Study of Carotenoid Composition and Fatty Acids of Astaxanthin Diester in Rainbow Trout *Salmo gairdneri* Fed the *Adonis* Extract

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1. The rainbow trout *Salmo gairdneri* were fed the diet containing *Adonis* extract or synthetic astaxanthin. Astaxanthin of both diets was incorporated in the skin and flesh of the fish.

2. Fatty acid composition of astaxanthin diester extracted from rainbow trout fed with diets containing the pigment extract from the flower *Adonis aestivalis* and racemic astaxanthin was determined.

3. Astaxanthin diester which was extracted from the fish fed the *Adonis* extract contained C18:1 (31.8%) and C22:6 (13.1%) as the major fatty acids.

4. Entirely different fatty acid profiles were found in the astaxanthin diester between the *Adonis* extract and the fish fed the *Adonis* extract. The result indicated that astaxanthin diester absorbed and hydrolyzed. After it was transferred into the skin, it was re-esterified.

5. Astaxanthin was extracted from the flesh and the skin of rainbow trout fed with racemic astaxanthin ((3S, 3'S)-(3S, 3'R)-(3R, 3'R)-astaxanthin (1/2/1)). In the flesh, 26.4% (3S, 3'S)-, 51.5% (3S, 3'R)- and 22.4% (3R, 3'R)-astaxanthin were detected. In the skin, 35% (3S, 3'S)-, 46.4% (3S, 3'R)- and 17.8% (3R, 3'R)-astaxanthin were detected.

It is well known that the xanthophylls which occur in nature are often present in biological tissues in the ester forms. Few studies on fatty acid composition of carotenoid esters have been published.1-4 According to these studies, it was found that the carotenoid esters in plants contained more saturated and shorter chain fatty acids than the total lipids of plants. The fatty acid composition of astaxanthin esters extracted from the algae *Ankistrodesmus braunii*5 and the green algae *Haematococcus pluvialis*6 were studied. Czygan and Eichenberger5 reported that the fatty acid profiles of astaxanthin diester were quite different from those of total lipid and concluded that these differences were due to a specific enzymatic reaction. The fatty acid composition of astaxanthin esters in shrimp *Pandalus borealis* has been studied by Renstrøm and Liaaen-Jensen.7 They found that sataxanthin esters in the shrimp had similar fatty acid profiles to those of the total lipids. There were no preferential selection of fatty acids in the astaxanthin extracted from the shrimp.

Hata and Hata7 fed 14C-zeaxanthin to goldfish and found that 14C-zeaxanthin was neither esterified in the intestine nor in the liver. They concluded that the only site where esterification of carotenoids had taken place was in the xanthophore of the skin. Hata7 also proposed that carotenid esters were absorbed in the intestine where they were hydrolyzed and the carotenoids were transferred into the skin. Re-esterification then occurred in the skin.

Recently, three optical isomers of astaxanthin were isolated from some marine organisms, such as lobster,8 shrimp9 and salmon.10 Foss et al.11 studied the pigmentation of rainbow trout using three optical isomers of astaxanthin and reported that the three isomers were equally utilized and deposited in trout without epimerization. Schiedt et al.12 studied absorption and metabolic transformation of optical isomers of astaxanthin. They reported that the ester hydrolases in the intestinal walls might have stereospecificity. However, esterification in the skin would take place without any stereospeci-
In the present paper, the pigment extracts of the flower, Adonis aestivalis, and chemically synthesized racemic astaxanthin were fed to rainbow trout Salmo gairdneri. The carotenoid composition of trout were analyzed. The fatty acid profiles of the astaxanthin diester extracted from the fish were analyzed, and possible mechanisms of absorption and transport of carotenoids are discussed.

Materials and Methods

Fish Culture
The rainbow trout (average weight 400 g) were purchased from American Fish Culture, Carolina, Rhode Island, U.S.A. Forty-five fish were divided into three groups, a control, a group fed the pigment extract of Adonis aestivalis (Adonis extract) and a group fed racemic astaxanthin. Each group was held in a 470-liter, fiber glass tank. The culture tanks were operated according to Kamata.* The fish were fed at a rate of 2% of their body weight per day for eight weeks.

Adonis aestivalis Flower
Dried Adonis aestivalis flower were provided by Dr. G. Neamtu of the Department of Biochemistry, Institute of Agronomy, Cluj-Napoca, Rumania.

Racemic Astaxanthin
Synthetic racemic astaxanthin was provided by F. Hoffmann-La Roche & Co., Basel, Switzerland.

Control Diet
The Oregon test diet for rainbow trout** was modified and used as a control diet. The formula of the diet is shown in Table 1 and pellets were formulated according to Kamata.*

Adonis Extract Diet
The pigments were extracted from A. aestivalis with acetone in a Waring blender. After the pigment were transferred into petroleum ether (PE) by the addition of water, PE was completely removed from the extracts by a rotary evaporator followed by storage under vacuum in a desiccator overnight. The pigments were then dissolved in cod liver oil and mixed with other diet ingredients.

Astaxanthin Diet
Synthetic astaxanthin was dissolved in a small amount of chloroform and mixed with cod liver oil. Chloroform was removed from the oil mixture by use of a stream of nitrogen, followed by storage in a desiccator under vacuum overnight. The cod oil containing astaxanthin was then mixed with other dietary ingredients.

Table 1. Composition of diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>Adonis Extract</th>
<th>Racemic Astaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Vitamin free)</td>
<td>30.68</td>
<td>30.68</td>
<td>30.68</td>
</tr>
<tr>
<td>Gelatin</td>
<td>1.95</td>
<td>1.95</td>
<td>1.95</td>
</tr>
<tr>
<td>Dextrin</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Carboxymethyl Cellulose</td>
<td>0.845</td>
<td>0.845</td>
<td>0.845</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Cod Liver Oil</td>
<td>9.75</td>
<td>9.75</td>
<td>9.75</td>
</tr>
<tr>
<td>Vitamin E (250 IU/g)</td>
<td>0.39</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>Cholin chloride</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.585</td>
<td>0.585</td>
<td>0.585</td>
</tr>
<tr>
<td>Alphacel</td>
<td>8.99</td>
<td>8.98</td>
<td>8.98</td>
</tr>
<tr>
<td>L-Arginine-HCl</td>
<td>0.76</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>Adonis Extract</td>
<td>—</td>
<td>0.01</td>
<td>—</td>
</tr>
<tr>
<td>Racemic Astaxanthin</td>
<td>—</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>Squid Extract</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td><strong>100.000</strong></td>
<td></td>
<td></td>
<td><strong>100.000</strong></td>
</tr>
</tbody>
</table>


Carotenoid Analysis

At the end of the feeding study, the rainbow trout were sacrificed. The skin was carefully separated from the flesh and the pigments were separately extracted from the skin and flesh with acetone in a Waring blender. The carotenoids were first separated on a Microcel-C column using 1-4% acetone in PE as a developing solvent. Each pigment fraction was then saponified with 10% KOH in methanol under nitrogen at room temperature. A MgO: Hyflo Supercel=1:2 column and Silicagel-G thin layer chromatography (TLC) plate were used for further separation and purification of pigments. The carotenoids were identified, based on a visible absorption spectrum, chemical tests and co-chromatography with authentic standards.14)

Purification of Astaxanthin Diester

Astaxanthin diester from the trout was separated on Microcel-C column using 2% acetone in PE as a developing solvent. Lipophilic Sephadex (LH-20) column (1.5 cm I. D. x 100 cm) chromatography was used for the elimination of other lipid materials such as steroids. The diester fraction was dissolved in a small amount of chloroform and passed through the Sephadex column by using chloroform as a developing solvent. For further purification, TLC was employed.

HPLC Analysis

Astaxanthin di-(−)-camphanate was prepared according to Müller et al.15) The resultant astaxanthin di-(−)-camphanate was analyzed by HPLC equipped with Uvikon LCD725 detector. Spherisorb S-5CN column (3.2 × 500 mm) was the stationary phase and n-hexane/isopropyl acetate/acetone (76/17/7) was used as eluting solvent at flow rate 1 ml/min.16)

Fatty Acid Analysis

The purified astaxanthin diester and lipid extract were saponified according to the method of Schauer and Simpson.* Fatty acid methyl esters were analyzed by a single column gasliquid chromatography (Varian Aerograph 1200) unit operated isothermally at 200°C equipped with a flame ionization detector. The temperature of the injector and the detector were 245°C and 270°C, respectively. A 10% SP–2330 silicon column (2.1 m × 3.2 mm) was used. Fatty acids were identified with an electronic integrator (Hewlett Packard 3380A) programmed with relative retention times of authentic standards and literature values.17)

Results and Discussion

At the end of the feeding study, the carotenoid composition of each group of fish were analyzed. As shown in Table 2, the fish fed the pigment containing diets had more carotenoids than the control fish. Astaxanthin diester was found as a major pigment in the skin of both fish fed with Adonis extract and synthetic astaxanthin. Free astaxanthin was a dominant pigment in the flesh. The presence of free astaxanthin in the flesh and astaxanthin esters in the skin of the fish fed synthetic astaxanthin could indicate that the astaxanthin was incorporated as a free form in the flesh and, after being transferred to the skin, was converted to esters in the skin.

Table 2. Carotenoid composition of the fish fed with Adonis aestivalis extract and racemic astaxanthin for eight weeks (μg/g wet basis)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Adonis Extract</th>
<th>Astaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin</td>
<td>Flesh</td>
<td>Skin</td>
</tr>
<tr>
<td>Unknown</td>
<td>—</td>
<td>—</td>
<td>0.04</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.98</td>
<td>0.05</td>
<td>2.01</td>
</tr>
<tr>
<td>Adonixinth</td>
<td>—</td>
<td>—</td>
<td>trace</td>
</tr>
<tr>
<td>Astaxanthin diester</td>
<td>0.21</td>
<td>—</td>
<td>1.18</td>
</tr>
<tr>
<td>Astaxanthin monoester</td>
<td>0.05</td>
<td>—</td>
<td>0.45</td>
</tr>
<tr>
<td>Astaxanthin</td>
<td>0.01</td>
<td>—</td>
<td>0.32</td>
</tr>
<tr>
<td>Total Astaxanthin</td>
<td>0.27</td>
<td>—</td>
<td>1.95</td>
</tr>
<tr>
<td>Total Carotenoids</td>
<td>1.25</td>
<td>0.05</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Table 3. Fatty acid composition of astaxanthin diester extracted from rainbow trout fed with *Adonis aestivalis* extract and racemic astaxanthin

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Astaxanthin Diester in Adonis</th>
<th>Astaxanthin Diester*1 in fish</th>
<th>Astaxanthin Diester*2 in fish</th>
<th>Whole Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>10.4</td>
<td>0.1</td>
<td>0.4</td>
<td>10.4</td>
</tr>
<tr>
<td>14:0</td>
<td>17.2</td>
<td>2.3</td>
<td>2.0</td>
<td>19.5</td>
</tr>
<tr>
<td>15:0</td>
<td>3.8</td>
<td>0.5</td>
<td>0.6</td>
<td>4.3</td>
</tr>
<tr>
<td>16:0</td>
<td>22.7</td>
<td>8.1</td>
<td>7.1</td>
<td>30.1</td>
</tr>
<tr>
<td>16:1</td>
<td>3.8</td>
<td>8.9</td>
<td>7.8</td>
<td>9.5</td>
</tr>
<tr>
<td>16:2/17:1</td>
<td>3.7</td>
<td>0.5</td>
<td>0.6</td>
<td>4.3</td>
</tr>
<tr>
<td>18:0</td>
<td>3.7</td>
<td>3.0</td>
<td>3.7</td>
<td>4.5</td>
</tr>
<tr>
<td>18:1</td>
<td>23.2</td>
<td>31.8</td>
<td>33.9</td>
<td>45.9</td>
</tr>
<tr>
<td>18:2</td>
<td>7.2</td>
<td>8.3</td>
<td>9.5</td>
<td>15.0</td>
</tr>
<tr>
<td>18:3</td>
<td>11.2</td>
<td>9.6</td>
<td>9.1</td>
<td>19.9</td>
</tr>
<tr>
<td>20:1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>20:2</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>20:3</td>
<td>4.3</td>
<td>3.5</td>
<td>2.8</td>
<td>4.3</td>
</tr>
<tr>
<td>20:5</td>
<td>5.7</td>
<td>2.2</td>
<td>2.8</td>
<td>7.7</td>
</tr>
<tr>
<td>22:0</td>
<td>1.2</td>
<td>0.9</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>22:1</td>
<td>13.1</td>
<td>12.0</td>
<td>10.0</td>
<td>25.1</td>
</tr>
<tr>
<td>24:1</td>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

99.4 99.4 98.8 100.2

*1 Astaxanthin diester extracted from trout fed *Adonis aestivalis* extract.

*2 Astaxanthin diester extracted from trout fed racemic astaxanthin.

Fig. 1. HPLC analysis of astaxanthin di-(-)-camphanate isolated from the flesh of rainbow trout fed with racemic astaxanthin. Stationary phase: Spherisorb S-5CN; Mobil phase: n-hexane/acetic acid isopropy-ester/acetone (76:17:7)
was esterified and stored. In the fish fed with Adonis extract, free astaxanthin was found in the flesh and astaxanthin esters were detected in the skin. These results showed that the absorbed astaxanthin diester was hydrolyzed and transported as the free form. After transformation of astaxanthin into the skin, it was re-esterified and stored. Hata proposed that the carotenoid esters were absorbed from the intestine where they were hydrolyzed to free astaxanthin and fatty acids, then transferred into the skin where they were re-esterified and stored. The results of carotenoid analysis of the fish fed Adonis extract supports his proposal.

The fatty acid composition of astaxanthin diester extracted from the fish fed the Adonis extract and synthetic astaxanthin was analyzed. Astaxanthin diester of the flower A. aestiralis contained C18:1 (23.2%), C16:0 (22.7%), C14:0 (17.2%), C18:3 (11.2%), C12:0 (10.4%), and C18:2 (7.2%) (Table 3). There were no fatty acids detected with carbon chain lengths greater than 20, nor with high levels of unsaturation.

It was found that the fatty acid profiles of astaxanthin diester extracted from the fish were entirely different from those of flower astaxanthin diester (Table 3). Astaxanthin diester extracted from the fish contained C18:1 (31.8%), as a major fatty acid as well as C22:6 (13.1%), C20:1 (9.6%), C18:2 (8.3%), C16:0 (8.1%), C22:1 (5.7%), and C20:5 (4.3%). The big differences in fatty acid profiles of these two astaxanthin diester were that long chain and highly unsaturated fatty acids such as C20:5 (4.3%), C22:5 (1.2%), and C22:6 (13.1%) were detected in the astaxanthin diester extracted from the fish, however, those fatty acids were not found in the flower astaxanthin diester.

The fatty acid profiles astaxanthin diester extracted from the fish fed with 10 mg/§ synthetic astaxanthin are shown in Table 3. It was found that the fatty acid profiles of this astaxanthin diester were identical to those of astaxanthin diester extracted from the fish fed Adonis extract. The results also indicated that no preferential selection of fatty acids in the sesterified astaxan-

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**Fig. 2.** HPLC analysis of astaxanthin di(-)-camphanate isolated from the skin of rainbow trout fed with racemic astaxanthin. Stationary phase: Sperisorb S-5CN; Mobile phase: n-hexane/acetic acis isopropyl-ester/acetone (76: 17: 7).
thin in fish appeared when they were compared with the fatty acid profiles in the total lipids. Similar results were reported by Renström and Liaaen-Jensen who studied the fatty acid composition of astaxanthin mono and diester extracted from the shrimp *Pandalus borealis*.

Astaxanthin was extracted from the flesh and the skin of the fish fed synthetic astaxanthin. HPLC analysis showed the presence of 22.4% (3R, 3\'R)-, 51.3% (3S, 3\'R) and 26.4% (3S, 3\'S)-astaxanthin in the flesh (Figure 1). In the skin, 17.8% (3R, 3\'R)-, 46.4% (3S, 3\'R)- and 35% (3S, 3\'S)-astaxanthin were detected (Figure 2). These results indicated that astaxanthin in the diet was directly incorporated in the flesh without any epimerization at C-3 and C-3′ position. The same results were reported by Schiedt et al. and Foss et al. However, in the skin, the ratio of (3S, 3\'S)- and (3R, 3\'R)-astaxanthin was changed to 2 to 1. The increased (3S, 3\'S)-astaxanthin ratio in the skin might be an indication of stereospecificities of enzymatic reactions. Schiedt et al. however, reported that the esterification took place without any stereospecificities in the skin.

In the present paper, it was found that astaxanthin diester in the diet was absorbed and hydrolyzed to free astaxanthin and fatty acids. After it was transported into the skin, it was reesterified by enzymatic reactions. No preferential selection of fatty acids took place during the esterification of astaxanthin in fish. If the above conversion had not occurred, then the fatty acid profiles of astaxanthin diester in the diet and fish would have been expected to be identical. It was also concluded that astaxanthin was first incorporated in the flesh without any epimerization, then transferred into the skin where esterification with fatty acids took place. The enzymes, esterases, in the skin might have stereospecificities at C-3 and C-3′ position of astaxanthin.

**Acknowledgements**

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**References**