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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.

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 can be linked to water-soluble groups (e.g., disodium disuccinate) through hydroxyl groups to form an antioxidant drug useful in the

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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

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