast} - C_{Ast}^{\ 0} \tag{4}$$

Substitution of this equation into eq 3 leads to

$$\ln[1 - (C_{Ast} - C_{Ast}^{\ 0})/C_{AE}^{\ 0}] = -kt \tag{5}$$

Graphical methods can be employed to test the first-order equations and obtain the rate constant *k*. If the hydrolysis reaction of astaxanthin esters is first order, a plot of $\ln[1 - (C_{Ast} - C_{Ast}^0)/C_{AE}^0]$ versus *t* will give a straight line. The slope of this plot is the rate constant, that is, slope = $-k$.

RESULTS AND DISCUSSION

Optimization of Sodium Hydroxide Concentration. The pigment extract of *H. pluvialis* was saponified for hydrolyzing astaxanthin esters. Because saponification might result in the destruction and structural transformation of carotenoids (Lietz and Henry, 1997), mild saponification of astaxanthin esters should be used. The addition of methanolic NaOH was necessary for the hydrolysis reaction of astaxanthin esters. Although some carotenoids were stable to alkaline solutions (Minguez-Mosquera and Perez-Galvez, 1998), our result indicated that astaxanthin was not stable to high concentrations of methanolic NaOH. Therefore, it is desirable to optimize the concentration of NaOH so as to facilitate the hydrolysis of astaxanthin esters without giving rise to the destruction or structural transformation of astaxanthin.

The HPLC method was used to measure the changes in content of astaxanthins in the process of saponification. Figure 1 shows the typical HPLC chromatograms

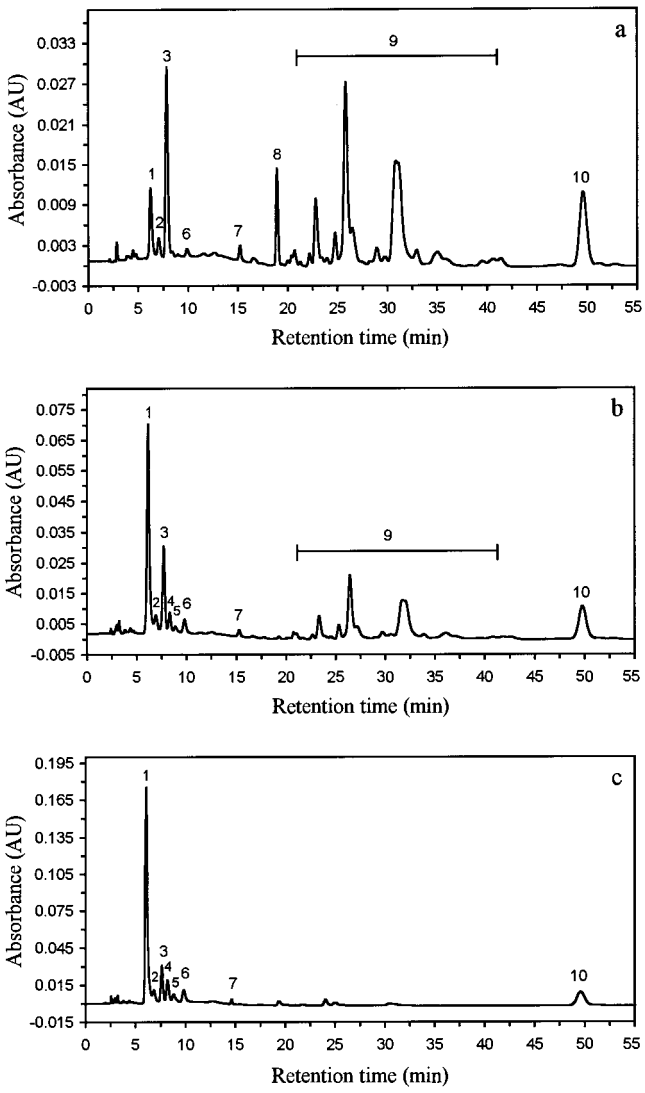


Figure 1. HPLC chromatograms of the pigment extracts before saponification (a), during saponification (b), and after saponification (c). Peaks: 1, *trans*-astaxanthin; 2, adonirubin; 3, lutein; 4, 9-*cis*-astaxanthin; 5, 13-*cis*-astaxanthin; 6, (3*R*,3′*R*)-*trans*-astaxanthin; 7, canthaxanthin; 8, chlorophyll *b*; 9, astaxanthin esters; 10, *β*-carotene.

of the pigment extracts before, during, and after saponification.

NaOH solutions in methanol (0.1 mL) at concentrations of 0.023, 0.047, 0.057, 0.072, 0.074, 0.090, 0.106, 0.117, 0.132, 0.143, and 0.181 M were mixed with 0.5 mL of the pigment extract solutions, respectively. After concentration to 0.5 mL, the concentrations of NaOH in reaction mixtures were 0.0046, 0.0094, 0.0115, 0.0144, 0.0148, 0.0180, 0.0212, 0.0234, 0.0264, 0.0284, and 0.0362 M, respectively. Figure 2 shows changes in free *trans*-astaxanthin content in the reaction mixtures during the process of saponification at different concentrations of NaOH. As can be seen from the figure, the application of different concentrations of NaOH could result in significant differences in the degree of hydrolysis reaction. A high concentration of NaOH would increase the rate of hydrolysis reaction, but the final concentrations of free astaxanthin were lower than at a low concentration of NaOH. While the concentrations of NaOH in reaction mixtures were >0.018 M, the contents of astaxanthin decreased significantly after reaching a maximum value, which was lower than the

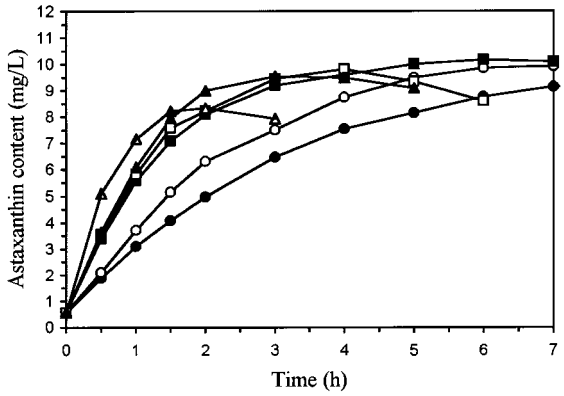


Figure 2. Changes in free *trans*-astaxanthin content during saponification with NaOH concentrations of 0.0094 (●), 0.0148 (○), 0.0180 (■), 0.0210 (□), 0.0264 (▲), and 0.0362 M (△) in the reaction mixtures at 22 °C. (SE ± 2.1%; $n = 3$.)

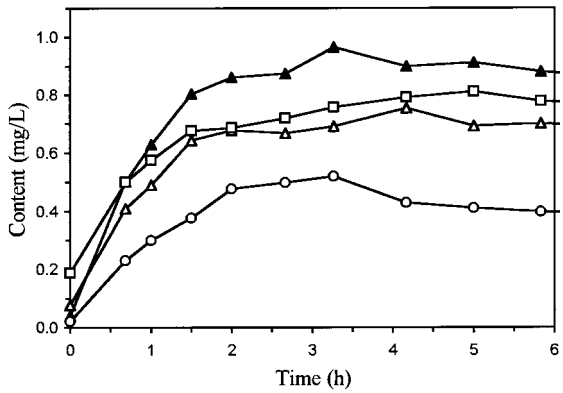


Figure 3. Changes in contents of 9-*cis*-astaxanthin (▲), 13-*cis*-astaxanthin (○), (3R,3′R)-*trans*-astaxanthin (△), and adonirubin (□) during the process of hydrolysis with 0.0180 M NaOH in the reaction mixtures at 22 °C. (SE ± 4.6%; $n = 3$.)

maximum concentration (10.2 mg/L) of astaxanthin while the concentration of NaOH in reaction mixtures was 0.018 M. The fact that the content of free astaxanthin decreased indicated that the degradation reaction of astaxanthin might occur, especially at a higher concentration of NaOH solution. While the concentration of NaOH in reaction mixtures was low, for example, 0.0094 M, a long reaction time was necessary for the complete hydrolysis of astaxanthin esters at 22 °C. Therefore, for the hydrolysis of astaxanthin esters, it is necessary to add moderate NaOH concentrations to obtain the maximum productivity of astaxanthin. The results suggested that a NaOH concentration of 0.0175−0.020 M in the reaction mixtures was optimum for complete hydrolysis of astaxanthin esters without causing significant degradation of astaxanthin at 22 °C.

During the hydrolysis reaction of astaxanthin esters, the isomers of astaxanthin esters in the extract of *H. pluvialis* were hydrolyzed simultaneously. Figure 3 shows changes in the content of astaxanthin isomers during hydrolytic process with 0.018 M NaOH at 22 °C. The change in the content of another carotenoid, which was tentatively identified as adonirubin, is also shown in Figure 3. The increase in adonirubin content during the saponification indicated that adonirubin existed mainly as the esters in *H. pluvialis*.

Hydrolysis Kinetics of Astaxanthin Esters. As can be seen in Figure 4a, there is a linear relationship between $\ln[1 - (x/C_{AE}^0)]$ and time, indicating that the hydrolysis of astaxanthin esters is a first-order reaction.

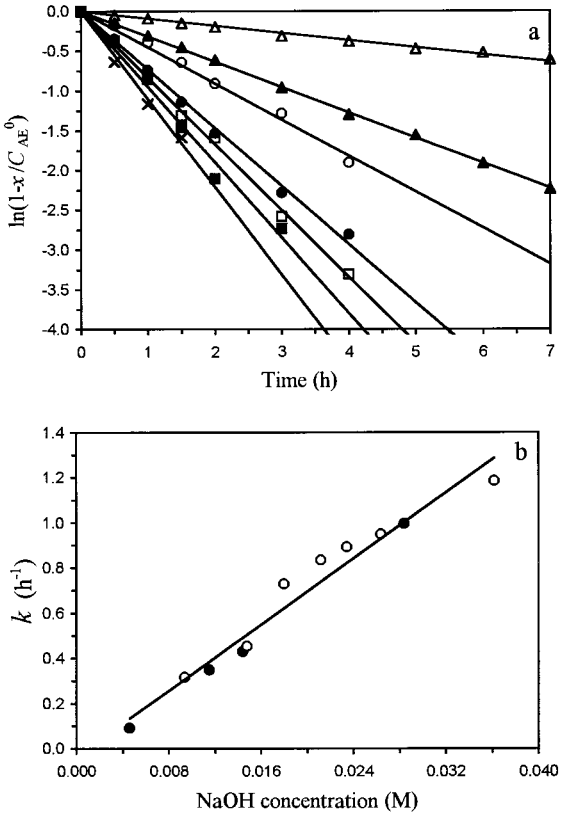


Figure 4. (a) First-order kinetics for the hydrolysis reaction of astaxanthin esters at concentrations of 0.0046 (△), 0.0094 (▲), 0.0148 (○), 0.0180 (●), 0.0210 (□), 0.0264 (■), and 0.0362 M (×) of NaOH in the reaction mixtures at 22 °C. (b) Effect of NaOH concentration in reaction mixture on rate constant of hydrolysis reaction of astaxanthin esters with the total astaxanthin concentrations of 10.2 (○) and 29.5 mg/L (●) in pigment extracts. (SE ± 2.1%; $n = 3$.)

Figure 4b shows the effect of NaOH concentration in reaction mixtures on the rate of hydrolysis reaction of astaxanthin esters ($k = 36.576C_{NaOH} - 0.0355$; $R = 0.9804$). Different concentrations of pigment extracts (the astaxanthin concentrations were between 10 and 100 mg/L) were used to test the effect of the initial concentration of astaxanthin esters on the rate constant of the hydrolysis reaction. The best fit of the experimental data at two initial concentrations of astaxanthin esters shows that the rate constant of the hydrolysis reaction of astaxanthin esters is independent of the initial concentration of astaxanthin esters (Figure 4b). The results indicate that for the saponification of different concentrations of pigment extracts (10−100 mg/L astaxanthin esters), the amount of NaOH and the saponification time needed to complete the hydrolysis should be similar.

Degradation Reaction of Astaxanthin. Figure 5a shows the changes in astaxanthin content in the *trans*-astaxanthin solution during the degradation process with different concentrations of NaOH. The results indicated that NaOH could result in significant degradation of astaxanthin. A higher concentration of NaOH would result in a higher rate of astaxanthin degradation reaction. As shown in Figure 5a, the plots of astaxanthin content versus time give a straight line, indicating that the degradation of astaxanthin is a zero-order reaction; that is, the rate constant k can be represented by

$$k = x/t = (C_{Ast}^{\ 0} - C_{Ast})/t \qquad (6)$$

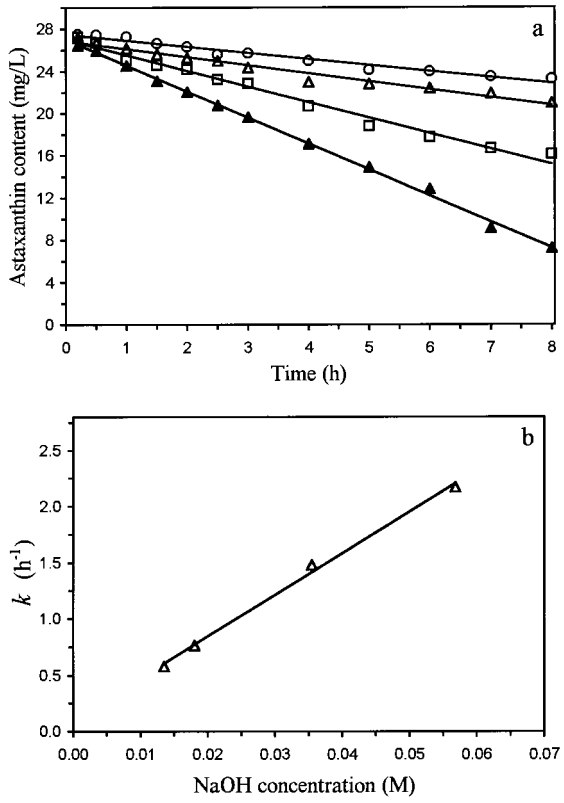


Figure 5. (a) Changes in *trans*-astaxanthin content during degradation at concentrations of 0.0135 (○), 0.0180 (△), 0.0355 (□), and 0.0568 M (■) of NaOH in the reaction mixtures at 22 °C. (b) Effect of NaOH concentration in reaction mixture on rate constant of degradation reaction of astaxanthin. (SE ± 1.8%; $n = 3$.)

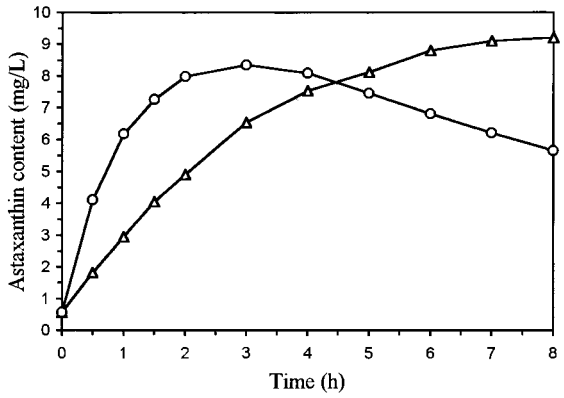


Figure 6. Changes in free *trans*-astaxanthin content during saponification with 0.0094 M NaOH in the reaction mixtures at 22 (△) and 40 °C (○). (SE ± 3.3%; $n = 3$.)

The result was similar to the degradation of β-carotene and capsanthin in anhydrous media (Minguez-Mosquera and Perez-Galvez, 1995). Figure 5b shows the effect of NaOH concentration in reaction mixtures on the reaction rate of astaxanthin degradation ($k = 36.937C_{NaOH} + 0.1055$; $R = 0.9981$).

Effect of Temperature on Astaxanthin Productivity. To test the effect of temperature on the hydrolysis of astaxanthin esters and the stability of astaxanthin, an experiment to hydrolyze astaxanthin esters at 40 °C was carried out. Figure 6 shows the progress of hydrolysis of astaxanthin esters at 22 and 40 °C, respectively, with 0.0094 M NaOH in reaction mixtures. The results indicated that a higher temperature (e.g., 40 °C) favored the hydrolysis of astaxanthin esters, but

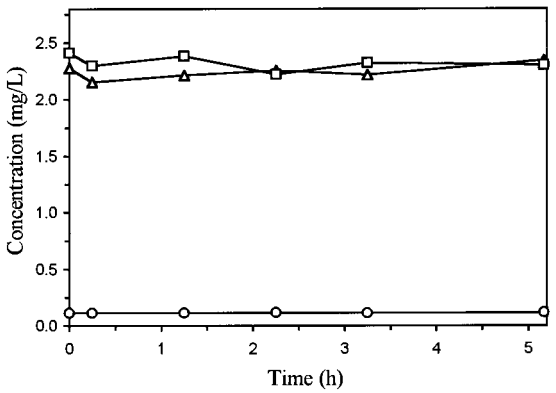


Figure 7. Changes in contents of lutein (△), β-carotene (□), and canthaxanthin (○) during saponification with 0.0264 M NaOH in the reaction mixtures at 22 °C. (SE ± 3.8%; $n = 3$.)

the degradation of astaxanthin was also promoted markedly at the same time (Figure 6). Therefore, high temperature should be avoided during the hydrolysis of astaxanthin esters.

Stability of Carotenoids to NaOH. Changes in the concentrations of lutein, β-carotene, and canthaxanthin during saponification were determined for monitoring the stability of these carotenoids to NaOH. Figure 7 indicated that, unlike astaxanthin, other carotenoids such as lutein, β-carotene, and canthaxanthin were stable to hydroxide, which is in good agreement with the results reported by Ittah et al. (1993) and Minguez-Mosquera and Perez-Galvez (1998). Because the degradation of carotenoids can be relieved by applying antioxidants to the solution during saponification (Lietz and Henry, 1997), the presence of a large amount of astaxanthin, the strong antioxidant, might be used to explain the phenomenon that no losses of lutein, β-carotene, and canthaxanthin were observed.

Free astaxanthin is preferable to astaxanthin esters in terms of the utilization by animals (Johnson and An, 1991). It is not clear how astaxanthin esters are synthesized within the cells. Nevertheless, esterification of astaxanthin with fatty acids may be enhanced by light induction because the astaxanthin concentrated within lipid bodies in the cytosol of the algal cell can maximize the photoprotective efficiency (Yong and Lee, 1991; Rise et al., 1994). Consequently, it is likely a biological process can be developed to maximize the production of free astaxanthin through controlling the environmental conditions or isolating mutants that do not esterify the astaxanthin, which would be preferred over a downstream processing method.

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