Carotenoids and Carotenoproteins in *Asellus aquaticus* L. (Crustacea: Isopoda)

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Accepted September 6, 2005

Column (CC), thin-layer (TLC), high-performance liquid (HPLC) and ion-exchange chromatography (IEC), were used to investigate carotenoid and carotenoprotein complexes in *Asellus aquaticus* specimens from the Narew river. The following carotenoids were found: $\alpha$-carotene, $\beta$-carotene, $\beta$-cryptoxanthin, lutein, zeaxanthin, diadinoxanthin, mutatoxanthin, crustaxanthin, echinene, hydroxyechinone, phoenicoxanthin, canthaxanthin and astaxanthin. Astaxanthin (37.5%), canthaxanthin (21.4%) and phoenicoxanthin (12.3%) were found in the largest amounts. The total carotenoid content was $13.82 \mu g g^{-1}$ of dry mass.

Carotenoprotein complexes containing astaxanthin as the prosthetic group were purified from *Asellus aquaticus*. The carotenoprotein complexes belonged to the crustacyanins group as $\alpha$- and $\gamma$-crustacyanin. The protein forming the $\alpha$-crustacyanin contained large amounts of such amino-acids as asparic acid, glutamic acid and leucine, whereas the protein of the $\gamma$-crustacyanin contained primarily glutamic acid, glycine and lysine.

Key words: Crustacea, Isopoda, carotenoids, carotenoproteins, crustacyanins, amino acids.

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*Asellus aquaticus* is one of the commonest Isopoda crustaceans in European waters. The carapace of this crustacean is of a grey colour which suggests the presence of a carotenoprotein complex.

Studies concerning carotenoprotein complexes in marine representatives of Isopoda of the *Idothea* genus (Lee 1966a,b; Lee & Gilchrist 1972) and of the *Mesidotea* genus (Czeczuga 1996) showed that they contain carotenoprotein complexes, the prosthetic group of which does not consist of astaxanthin, as in other such complexes in other crustacean species, but of a different ketocarotenoid, canthaxanthin.

It was in view of this, and the fact that no investigations of carotenoprotein pigments in fresh-water representatives of the Isopoda have been carried out, that this study was undertaken in the hope that the results would enrich our knowledge of these complexes in the Isopoda.

Material and Methods

*Asellus aquaticus* L. individuals (approx. 100 g of dry weight) were collected in May and June of 1999 from the river Narew near Suraż. The material was kept in the freezer compartment of a refrigerator until removed for chromatographic analysis.

Investigation of carotenoids

The presence of respective carotenoids in *Asellus aquaticus* L. specimens was determined using column chromatography (CC), thin-layer chromatography (TLC) with different systems of solvents (Czeczuga 1984a) and high-performance liquid chromatography (HPLC). Prior to chromatography, the material was homogenized with acetone under nitrogen in dark glass bottles and the extracts kept in a refrigerator until analysis. Saponification was carried out with 10% KOH in ethanol at 20°C for 24 h in the dark under nitrogen. Column and thin-layer chromatography (Czeczuga 1984a; Kraus & Koch 1996) were used to separate the carotenoids, which were identified by comparison with standard compounds by (a) the behaviour on column chromatography; (b) their UV-VIS spectra (Beckman 2400 spectrophotometer); (c) their
partition between n-heksane and 95% ethanol; (d) their Rf-values on thin-layer chromatography; (e) the presence of allylic OH-groups determined by the acid CHCl₃ test; (f) the epoxide test and (g) the mass spectrum (cf. VETTER et al. 1971). Carotenoid pigments were also determined by ion – pairing, reverse – phase HPLC. To 1000 μl of the clear extract, 300 μl of ion – pairing reagent was added according to MANTOURA & LLEWELLYN (1983). The HPLC equipment consisted of a Shimadzu LC – 6A double – system pump, driven by a gradient programmer Shimadzu SCL-6B and Rheodyne 7125 injector equipped with a 20 μl loop. Detection was performed by a Shimadzu SPD – 6AV UV – VIS spectrophotometric detector set on 440 nm and Shimadzu RF – 535 fluorescence detector.

Carotenoid pigment standards were obtained from Hoffman – La Roche Company, Switzerland, International Agency for ¹⁴C Determinations, Denmark and Sigma Chemical Company, USA.

Quantitative determinations were performed by UV, VIS spectroscopy according to the Davies method (CZECZUGA 1985). For the structures of carotenoids see STRAUB (1987) and CZECZUGA (1988).

Investigation of carotenoproteins

The material was dissected, homogenized and centrifuged under refrigeration in a Janetzki K-24 centrifuge and suspended in EDTV solution. The carotenoprotein complex was precipitated with ammonium sulphate (ZAGALSKY et al. 1970). The precipitate was centrifuged again and dissolved in 0.05 M phosphate buffer (pH 7.0). After an overnight dialysis, also under refrigeration, in the presence of phosphate buffer (pH 7.0), the material was centrifuged once and then purified by means of ion-exchange chromatography with a DEAE-
cellulose carrier. Elution was made with phosphate buffer (pH 7.0) using a linear concentration gradient of 0.02-0.35 M. The measurements of extinction in the eluent were taken in the range of 300-800 nm, using a “Spectroma” spectrophotometer Model 203.

Ketocarotenoid was identified as the prosthetic group of the carotenoproteins examined by means of thin-layer chromatography of the extracted carotenoid, alone or admixed with a ketocarotenoid (astaxanthin) standard (Hoffman-La Roche and Co. Ltd., Basle), on a thin-layer of silica gel-G with 15% acetone in petroleum ether (ZAGALSKY et al. 1967).

Carotenoids were liberated from carotenoproteins with acetone (SHONE et al. 1979).

Samples for the analysis of the amino acid composition were prepared after the methods described by ZAGALSKY et al. (1967). They were hydrolyzed for 36 hrs, at a temperature of 110°C.

Amino acids were separated on a two-column system, using a JEOL JLC-6 AH automatic amino acids analyser, under the standard conditions recommended by the makers (JEOL Instructions, Tokyo).

The columns were filled with ICR-2 resin, separation temperature -52°C, 0.8 ml samples of the material used for the analyses. The speed of flow of buffer solutions was 25.2 ml/hr and of the anihindrine dye 12.6 ml/hr.

The alkaline amino acids (Lys, His, Arg) were separated in 8×150 mm column, in 0.33 Na-citrate buffer solution, at pH 5.28, under the pressure of approximately 8 atm.

Acidic and neutral amino acids were separated on 8×500 mm column, in buffer solutions No. 2:0.2 n Na-citrate, at pH 4.2, under the pressure of approximately 20 atm.

### Table 1

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Summary Formula</th>
<th>Structure (see Fig. 1)</th>
<th>Semisystematic name</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. α-carotene</td>
<td>C₄₀H₅₀</td>
<td>A - R - B</td>
<td>β,β-Carotene</td>
<td>0.8</td>
</tr>
<tr>
<td>2. β-carotene</td>
<td>C₄₀H₅₀</td>
<td>A - R - A</td>
<td>β,β-Carotene</td>
<td>3.7</td>
</tr>
<tr>
<td>3. β-lycopene</td>
<td>C₄₀H₅₀</td>
<td>A - R - C</td>
<td>β,β-Caroten - 3-ol</td>
<td>4.1</td>
</tr>
<tr>
<td>4. lutein</td>
<td>C₄₀H₅₀O₂</td>
<td>C - R - D</td>
<td>β,ε-Carotene-3,3’-diol</td>
<td>2.7</td>
</tr>
<tr>
<td>5. zeaxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>C - R - C</td>
<td>β,β-Carotene-3,3’-diol</td>
<td>2.8</td>
</tr>
<tr>
<td>6. mutatoxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>F - R₁ - G</td>
<td>5,8-Epoxo-5,8-dihydro-β,β-carotene-3,3’-diol</td>
<td>0.8</td>
</tr>
<tr>
<td>7. cryptoxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>H - R - H</td>
<td>Trans-β,β-carotene-3,4,3’,4’-tretol</td>
<td>6.5</td>
</tr>
<tr>
<td>8. diadinoxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>E - R₁ - G</td>
<td>5,6-Epoxo-7,8-dihydro-5,6-dihydro-β,β-carotene-3,3’-diol</td>
<td>2.4</td>
</tr>
<tr>
<td>9. echinonone</td>
<td>C₄₀H₅₀O₂</td>
<td>A - R - I</td>
<td>β,β-Caroten-4-one</td>
<td>1.2</td>
</tr>
<tr>
<td>10. hydroxyechinone</td>
<td>C₄₀H₅₀O₂</td>
<td>A - R - K</td>
<td>3-Hydroxy-β,β-caroten-4-one</td>
<td>3.8</td>
</tr>
<tr>
<td>11. phoenicoxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>I - R - K</td>
<td>3-Hydroxy-β,β-caroten-4,4’-dione</td>
<td>12.3</td>
</tr>
<tr>
<td>12. canthaxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>I - R - I</td>
<td>β,β-Caroten-4,4’-dione</td>
<td>21.4</td>
</tr>
<tr>
<td>13. astaxanthin</td>
<td>C₄₀H₅₀O₂</td>
<td>K - R - K</td>
<td>3,3’-Dihydroxy-β,β-caroten-4,4’-dione</td>
<td>37.5</td>
</tr>
</tbody>
</table>
Fig. 1. Structural features of carotenoids from investigated materials (see Table 1).

Table 2

Amino acid composition of the carotenoproteins of *Asellus aquaticus*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount in mol % (\alpha)-crustacyanin</th>
<th>Amount in mol % (\gamma)-crustacyanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>5.45</td>
<td>8.52</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.07</td>
<td>2.06</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.11</td>
<td>6.24</td>
</tr>
<tr>
<td>Asparic acid</td>
<td>12.78</td>
<td>3.48</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.79</td>
<td>2.24</td>
</tr>
<tr>
<td>Serine</td>
<td>4.77</td>
<td>5.01</td>
</tr>
<tr>
<td>Proline</td>
<td>5.96</td>
<td>2.47</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.22</td>
<td>12.63</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.98</td>
<td>8.92</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.16</td>
<td>6.12</td>
</tr>
<tr>
<td>Valine</td>
<td>7.50</td>
<td>4.19</td>
</tr>
<tr>
<td>Cystine/Cystine</td>
<td>1.19</td>
<td>0.84</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.17</td>
<td>3.07</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.62</td>
<td>4.26</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.18</td>
<td>6.18</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.41</td>
<td>1.86</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.62</td>
<td>4.11</td>
</tr>
</tbody>
</table>

Table 3

Comparison of composition (mol %) of carotenoproteins in some species of Isopoda

<table>
<thead>
<tr>
<th>Amino acid sets</th>
<th><em>Asellus aquaticus</em></th>
<th><em>Idothea resecata</em></th>
<th><em>Mesidotea entomon</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolar residues (A): Val, Leu, Ileu, Phe, Met</td>
<td>25.0</td>
<td>27.3</td>
<td>26.4</td>
</tr>
<tr>
<td>Polarity index (P): sum of values of Asp, Thr, Ser, Glu, Lys, His, Arg</td>
<td>43.7</td>
<td>46.0</td>
<td>50.2</td>
</tr>
<tr>
<td>P/A ratio</td>
<td>1.75</td>
<td>1.70</td>
<td>1.90</td>
</tr>
<tr>
<td>Small amino acids: Ala, Gly</td>
<td>14.6</td>
<td>15.2</td>
<td>14.3</td>
</tr>
<tr>
<td>Charged amino acids: Asp, Glu, Arg, Lys</td>
<td>32.3</td>
<td>31.1</td>
<td>30.4</td>
</tr>
</tbody>
</table>

* mean for sum of \(\alpha\)-crustacyanin and \(\gamma\)-crustacyanin
The results were calculated on the basis of the data from a two-channel integrator. A standard amino acid solution, produced by Pierce Ltd., U.S.A., was used.

Results

Chromatographic analysis revealed the presence of 13 carotenoids in Asellus aquaticus individuals, with the largest amounts of astaxanthin, canthaxanthin and phoenicoxanthin. The total carotenoid content was 13.824 µg g⁻¹ of dry mass (Table 1, Fig. 1).

A carotenoprotein complex was isolated from specimens of Asellus aquaticus which, in a phosphate buffer at pH 7.0, gave two fractions, absorption maxima first fraction at 608 and the second fraction at 636 nm. The carotenoid separated from these fractions was identified as astaxanthin. The proteins of the first fraction contained large amounts of amino-acids such as asparagine, glutamic acid, glycine and leucine. Considerable amounts of valine, alanine and glycine were also found. Methionine and cysteine constituted the smallest proportions. Proteins of the second fraction contained large amounts of glutamic acid, glycine and lysine (Table 2).

Discussion

Three basic groups of carotenoproteins can be distinguished in aquatic animals. The first group includes blue-coloured crustacyanins, a combination of carotenoid and protein found mainly in crustacean armour. BUCHWALD and JENCKS (1968) demonstrated that crustaceans contain three types of crustacyanine: α-, β-, and γ-, all with astaxanthin as the prosthetic group. Presently it is known that the prosthetic group of crustacyanins may include other ketocarotenoids such as canthaxanthin and phoenicoxanthin (CZECZUGA & CZECZUGA-SEMENIUK 1998). Crustacyanins can be found both in marine crustacean species and fresh water ones. α-Crustacyanine was detected in Eudiaptomus amblyodon and Palaemon adspersus, β-crustacyanine in Gammarus lacustris and Palaemon adspersus and γ-crustacyanine in Cran gon crangon and Cyclops kolensis (CZECZUGA 1997; CZECZUGA & CZECZUGA-SEMENIUK 2000). Carotenoproteines such as crustacyanines were also found in other marine (Euphausia superba, Mesidotea entomon) and fresh water (Chirocephalus diaphanus, Daphnia magna, Orconectes limosus) crustacean species (ZAGALSKY et al. 1983; CZECZUGA 1984a, b; 1996; CZECZUGA & KRYWUTA 1981a; CZECZUGA & CZECZUGA-SEMENIUK 1998). The second group contains oovorubins, a combination of carotenoid and glycolypoprotein, which gives a green pigmentation to crustacean eggs and ovaries. The third group consists of red oovorubins, a combination of carotenoid and glycoprotein, found in the albumen gland and shellfish eggs (CHEESMAN et al. 1967; KE 1971; ZAGALSKY 1976; LEE 1977; NAKAGAWA 1978; BRITTON et al. 1982; RENSTRÖM et al. 1982; GOODWIN 1984). According to ZAGALSKY et al. (1990) and KEEN et al. (1991), the large molecule of the carotenoprotein complex consists of a number of smaller protein subunits, each cup-shaped with edges formed by a coiled protein chain, in which a pigment (astaxanthin) molecule may be attached to the hydrophobic part of the protein and situated on the cup bottom. Apart from grey, green and a variety of red shades, the most common colour is blue, an effect of crustacyanins, particularly α-crustacyanin. A blue colour of carotenoproteids due to crustacyanins also occurs in marine species of coelenterates (ZAGALSKY & HERRING 1977; ZAGALSKY & JONES 1982), starfish (CZECZUGA 1983; CLARK et al. 1990) and Tunicata representatives (HERRING 1978).

α-Crustacyanine is more common in crustaceans (HOISCHEN et al. 1998). The same individual may contain more than one protein-carotenoid complex simultaneously: red (MILICUA et al. 1985), pink (GOMEZ et al. 1988), yellow (ZAGALSKY 1982; MILICUA et al. 1986 a, b) and blue (GARATE et al. 1984, RIVAS et al. 1988). This also refers to the eggs of Cyclops vernalis females. The colour can be grey, brown, green, purple, blue and even black (DU PRAW 1958). Different proportions of these protein-carotenoid complexes result in various colours of crustacean armour. A greyish colour due to a carotenoprotein complex in isopods produces protective coloration to environments in which they live (LEE 1966a, b; CASTILLO et al. 1982; CZECZUGA 1996).

In view of the absorption maxima of the carotenoprotein complex isolated from Asellus aquaticus, it seems that two varieties of the same complex occur in these specimens. The literature (JENCKS & BUTEN 1964; BUCHWALD & JENCKS 1968; CHEESMAN et al. 1966) indicates that absorption maxima at 608 and 636 nm are characteristic of crustacyanine. This type of crustacyanine has already been noted in the fresh-water crustacean, Gammarus lacustris (CZECZUGA & KRYWUTA 1981b). The absorption maximum at 608 nm indicates the presence of γ-crustacyanine in Asellus aquaticus, whereas the absorption maximum at 636 nm indicates α-crustacyanine. The absorption maximum of γ-crustacyanine is within 603-616 nm. Absorption maxima approaching 636 nm were noted by other authors in studies of other crustaceans. CECCALDI & ALLEMAND (1964a, b) iso-
lated a carotenoprotein complex with maximum absorption within the range of 625-635 nm from *Homarus gammarus* individuals and from the shrimp *Aristeus antennatus* (CECCALDI and ZAGALSKY 1967). In both cases, the authors identified this complex as α-crustacyanin, with astaxanthin as the prosthetic group.

In this study the main maximum was at 636 nm, it can be assumed that this crustacyanin was the main component of the complex. It would therefore seem more advisable to compare the amino acid composition of the protein in the complex contained in the *Asellus aquaticus* with that of α-crustacyanin from other crustaceans. According to BUCHWALD and JENCKS (1968), the protein of α-crustacyanin from *Homarus gammarus* consisted mainly of aspartic acid, glutamic acid and tyrosine. In the material examined in this study, the first two constituted the greatest proportions of the amino acid composition whereas tyrosine, on the other hand, constituted one third of the quantity of aspartic- and glutamic acids. It should be emphasized that in eight species of crustaceans belonging to various systematic classes, the tyrosine content in protein of the carotenoprotein complexes was found to be approximately 3 mol% (ZAGALSKY 1976). Furthermore, in certain fresh-water crustaceans in which crustacyanin was determined, tyrosine was found to constitute 1.80-2.42 mol% (CZECZUGA & KRYWUTA 1981a,b,c).

When the proteins of the complexes isolated from *Asellus aquaticus* are compared with those obtained from other species of Isopoda (Table 3), it was found that the P/A ratio and the smaller index are the same as those noted in *Idothea resecata* (LEE 1977) and *Mesidotea entomon* (CZECZUGA 1996). On the other hand, the apolar index and the polarity index and sum of values of charged amino acids are specific for *Asellus aquaticus* specimens.

Unlike that of the marine representatives of the Isopoda, the carotenoprotein complex of *Asellus aquaticus* contained an astaxanthin prosthetic group. It should be noted that astaxanthin was found to occur in large amounts in the *Asellus aquaticus* throughout a one year cycle of investigations concerning the presence of various carotenoids on a population in the River Narew (CZECZUGA 1975). Astaxanthin is also the dominant carotenoid in land crustaceans belonging to isopods (CZECZUGA 1975).

**References**


