Enzymatic Synthesis of Astaxanthin *n*-Octanoic Acid Esters

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Abstract: We examined the enzymatic synthesis of astaxanthin *n*-octanoic acid esters. Carriers for the immobilized enzyme and reaction conditions such as water content, reaction temperature, and time were examined using *Candida cylindracea* lipase (Lipase OF[®]). Lipase OF[®] immobilized by a hydrophobic anion exchange resin (10% w/w content of lipase) gave the best yield in the esterification reaction of astaxanthin. Two milligrams of astaxanthin per 750 μ L tri-*n*-octanoin (ca. 0.3%) was optimum because of the low solubility of tri-*n*-octanoin. The esters were obtained in a yield of 36.4% under the optimal reaction conditions.

Key words: astaxanthin, *n*-octanoic acid, *Candida cylindracea* lipase, esterification.

1 INTRODUCTION

Astaxanthin (3,3'-dihydroxy- β,β' -carotene-4,4'-dione, Fig. 1) and related compounds are one class of carotenoids. They are tangerine-colored pigments that are found widely in *Crustacea* such as shrimps or crabs, in flesh or the eggs of redfish or trout, and on the body surface of sea bream, carp, and goldfish among others¹. These compounds have remarkable antioxidant activities and are used as health foods^{2,3}.

For their color and physiological function (antioxidant action), astaxanthins are used as natural colorants, cosmetics, and in health foods. For such use, astaxanthins are extracted from *Euphausiacea*, shrimp, crab, *Phaffia* yeast or *Chlorophyceae*, and *Haematococcus*¹. Astaxanthin has been synthesized chemically and is used as a feed additive for the purpose of coloring cultured fish⁴. In nature, astaxanthin is present as free astaxanthin or astaxanthin fatty acid esters (monoester or diester) singly or in a mixture⁵. These astaxanthin esters are known to be usually long chain fatty acid esters including saturated fatty acids such as palmitic acid and stearic acid and unsaturated fatty acids such as oleic acid, linoleic acid, and linolenic acid⁶.

Several methods of obtaining ester derivatives from free

astaxanthin have been published, which consist of esterification reactions of astaxanthin and a long chain fatty acid using lipase as a catalyst⁷ and chemically synthesizing palmitic acid ester⁸. By using lipase, astaxanthin diesters were also converted to the corresponding monoesters⁹. In our previous report¹⁰, we chemically synthesized astaxanthin *n*-octanoic acid monoester and diester (Fig. 1), and we found the esters are better oral-absorbability than free astaxanthin or *Haematococcus* algae derived astaxanthin (an astaxanthin derivative mixture esterified by long-chain fatty acid). So far, it is known that medium-chain fatty acid astaxanthin esters such as *n*-octanoic acid or *n*-dodecanoic

Fig. 1 The Chemical Structure of Astaxanthins.

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acid are present in certain kinds of *Chlorophyceae* and plants although in very small amounts⁶. In particular, the presence of *n*-decanoic acid astaxanthin ester has only been suggested, but it has not been specified as a compound¹¹.

In this report, we examined the enzymatic synthesis of *n*-octanoic acid esters of astaxanthin for use as food additives.

2 EXPERIMENTAL

2.1 Chemicals

Free astaxanthin was purchased from Wako Pure Chemical Industry, Ltd. The carriers for enzyme immobilization were a hydrophobic anion exchange resin (Dowex MARATHON® WBA, Dow Chemical), a hydrophobic adsorptive resin (TOYOPEARL® Phenyl-650S, Tosoh Corporation), an aromatic adsorptive resin (DIAION® HP20, Mitsubishi Chemical Corporation), a methacryl adsorptive resin (DIAION® HP1MG; Mitsubishi Chemical Corporation), a high porous-type aromatic adsorptive ion exchange resin (DIAION® HPA25; Mitsubishi Chemical Corporation), and Celite® (Celite Corporation). A *Candida cylindracea* lipase (Lipase OF®; powder; Meito Sangyo Co. Ltd.) was used as the lipase.

2.2 Immobilization of lipase

In each case the suspension of the resin or gel and Lipase OF® solution was dried under reduced pressure to obtain the indicated amounts of immobilized enzyme product. One hundred grams of anion exchange resin (Dowex MARATHON® WBA) was suspended in 80 mL (5,760,000 units) of Lipase OF® solution to yield 71.0 g WBA-OF. One hundred mL of a hydrophobic adsorptive resin (TOYOPEARL® Phenyl-650S) was suspended in 80 mL (5,760,000 units) of Lipase OF® solution to yield 24.9 g Phenyl-OF. One hundred grams of an aromatic adsorptive resin (DIAION® HP20) was suspended in 80 mL (5,760,000 units) of Lipase OF® solution to yield 60.3 g HP20-OF. Twenty five grams of a high porous-type aromatic adsorptive ion exchange resin (DIAION® HPA25) was suspended in 20 mL (1,440,000 units) of Lipase OF® solution to yield 12.5 g HPA25-OF. Twenty five grams of a methacryl adsorptive resin (DIAION® HP1MG) was suspended in 20 mL (1,440,000 units) of Lipase OF® solution to yield 13.1 g HP1MG-OF. Forty three grams of Celite® was suspended in 80 mL (5,760,000 units) of Lipase OF® solution to yield 45.5 g Celite-OF.

2.3 General enzyme reaction

Free astaxanthin and an acid donor were placed in a brown glass bottle. Immobilized *Candida cylindracea* lipase (Lipase OF®) and a small amount of water were

added, and the mixture was then stirred for 3 or 4 days at 30°C to 50°C. The reaction solution was then taken out, and the same volume of acetone was added. The composition ratio of astaxanthin was analyzed by HPLC (L7000 (Hitachi Co. Ltd., Japan); column: Develosil C30-UG5 (Nomura Chemical Co. Ltd., Japan, 4.6 × 150 mm, 5 μm); mobile phase: a gradient program of 75% aq. acetone for 1 min, and from 75% aq. acetone to 100% acetone over 15 min, then 100% acetone for 10 min; flow rate: 1.0 mL/min; column temperature: ambient; detection wavelength: 480 nm). An area ratio of astaxanthin esters was calculated as the ratio (%) of the total area of astaxanthin monoester and diester to the total area of astaxanthin esters and unreacted free astaxanthin.

2.4 The effect of water content in esterification

Free astaxanthin (3 mg) and WBA-OF (10% lipase, 300 mg) were added to tri-*n*-octanoin (750 μL) in a brown glass bottle, and 3.75 (0.5%), 7.5 (1%), 15 (2%), 22.5 (3%), 37.5 (5%), or 75 μL (10%) of water was added to the reaction mixture, or without water (0%) as a control. The reaction mixture was stirred for 72 h at 40°C.

2.5 The effect of carriers for immobilization

Free astaxanthin (3 mg) and the respective immobilized enzyme (300 mg) preparations were added to tri-*n*-octanoin (750 μL) in a brown glass bottle, water (3.75 μL) was added, and then the reaction mixture was stirred for 72 h at 40°C.

2.6 The effect of acid donor

Free astaxanthin (2 or 3 mg) and WBA-OF (10% lipase, 300 mg) were added to tri-*n*-octanoin (750 μL) or *n*-octanoic acid (750 μL) in a brown glass bottle, water (3.75 μL) was added, and then the reaction mixture was stirred for 72 h at 40°C.

2.7 The effect of lipase content in immobilized enzyme preparations

Free astaxanthin (3 mg) and WBA-OF containing a range of lipase contents (300 mg) were added to tri-*n*-octanoin (750 μL) in a brown glass bottle, water (3.75 μL) was added, and then the reaction mixture was stirred for 72 h at 40°C.

2.8 Time course of the reaction at the respective temperature

Free astaxanthin (3 mg) and WBA-OF (10% lipase, 300 mg) were added to tri-*n*-octanoin (750 μL) in a brown glass bottle, water (3.75 μL) was added, and then the reaction mixture was stirred for 96 h at 30, 40, or 50°C. The area ratio of astaxanthin esters was analyzed at 24, 48, 72, and 96 h.

3 RESULTS AND DISCUSSION

3.1 Water content for enzyme reaction

In the enzyme reaction using an immobilized lipase, water has to be added to the reaction mixture to activate the lipase¹². The optimal amount of water was determined as the percentage volume of water per volume of tri-*n*-octanoic acid (MCT). Zero percent means without the addition of water. As shown in Fig. 2, the addition of 0.5% (3.75 μ L) water was optimal, and the area ratio of esterified product was ca. 26%. The addition of larger percentage volumes of water reduced the area ratio.

3.2 The effect of carriers for lipase immobilization

A hydrophobic anion exchange resin (WBA), Celite[®], a hydrophobic resin (HP20), a hydrophobic adsorptive resin (Phenyl-650S), a high porous-type aromatic adsorptive ion exchange resin (HPA25), and a methacryl adsorptive resin (HP1MG) were tested as carriers for immobilizing the lipase enzyme. As shown in Table 1, WBA was the best support matrix for the immobilized enzyme. WBA is a hydrophobic anion exchange resin consisting of a styrene polymer with trimethylamine residue attached and is sold as a free form. The surface of Lipase OF[®] has acidic regions and it bound in a regio-specific manner to the anionic residue of the resin leaving the active site free to interact with the substrate. The other carriers did not have anionic residues and were unable to bind to the lipase in a manner that retained its activity. Although Celite[®] is sometimes used as a carrier to immobilize lipase¹³, in this case the immobilized enzyme exhibited lower activity than when it was bound to WBA.

Fig. 2 The Effect of Water Content in Esterification.

Free astaxanthin (3 mg) and WBA-OF (10% Lipase OF[®] immobilized with an anion exchange resin, 300 mg) were added to tri-*n*-octanoic acid (750 μ L) in a brown glass bottle, and 0%-10% of water was added to the reaction mixture. The reaction mixture was stirred for 72 h at 40°C.

3.3 The effect of acid donor

MCT and *n*-octanoic acid were examined as acid donors and as reaction solvents. As shown in Table 2, MCT gave a much better yield. Transesterification is preferred to acidolysis as the reaction in the esterification of astaxanthin.

3.4 The effect of lipase content on the immobilized enzyme

The Lipase OF[®] content in WBA was tested across a range from 2.5% to 40%. The immobilized enzyme was then used in the esterification of astaxanthin in MCT. As shown in Table 3, 7.5% and 10% immobilized enzyme gave the best yields of astaxanthin esters. On the other hand, both lower and higher contents of Lipase OF[®] gave lower yields. In the case of 40% immobilized enzyme, it was speculated that the lipase could not display high activity because most of the Lipase OF[®] was stuck to the resin.

3.5 Time course of the reaction at a range of temperatures.

A time course for 4 days at 30°C, 40°C, and 50°C was examined. As shown in Fig. 3, 40-50°C was the optimum temperature. The yield increased up to 3 days, but did not

Table 1 The Effect of Carriers for Immobilization.

Resin	Area ratio of astaxanthin esters (%)
WBA	26.39
Phenyl-650S	4.00
HP-20	2.07
HP1MG	3.61
HPA25	0.43
Celite [®]	3.91

Free astaxanthin (3 mg), the respective immobilized enzymes (300 mg), and water (3.75 μ L) were added to tri-*n*-octanoic acid (750 μ L) in a brown glass bottle and stirred for 72 h at 40°C. WBA, Phenyl, HP20, HP1MG, HPA25, and Celite[®] denote a hydrophobic anion exchange resin, a hydrophobic adsorptive gel, an aromatic adsorptive resin, a methacryl adsorptive resin, a high porous-type aromatic adsorptive ion exchange resin, and clay, respectively.

Table 2 The Effect of Acid Donor.

	Area ratio of astaxanthin esters (%)	
	MCT	<i>n</i> -Octanoic acid
Asta 2mg	36.40	2.47
Asta 3mg	26.22	1.31

Free astaxanthin (2 or 3 mg), WBA-OF (10% lipase, 300 mg), and water (3.75 μ L) were added to tri-*n*-octanoic acid (750 μ L) or *n*-octanoic acid (750 μ L) in a brown glass bottle and stirred for 72 h at 40°C.

change further even though the reaction was continued for a further 24 h.

From these results, Lipase OF[®] immobilized by WBA (10% w/w content of lipase) gave the best yield in the esterification reaction of astaxanthin. Two mg of astaxanthin with 750 μ L MCT (ca. 0.3%) was optimum because of the low solubility of MCT. Astaxanthin esters were obtained at a yield of 36.4% under optimal reaction conditions (Table 2, free astaxanthin (2 mg), WBA-OF (10% lipase, 300 mg), water (3.75 μ L) and tri-*n*-octanoin (MCT, 750 μ L) for 72 h at 40°C). Further studies are needed for additional optimization of the reaction conditions and to increase the yield of astaxanthin ester products.

Table 3 The Effect of Lipase Content in Immobilized Enzyme Preparations.

Lipase content ^{*1}	Area ratio of astaxanthin esters (%)
2.5%	13.08
5%	16.95
7.5%	26.86
10%	25.42
20%	18.61
40%	10.77

^{*1} Lipase content (%) in 300 mg of immobilized enzyme.

Free astaxanthin (3 mg), WBA-OF containing a variety of lipase contents (300 mg), and water (3.75 μ L) were added to tri-*n*-octanoin (750 μ L) in a brown glass bottle and stirred for 72 h at 40°C.

Fig. 3 Time course of the Reaction at a Range of Temperatures.

Free astaxanthin (3 mg), WBA-OF (10% lipase, 300 mg), and water (3.75 μ L) were added to tri-*n*-octanoin (750 μ L) in a brown glass bottle and stirred for 96 h at the indicated temperatures.

4 CONCLUSION

WBA-OF, in which Lipase OF[®] was immobilized by a hydrophobic anion resin (Dowex MARATHON[®] WBA) gave the best yield (36.4%) in the esterification reaction of astaxanthin. The optimal reaction was conducted in the following condition; free astaxanthin (2 mg), WBA-OF (10% lipase, 300 mg), water (3.75 μ L) in tri-*n*-octanoin (MCT, 750 μ L) for 72 h at 40°C.

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