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Research article

Hydrolytic efficiency and isomerization during de-esterification of natural astaxanthin esters by saponification and enzymolysis

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ABSTRACT

Background: Astaxanthin from natural sources is typically esterified with fatty acids; hence, it must be hydrolyzed to remove esters before identification and quantification by conventional HPLC. Alkaline-catalyzed saponification and enzyme-catalyzed enzymolysis are the most commonly used de-esterification methods. However, information on the efficiency and isomerization during de-esterification of natural astaxanthin esters by these two methods remains scarce. Therefore, we conducted two HPLC-based experiments to determine which method is better for hydrolyzing astaxanthin esters.

Results: To assess the effect of enzymolysis (0.67 U/mL cholesterol esterase, at 37°C) and saponification (0.021 M NaOH, at 5°C) conditions on free astaxanthin recovery and destruction or structural transformation of astaxanthin, we varied the total treatment time across a range of 195 min. The results showed that enzymolysis and saponification were complete in 60 min and 90 min, respectively. After complete hydrolysis, the maximum free astaxanthin recovery obtained by enzymolysis was 42.6% more than that obtained by saponification. The identification of by-products, semi-astacene and astacene, during the process of saponification also indicated that a more severe degradation of astaxanthin occurred during saponification. Moreover, the composition of astaxanthin isomers during saponification was similar to that of the isomers during enzymolysis between 30 min and 75 min (all-*trans*:9-*cis*:13-*cis* = 21:3:1, approximately) but dramatically changed after 90 min, whereas the composition in the enzymolysis treatment remained relatively stable throughout.

Conclusion: Compared with saponification, enzymolysis with cholesterol esterase was recommended as a more accurate method for de-esterification of natural astaxanthin esters for further qualitative and quantitative HPLC analysis.

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1. Introduction

Astaxanthin (3,3-dihydroxy-β, β-carotene-4,4-dione) is a carotenoid with significant antioxidant activity [1,2,3,4] and hence may play a role in human health, such as delaying or preventing degenerative diseases [5,6,7]. Natural astaxanthin occurs in three forms: the free form, monoester, and diester [8,9]. The astaxanthin esters predominate in the pigments of most astaxanthin sources such as *Haematococcus pluvialis*, Adonis, and most crustaceans [10,11,12,13]. Owing to the lack of standards and the difficulty in synthesizing astaxanthin esters, the determination of astaxanthin esters was done using high-performance liquid chromatography–mass spectrometry (HPLC–MS) [14,15]. However, determination by HPLC–MS is expensive and time consuming because without standards, each broken molecular fragment must be examined by MS to provide conclusive data. In addition, HPLC cannot completely separate different astaxanthin esters, which makes the analysis by MS incomplete [13]. Natural astaxanthin may exhibit diverse geometrical isomers such as all-*trans* astaxanthin, 9-*cis* astaxanthin, and 13-*cis* astaxanthin [16,17]. A distinction between esterified isomers is not possible by MS. After removing the fatty acid chains of the astaxanthin esters, free astaxanthin isomers are then easy to separate by HPLC. Free astaxanthin cleaved from astaxanthin esters

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can be linked to water-soluble groups (e.g., disodium disuccinate) through hydroxyl groups to form an antioxidant drug useful in the therapy for cardiovascular disease [18,19,20]. De-esterifying the astaxanthin esters is a very important step because it can facilitate the accurate determination of astaxanthin by HPLC. Production of free astaxanthin with fewer structural changes and by-products will facilitate the determination of astaxanthin concentration, yield a superior product, and hence facilitate experiments using astaxanthin.

Methods for de-esterifying astaxanthin esters fall into two categories: alkaline saponification and enzymolysis. In 1999, Yuan and Chen [21] completely hydrolyzed astaxanthin esters in pigment extracts of *H. pluvialis* by methanolic NaOH. However, saponification of astaxanthin esters under alkaline conditions produced by-products such as astacene and resulted in severe degradation [20]. Additionally, there are limited data on the recovery and structural (geometrical and optical) changes of astaxanthin during saponification. Methods based on enzymolysis are milder. Cleavage of astaxanthin esters by alkaline lipase from *Penicillium cyclopium*, which was expressed in *Pichia pastoris*, has also been described by Zhao et al. [20]. However, only partial cleavage of the esters was obtained. Cholesterol esterase (EC 3.1.1.13) from *Pseudomonas fluorescens* is commonly used to cleave carotenoid esters [22]. Many *H. pluvialis*–producing enterprises such as Fuji Chemical Industry Co., Ltd. and Cyanotech Corporation use cholesterol esterase to cleave astaxanthin esters from *H. pluvialis*. A similar method was included in the 8th edition of Food Chemicals Codex (FCC) [23]. However, no data were presented on the recovery and structural stability of the free astaxanthin cleaved from esters. This raises the question of the differences between these two types of methods for hydrolyzing astaxanthin esters.

To assess the relative efficiency of enzymolysis and saponification for de-esterifying natural astaxanthin esters, we compared enzymolysis conditions derived from previous studies [23] to a method that has been used to recover esterified astaxanthin from *H. pluvialis* by saponification [24]. Free astaxanthin was recovered in both methods, but the carotenoid profiles observed in the HPLC chromatogram and the quantitative analysis result of free astaxanthin showed significant differences between the two methods. Therefore, we conducted a 195-min experiment by collecting samples for analysis at 30, 45, 60, 75, 90, 105, 120, 135, and 195 min during the 195-min enzymolysis or saponification treatments, with the goal of achieving optimal recovery of free astaxanthin and observing the stability of the geometrical and optical structures of the astaxanthin esters by both methods. The practicability of the enzymolysis was verified by hydrolysis of the astaxanthin esters extracted from Prawn (*Litopenaeus vannamei*).

2. Materials and methods

2.1. Preparation of astaxanthin esters

The alga *H. pluvialis* (strain H_2) was grown in a photo-bioreactor outdoors and harvested by centrifugation. A pigment mixture with a high content of astaxanthin esters was extracted using supercritical carbon dioxide extraction technology. Astaxanthin extraction was done in collaboration with the Yunnan Alphy Biotech Co., Ltd. (China). Fresh *L. vannamei* were purchased from local markets (Qingdao, Shangdong, China) and the extraction procedures were based on those used by Wade et al. [25].

2.2. Chemicals and reagents

Analytical pure sodium hydroxide, trihydroxymethyl aminomethane (Tris), and hydrochloric acid were obtained from Sinopharm Chemical Reagent Co., Ltd. HPLC-grade n-hexane and acetone were obtained from Thermo Fisher Scientific Inc., UK, and the all-*trans* astaxanthin standard was purchased from Dr. Ehrenstorfer GmbH, Germany.

2.3. Hydrolysis of astaxanthin esters

2.3.1. Enzymolysis

The enzymolysis procedure was adapted from the 8th edition of Food Chemicals Codex (FCC) [23]. One milliliter of the acetone-dissolved astaxanthin solution was mixed with 2 mL of acetone and 2 mL of 0.05 M Tris–HCl buffer (pH = 7.0) and 1 mL of 4 U/mL cholesterol esterase in a 15-mL graduated test tube with a stopper (the final cholesterol esterase concentration in the reaction system was 0.67 U/mL). Then, the tube was placed in a thermostatically controlled water bath at 37°C in darkness, with gentle mixing every 10 min.

2.3.2. Saponification

The results of Yuan and Chen [24] indicated that 0.021 M NaOH sodium in the reaction mixtures at 5°C was optimum for complete saponification of astaxanthin esters without causing degradation. Thus, the saponification conditions (temperature and alkali concentration) suggested by Yuan and Chen [24] were used. Five milliliters of the acetone-dissolved astaxanthin solution were mixed with 1 mL of 0.107 M NaOH-methanol solution under nitrogen atmosphere in darkness and then concentrated to 5 mL by flushing with nitrogen (the final NaOH concentration in the reaction system was 0.021 M). Astaxanthin esters were hydrolyzed in a 5°C water bath in darkness, with gentle mixing every 10 min.

Thereafter, to investigate the effect of reaction time on de-esterification of natural astaxanthin esters and to find the optimal reaction times for enzymolysis and saponification, reaction mixtures in both treatments were sampled at 0, 30, 45, 60, 75, 90, 105, 120, 135, and 195 min. The hydrolysis reaction was terminated by adding 2 mL of deionized water. The pigment in the reaction mixture was extracted by petroleum ether until the lower aqueous phase and the upper organic phase were uncolored. The combined petroleum ether extracts were dried by flushing with nitrogen gas at room temperature and were then redissolved in 1 mL of a solution of n-hexane:acetone (9:1) for HPLC analysis. Each set of experiments was conducted in triplicate.

2.4. Analysis of pigments

UV spectrophotometry was used to determine the content of total carotenoids. The absorbance of samples at 478 nm was determined against an n-hexane: acetone (9:1) blank on a TU1900 dual-beam UV–VIS spectrometer (Beijing Purkinje General Instrument Co., Ltd., China). Subsequently, the content of total carotenoids was calculated using [Equation 1]:

$$C = A_{478\ nm} \times V/(2100 \times W) \quad \text{[Equation 1]}$$

where $A_{478\ nm}$ is the absorbance of the sample at 478 nm; V is the diluted volume of the sample; 2100 is the extinction coefficient of 1% (g/mL) carotenoids in a solution of hexane:acetone (9:1), in a 1-cm cuvette at 478 nm; and W is the weight in grams of the sample.

HPLC was performed on an Elite liquid chromatograph equipped with a 1201 UV–VIS detector (Dalian Elite Analytical Instruments Co., Ltd., China). Twenty microliters of the hydrolyzed extracts prepared by saponification and enzymolysis were injected into the HPLC system. The hydrolyzed extract solution was separated and analyzed using a Phenomenex silica gel column (Luna 3 μ Silica (2) 100A 150 × 4.6 mm) at 25°C. An isocratic elution with a solution of n-hexane:acetone at 83:17 (*v*/v) was performed for 14 min at a flow rate 1.0 mL/min. Astaxanthin absorbance was detected at 478 nm. The carotenoids were identified by their retention times. Astaxanthin was quantified against peak areas of an all-*trans* astaxanthin standard. Other carotenoids without standards were identified by comparing their retention times with published data [22,26] and quantified by area comparison with the astaxanthin standard. As the basis for the

determination of hydrolysis efficiency, the total amount of starting carotenoids was determined from the 0-min samples. The percentage recovery of free astaxanthin was defined as the ratio between the amount of astaxanthin de-esterified at different time points and the total amount of astaxanthin in the 0-min sample according to [Equation 2]:

$$R_t = A_t \times 100/T \quad \text{[Equation 2]}$$

where R is the percentage recovery, A is the free astaxanthin content, T is the total amount of carotenoids before hydrolysis, and t is the time of sampling.

Further separation of optical isomers of astaxanthin was according the method of Wang et al. [27] with some improvements. Baseline separation of three stereomers of astaxanthin was using a Chiralpack IC column (25 cm × 4.6 cm; Daicel chiral technologies (China) Co., Ltd., China) with methyl tert-butyl ether and acetonitrile (35:65, *v*/*v*) at a flow rate of 1.0 mL/min at 470 nm under room temperature. Peaks were identified by comparing the retention times of sample peaks with (3*S*, 3′*S*) astaxanthin and racemic astaxanthin (1:2:1 mixture of the (3*S*, 3′*S*), (3*R*, 3′*S*), and (3*R*, 3′*R*) isomer standards, which were purchased from Carotenature, Lupsingen, Switzerland). Replicate HPLC runs showed high reproducibility, and peak areas were averaged for the two runs before assessing the variation between individuals.

3. Results and discussion

3.1. Effects of hydrolysis on total carotenoid content

The study investigated the degradation of carotenoids in the samples during the hydrolysis processes. UV spectrophotometry and HPLC were used to quantify the total carotenoid content. A detection wavelength of 478 nm was used in the UV and HPLC analyses. The total carotenoid content in samples with cholesterol esterase decreased by 13.9%, whereas the samples with NaOH decreased by 57.8% after complete hydrolysis of astaxanthin esters (Fig. 1). After enzymolysis, the total carotenoid values obtained by the UV and HPLC methods were in good agreement ($r = 0.889$), and the absolute differences were low (Fig. 1a). However, results obtained by saponification showed a high discrepancy between the UV and HPLC methods (Fig. 1b), especially when the reaction time was prolonged. The higher values obtained by the UV method indicated that pigments with a UV–Visible absorption similar to that of astaxanthin were generated in the saponification process. Their presence was evident on the HPLC chromatograms (Fig. 2). The results also showed that the UV method was not suitable for the determination of astaxanthin and other carotenoids in natural astaxanthin ester samples. Thus, the HPLC method was used for the qualitative and quantitative analyses of astaxanthin and other carotenoids after hydrolysis of astaxanthin esters.

Fig. 2 shows typical HPLC chromatograms of no, partial, complete, and excessive hydrolysis of the astaxanthin esters. Three free astaxanthin fractions were identified according to their retention time by both methods. All-*trans* astaxanthin was the dominant astaxanthin isomer, followed by 9-*cis* and 13-*cis* astaxanthin isomers. Data from the HPLC assay indicated that, with saponification (Fig. 1b), semi-astacene (peak 4) and astacene (peak 3) were present and increased remarkably during the hydrolysis.

The increase in semi-astacene and astacene indicates that there had been degradation of astaxanthin. Furthermore, the loss of astaxanthin isomers and increase in semi-astacene and astacene indicates that during degradation with NaOH, astaxanthin may first oxidize to semi-astacene, then to astacene, and subsequently to other degradation products. Although the concentration of NaOH in reaction mixtures was low, the degradation of astaxanthin after 195 min remained high. By contrast, no semi-astacene or astacene were observed during the 195-min enzymolysis process.

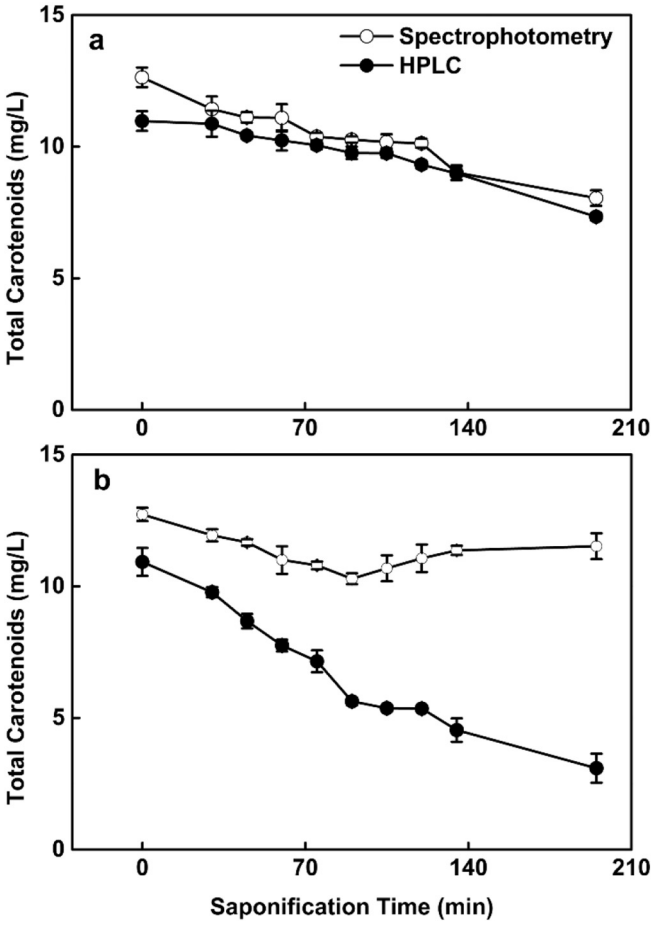

Fig. 1. Changes in the total carotenoid content during enzymolysis (a) and saponification (b). The content of total carotenoids was determined by spectrophotometry and HPLC. Means ± SE of three replicates are presented.

3.2. Effects of enzymolysis and saponification on free astaxanthin recovery

The enzymolysis and saponification methods differed significantly in astaxanthin recovery. As shown in Fig. 3, the application of different reaction times could result in significant differences in the degree of hydrolysis. With the enzymolysis method, free astaxanthin recovery increased initially at 30 min, but plateaued between 45 min and 120 min, and there were no significant differences in recovery (Games Howell, $P > 0.4$). After 120 min, recovery declined with increasing reaction time. Corroborating findings from HPLC chromatograms, the esters were completely hydrolyzed after 60 min, and free astaxanthin recovery reached 89.5%. The free astaxanthin reached its maximum concentration at 75 min. These results suggest that a reaction time of 60–75 min is optimum for complete hydrolysis of astaxanthin esters, with relatively high recovery of free astaxanthin at 37°C.

By the saponification method, the recovery of free astaxanthin increased in the first 60 min and reached the maximum and then declined sharply with extended time. The maximum free astaxanthin recovery (65.7%) was obtained at 60 min. However, esterified astaxanthin was still present in the samples. Complete saponification was observed after 90 min with a recovery of 48.4%. After 195 min, only 26.7% of free astaxanthin was obtained. These results indicate that astaxanthin was not stable in NaOH, even at a low concentration. We found that the time needed for complete hydrolysis of astaxanthin esters was shorter than that reported in previous studies under similar

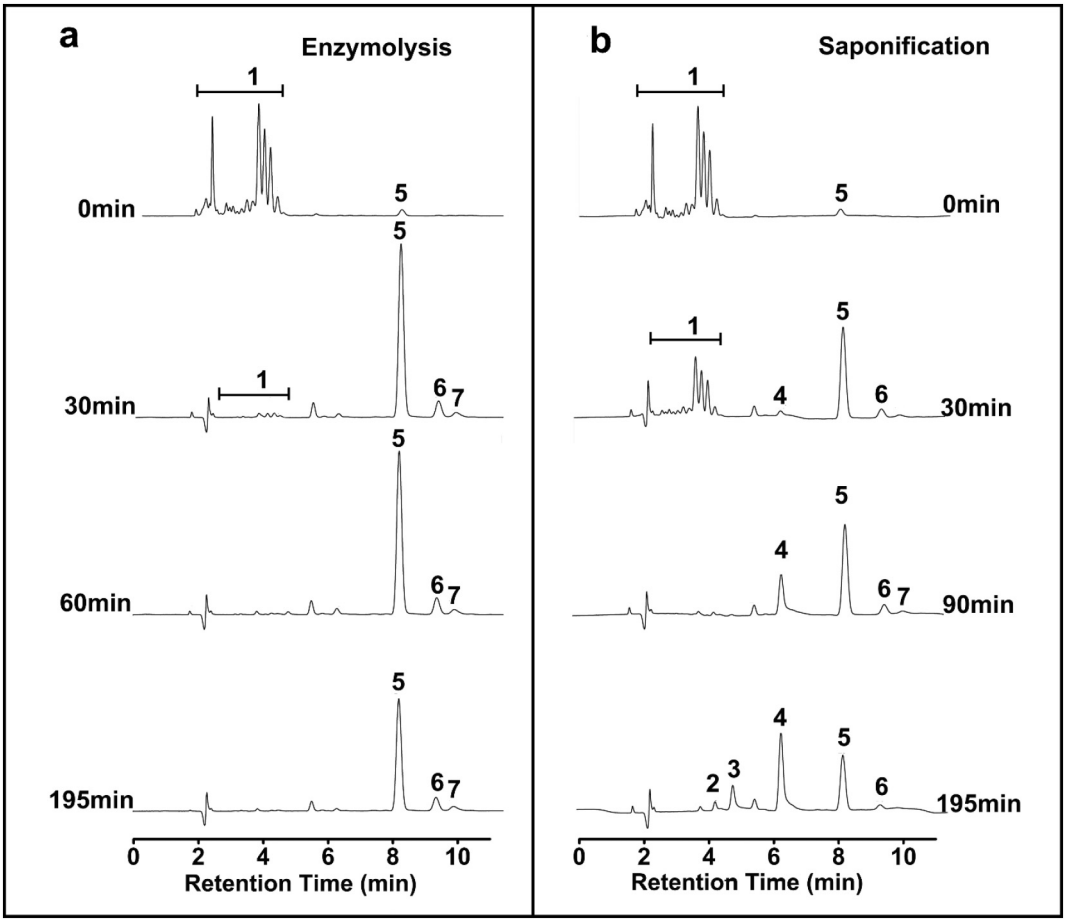

Fig. 2. HPLC chromatograms of enzymolysis (a) and saponification (b) of non, partial, complete, and excessive hydrolysis (see from top to down) of astaxanthin ester extracts. The initial substrate concentration in both methods was the same (12.73 mg/L). The reaction solution contained 0.67 U/mL cholesterol esterase at 37°C and 0.021 M NaOH in methanol at 5°C, respectively. Peaks: 1. Astaxanthin esters; 2. Unknown derivatives; 3. Astacene; 4. Semi-astacene; 5. All-*trans* astaxanthin; 6. 9-*cis* astaxanthin; and 7. 13-*cis* astaxanthin. Note that the peaks 2–4 are degradation products of astaxanthin.

saponification conditions (Table 1) [24,28,29,30]. For example, when we replicated the NaOH concentration and temperature conditions of Yuan and Chen [24], the reaction time was decreased to one-eighth of the time they used. As the rate constant of saponification of astaxanthin esters is independent of the initial concentration of astaxanthin esters [21], discrepancies between these two results may be attributed to the extra mixing step. That is, mixing during the hydrolysis helped increase molecular collision between reactants to increase the reaction rate [31,32]. However, this hypothesis needs to be verified in further studies. The results also showed that the reaction time of saponification should be carefully considered.

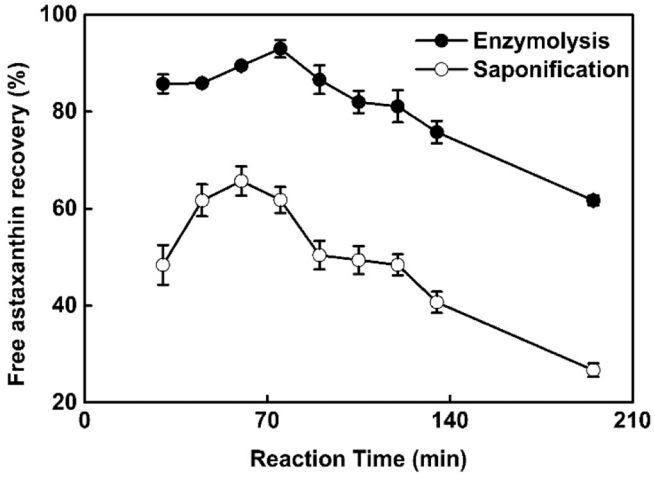

Fig. 3. Free astaxanthin recovery after saponification or enzymolysis for a 195-min hydrolysis as measured by HPLC detection. Means ± SE of three replicates are presented.

3.3. Effect of enzymolysis and saponification on astaxanthin isomerization

During the hydrolysis of astaxanthin esters, the isomers of astaxanthin esters were hydrolyzed simultaneously. Fig. 4 shows the changes in the ratios of each isomer to the total astaxanthin during enzymolysis (Fig. 4a) and saponification (Fig. 4b). In both methods, all-*trans*, 9-*cis*, and 13-*cis* astaxanthin isomers occurred in relatively stable proportions (all-*trans*:9-*cis*:13-*cis* = 21:3:1, approximately) between 30 and

Table 1
Comparison of the time for complete hydrolysis of astaxanthin esters and free astaxanthin recovery under saponification conditions.

Base	Concentration (M)	Temperature (°C)	Time (h)	Recovery (%)	Reference
NaOH	0.021	5	12	N/A	[24]
NaOH	0.020	RT	6	N/A	[28]
KOH	0.020	RT	Overnight	N/A	[29]
NaOH	0.020	5	12	N/A	[30]
NaOH	0.021	5	1.5	48.4	This study

RT, room temperature, approximately 22°C.

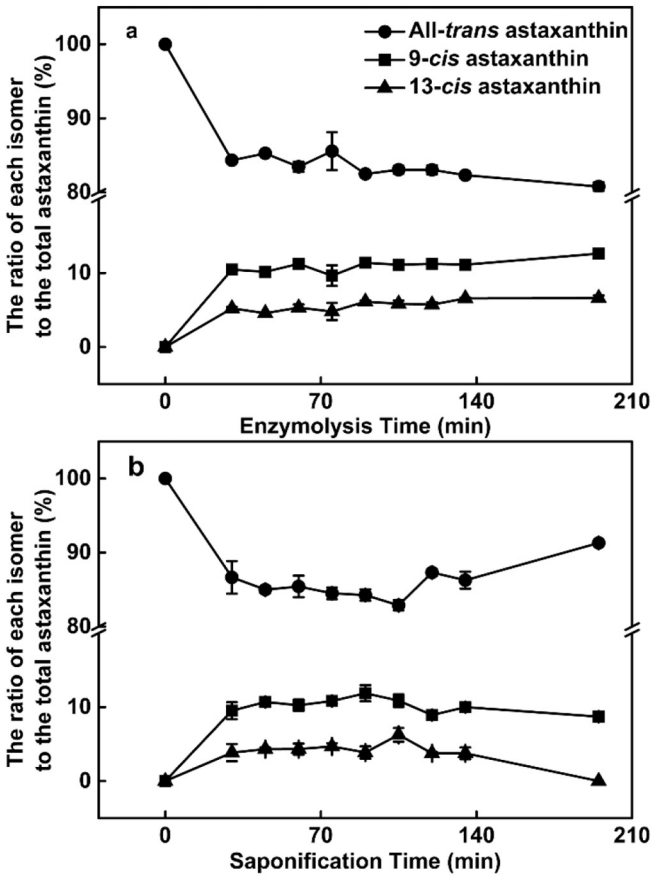

Fig. 4. Changes in the ratio of each isomer to the total astaxanthin during enzymolysis (a) and saponification (b): all-*trans* astaxanthin, 9-*cis* astaxanthin, and 13-*cis* astaxanthin. Means ± SE of three replicates are presented.

75 min. After prolonged treatment time, the isomer ratios changed sharply under saponification conditions (13-*cis* astaxanthin disappeared from the HPLC chromatogram after 195 min), whereas under enzymolysis conditions, the composition of isomers remained stable. Severe degradation and isomerization occurring after saponification but not after enzymolysis indicated that the astaxanthin was more sensitive to alkaline conditions. This finding is consistent with the conclusions reported by Schiedt and Liaaen-Jensen [26]. However, no data were present on the degradation and isomerization of the astaxanthin esters in the study by Schiedt and Liaaen-Jensen [26]. It was observed that extending saponification or enzymolysis time after complete hydrolysis increased the degradation and isomerization of all-*trans* astaxanthin and its *cis*-isomers. Similar changes were observed in the thermal treatment of astaxanthin [33]. The astaxanthin in *H. pluvialis* is the (3*S*, 3′*S*) stereoisomer. No isomerization of optical isomers was observed during the hydrolysis of astaxanthin esters by both methods (data not shown).

3.4. Practical application of enzymolysis in hydrolyzing the astaxanthin esters of prawn (L. vannamei)

The enzymolysis method developed here was used to hydrolyze the astaxanthin esters occurring in prawn (*L. vannamei*), which are known to produce astaxanthin esters [33,34,35,36]. Fig. 5 shows the chromatography changes with or without enzymolysis treatment. It was shown that the prawn produces astaxanthin predominantly in the form of astaxanthin esters (Fig. 5a). This result is identical to those reported in the literature, where the composition of astaxanthin was determined by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC-(APCI)-MS/MS) [33]. Five astaxanthin isomers were identified after enzymolysis

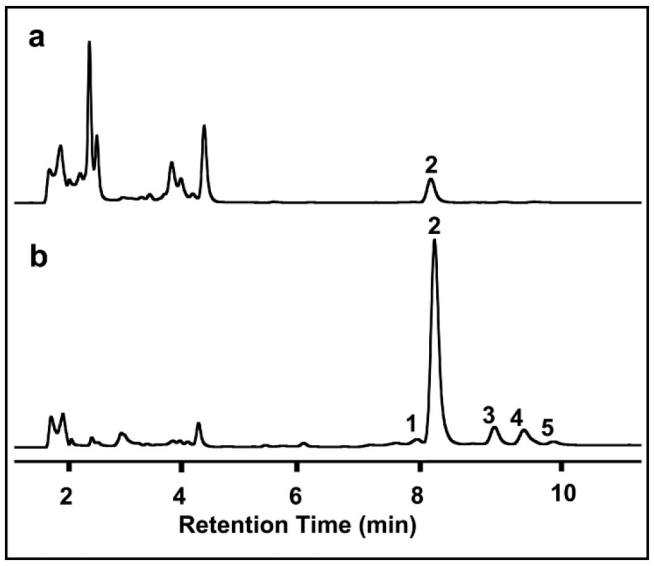

Fig. 5. HPLC chromatograms of the pigment extracts of prawn (*Litopenaeus vannamei*) before (a) and after (b) enzymolysis. Yield was 5.15 ± 0.06 mg of free astaxanthin/kg of prawns from the pigment extracts with 5.61 ± 0.22mg of total carotenoids/kg of prawns, after enzymolysis. Peaks: 1. Di-*cis* astaxanthin; 2. All-*trans* astaxanthin; 3. 9-*cis* astaxanthin; 4. 13-*cis* astaxanthin; and 5. 15-*cis* astaxanthin.

(Fig. 5b). A total of 5.15 ± 0.06 mg/kg free astaxanthin was obtained from the pigment extracts of prawn. The results indicate that the esterified astaxanthin originating from prawn was also accepted as substrates by cholesterol esterase.

4. Conclusion

Hydrolysis of astaxanthin esters is essential for HPLC analysis of natural astaxanthin samples. A comparison of saponification and enzymolysis for hydrolyzing astaxanthin esters of *H. pluvialis* indicates that enzymolysis using cholesterol esterase allowed complete hydrolysis of the astaxanthin esters with much less degradation and isomerization. The saponification treatment with weak alkaline conditions was found to be unsuitable for hydrolyzing astaxanthin esters because of the significant degree of oxidation and degradation occurring during ester hydrolysis. For the hydrolysis of naturally occurring astaxanthin esters in aquatic animals, the enzymolysis method is also suitable. By employing this method, an easy differentiation between the geometrical isomer compositions of astaxanthin from a variety of sources is possible.

Conflict of interests

The authors declare no conflict of interest.

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