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Comparative Study of the Endemic Freshwater Fauna of Lake Baikal-VII. Carotenoid Composition of the Deep-Water Amphipod Crustacean *Acanthogammarus (Brachyuropus) grewingkii*

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ABSTRACT. The carotenoid composition of the deep-water gammaride is reported. Astaxanthin and their derivatives were determined to be major components of the carotenoids (58.4%). Astaxanthin-glycoside-esters comprised 21.6% of total carotenoids. A new carotenoid glycoside ester (CGE) was isolated from the deep-water gammaride *Acanthogammarus (Brachyuropus) grewingkii* and its structure was elucidated. The structures were determined from spectra (^{1}H-NMR, ^{13}C-NMR, MS, IR) after their isolation and quantification by means of semipreparative RP-HPLC and capillary GC-MS. COMP BIOCHEM PHYSIOL 114B;4:383–387, 1996.

KEY WORDS. Lake Baikal, deep-water amphipod, carotenoids, astaxanthin glycoside esters, *Acanthogammarus grewingkii*

INTRODUCTION

The carotenoids are widespread, naturally occurring compounds the importance of which is related to their function (8). Esterified carotenoids occur in nature in algae, higher plants, fruits and marine animals (18). At present, about ten different CGEs have been isolated from among more than six hundred naturally occurring carotenoids (19,26).

CGEs have been isolated mainly from smile-moulds. Kleinig and co-workers have identified myxobactin, myxobactone and 3-hydroxy derivative from *Myxococcus fulvus* Mx-f2 (9,20) and *Stigmatella aurantiaca* (12). The ester of the 3-hydroxy derivative has been isolated only from two species, *Chondromyces apiculatus* (10) and *Sorangium compositum* So-c1 (13). Other glycoside esters have also been identified (6,11,14,17,28,29,30). Recently, a novel monocylic carotenoid glucoside ester, was found in the green filamentous bacterium *Chloroflexus aurantiacus* strain J-10-f1 as a major carotenoid component, and its chemical structure was determined to be 1′-[(6-0-acyl-β-D-glucopyranosyl)oxy]-1′,2′-dihydro-β,ψ-carotene (27).

Deep-water gammaride species found in the Lake Baikal usually live from 100 to 1300 m. The species which live in the depths, e.g., species of the *Acanthogammarus* genus, are armed with spikes and their carapace is a yellowish-pink-colour. The carotenoid composition of some Baikalian gammarides was analyzed some years ago (4); but neither CGEs nor carotenoid esters were found. We have previously examined the lipid composition of *A. grewingkii* (5) and identified some new cyclopropane monounsaturated fatty acids (23) and long-chain unsaturated ketones (24).

The present work is a continuation of our earlier studies detailing the carotenoid composition, their esters and the fatty acid composition of the Baikalian gammaride.

MATERIALS AND METHODS

Collection of Samples

Acanthogammarus (Brachyuropus) grewingkii Dyb., belonging to the genus Acanthogammarus, subfamily Brachyuropus, family Eulimnogammarus, class Malacostraca, subphylum Crustacea, the phylum Arthropoda, order Amphipoda (1), were sampled in the south-western part of Lake Baikal, in July 1992, at depths of 350–400 m. The animals were put in tanks under water at the site of collection and transported to the laboratory (Biological Station of Irkutsk University, Bay of Bolshye Koty). Nine samples of gammarides (7–9 cm in length, raw weight of animals vary from 4.3–8.2 gr) were collected and used for the lipid extraction.

Carotenoids Analysis

The total lipids (3.57 g) from gammaride were extracted with chloroform-methanol (1:1, v/v) and lipid extract was washed with 50 mM Tris buffer (29) and evaporated to 1/

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10 volume. The crude carotenoids (0.94 g) were separated by preparative TLC on silica gel G using acetone-hexane (30:70, v/v) and each fraction was submitted to further purification by HPLC.

The liquid chromatograph, i.e., the semipreparative Gradient LC System G-1 (Shimdzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (481 nm) a SIL-1A sample injector and a C-R3A data processor, with preparative column SGX (spherical silica gel) 250 mm × 8 mm ID: 7 μm particles (Tessek, Prague) and with an analytical column 250 mm × 4 mm ID, packed with SGX-C18 with 5 μm particles were used (Tessek). After injection of 100 μl solution of total crude extract (5 mg/ml) a gradient of aceto-nitrile-methanol (convex gradient from 100:0 to 90:10 for 20 min) was applied. The eluted peaks were collected manually and transferred directly to the second reverse phase column and the elution was then with a mixture acetonitrile-tetrahydrofurane-methanol using a linear gradient at 40:50:2 (v/v) during 10 min to 70:28:2 (v/v) and during additional 8 min to 70:20:10 (v/v) (3,22). Individual compounds were collected manually.

Individual compounds were identified by determining fatty acid oxazolines (by GC-MS) after hydrolysis (22). They were also identified by a direct injection into FAB-MS, measurement of ^{1}H-NMR, ^{13}C-NMR and UV-spectra (25).

All FAB-MS and GC-MS separation were performed by using a Finnigan MAT (San Jose, CA, USA) 1020B apparatus with electron impact and/or fast atom bombardment ionization. FAB-MS were obtained using Xe at 6 keV for ionization, accelerating voltage was 10 keV, the resolving power was 1000, a range of m/z 10–1200 was scanned every 10s. A mixture of 3-nitrobenzyl-alcohol and glycerol (50:10, v/v) was used as the matrix. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker AM 500 pulsed Fourier transform spectrometer (Karlsruhe, Germany) operating at 500 MHz for ^{1}H and 125.69 MHz for ^{13}C at ambient temperature. Absorption spectra in visible light were measured in EtOH with UV-160A (Shimadzu).

The oxazolines were prepared using a modification of published methods (21) and identified and quantified (by ion current) by means GC-MS equipped with a fused silica capillary column (60 × 0.32 mm ID × 0.25 μm (SPB-1, Supelco); splitless injection, carrier gas He. Oven temperature was programmed from 150–330°C at a rate of 5°C/min. Ionization energy was 70 eV, electron multiplier voltage 2.5 kV.

CGEs were dissolved in 1 ml 95% EtOH and reduced by $NaBH_4$ for 30 min. After evaporation, the compounds of the mixture were analysed by FAB-MS. CGEs were also dissolved in pyridine, acetic anhydride was added and the mixture was stirred 2 h at room temperature. The number of carbonyl groups and/or free hydroxyles were determined by the increasing mass unit of the chemically modified carotenoid by two (i.e., M.W. 1024 = 2 oxogroups), respective 42 amu for every hydroxyl group (i.e., M. W. 1188 = 4 hydroxyles).

Enzymatic hydrolysis of the CGEs was made as described (28). Carotenoid glycoside was measured by FAB-MS (M.W. 758). The sugars in the water layer were analyzed using color reaction with naphtoresorcinol, after separation by TLC.

TABLE 1. Composition of carotenoids from *A. grewingkii*

No.	Carotenoid's compounds	Distribution (%wt)[a,b]
1.	β-Carotene	5.4
2.	α-Carotene	7.8
3.	Astaxanthin esters	14.9
4.	Astaxanthin	8.2
5.	Astaxanthin-glycosides-esters	21.6
6.	Zeaxanthin	4.9
7.	Astacene	6.2
8.	Astaxanthin-glycosides	13.7

[a]Percentage of compounds from total carotenoids.
[b]Minor and unidentified compounds (17.3%).

RESULTS AND DISCUSSION

The present study is concerned with the carotenoid composition of *Acanthogammarus grewingkii*. The content and percentage composition of individual carotenoids are shown in Table 1. Pure astaxanthin comprised 8.2%, astaxanthin esters (the sum of mono- and di- esters), 14.9%. Astaxanthin-glycosides comprised 13.7% and the CGEs were present in the largest quantity of all the carotenoids (21.6%) (fraction No. 5).

Fraction No. 5 (see Table 1) was collected using semi-preparative HPLC and its contents are presented in Table 2. Fourteen different glycoside-esters were separated and identified. Two saccharides, glycose and galactose were

TABLE 2. Composition of fraction astaxanthin-(AST)-glycoside-esters

No.	Saccharide	Fatty Acid	% a	Molecular weight
i	AST glucose	6,9,12-octadecatrienoic	10.3	1018
ii	AST galactose	tetradecanoic	4.3	968
iii	AST glucose	9,12,15-octadecatrienoic	7.1	1018
iv	AST glucose	tetradecanoic	4.2	968
v	AST galactose	9-hexadecenoic	1.3	994
vi	AST glucose	9-hexadecenoic	2.9	994
vii	AST glucose	5,8,11,14-eicosatetraenoic	1.5	1036
viii	AST glucose	9,12-octadecadienoic	14.3	1020
ix	AST galactose	hexadecanoic	1.7	996
x	AST galactose	9-octadecenoic	2.2	1022
xi	AST glucose	hexadecanoic	14.7	996
xii	AST glucose	9- and 11-octadecenoic	22.6	1022
xiii	AST galactose	octadecanoic	1.7	1024
xiv	AST glucose	octadecanoic	11.2	1024

a, % total fatty acids from fraction No. 5.

TABLE 3. ^{1}H- and ^{13}C-NMR data of compound (viii)

Carbon No.	Carotenoid		Saccharide		Fatty acid	
	^{1}H-	^{13}C-	^{1}H-	^{13}C-	^{1}H-	^{13}C-
1	—	37.8	5.01 d	106.4	—	173.4
2	1.82 ax dd 2.16 eq dd	42.9	3.55 dd	76.9	2.30 t	34.1
3	4.32 ax dq	72.3	3.57 dd	78.1	1.62 m	25.0
3′	3.85 ax dq	64.4	—	—	—	—
4	—	196.2	3.38 dd	71.2	1.31 br s	29.3
5	—	128.4	3.30 ddd	75.6	1.31 br s	29.2
6	—	161.6	3.65 dd 3.80 dd	65.6	1.31 br s	29.2
7	6.22 d	123.4			1.31 br s	29.7
8	6.43 d	142.7			2.02	27.3
9	—	135.1			5.35	130.0
10	6.30 d	135.2			5.35	128.1
11	6.57 q	124.6			2.77	25.7
12	6.45 d	139.9			5.35	128.1
13	—	137.1			5.35	130.1
14	6.28 m	134.1			2.02	27.3
15	6.67 m	131.0			1.31 br s	19.4
16	1.21 eq s	31.1			1.31 br s	31.6
17	1.32 ax s	27.5			1.31 br s	22.7
18	1.95 s	15.5			0.89 t	14.1
19	2.00 s	12.2				
20	1.99 s	11.9				

3′-Nonesterified carbon atom on cyclohexane.

found to be present in the AST-glycoside-esters. The fatty acid composition of individual AST-glycoside-esters was also determined. Five major fatty acids such as 18:1 (two isomers, 22.6%), 16:0 (14.7%), 18:0 (11.2), γ18:3 (10.3%) and 18:2 (14.3%) consisted more than 73% of the total fatty acids.

The structure of the major peak (viii, see Table 2) after RP-HPLC separation was determined by means ^{1}H-NMR and ^{13}C-NMR spectra (see Table 3 for this and other signals). Comparison of our CGEs to one found in a previous study (6,9,11,14,28,29) clearly reveals very similar structures. Chemical shifts for appropriate to glycone are also very similar to earlier data (6,9,11,14,28,29), as are shifts for saccharide and fatty acids. The ^{1}H- and ^{13}C-NMR spectra of one of the AST-glycoside-esters is shown in Table 3. The ^{1}H-NMR spectrum indicated that the anomeric proton of the glucose gives a doublet at 5.01 ppm which supports a β-D-configuration of the glucoside (7). The structure of the CGEs was also determined by means of MS and UV methods.

The major CGE (14.3%) was isolated from the deep-water gammaride A. *grewingkii* and its structure was determined (see Fig. 1).

Astaxanthin and their esters are widely distributed in nature. It is the principal pigment in crustaceans, fishes, and many other aquatic organisms (15).

There have been comparatively few publications concerning the carotenoid content of the Baikalian Gammaride (4,16). Usually few carotenoids (from 6–11) were found (4). In some gammarides astaxanthin and their esters were the major pigments: *Crypturopus pachytus* (39% of total carotenoids), *Micruropus vortex* (26%), *Pallasea cancelloides* (37%), *Eulimnogammarus cruentus* (37%), *Eu. maaki* (51%) and *Macrohectopus branickii* (31%). According to Czeczuga (4) who analysed the carotenoid content of *Acanthogammarus albus*, astaxanthin esters comprised 13.5% of the total carotenoids, pure astaxanthin −5%, and the major carotenoid was astacene at 43.3%.

The *Eulimnogammarus* species taken for carotenoid analysis have usually various shades of red colour. It is known that such carotenoids as astaxanthin and/or its mono- and diesters, among others, form complexes with proteins which give the crustaceans a different coloration (2). The carotenoids which form part of the protein carotenoid complex in the crustaceans investigated have been identified. This probably explains the colour of the A. *grewingkii* and other Baikalian gammarides. The carotenoids found in A. *grewingkii* specimens are also worthy of note. It is known that this species leads a plankton way of life (1), as a result of which this crustacean has undergone a series of adaptations. The chromatographic analysis showed only five carotenoid species to be present in this species. Zeaxanthin comprised only 4.9% of the carotenoid content, carotene (both α- and β-forms)- 13.2%, whereas about 64.6% consisted astaxanthin, including its derivatives, and astacene which we know gives crustaceans their pink colour. In the case A.

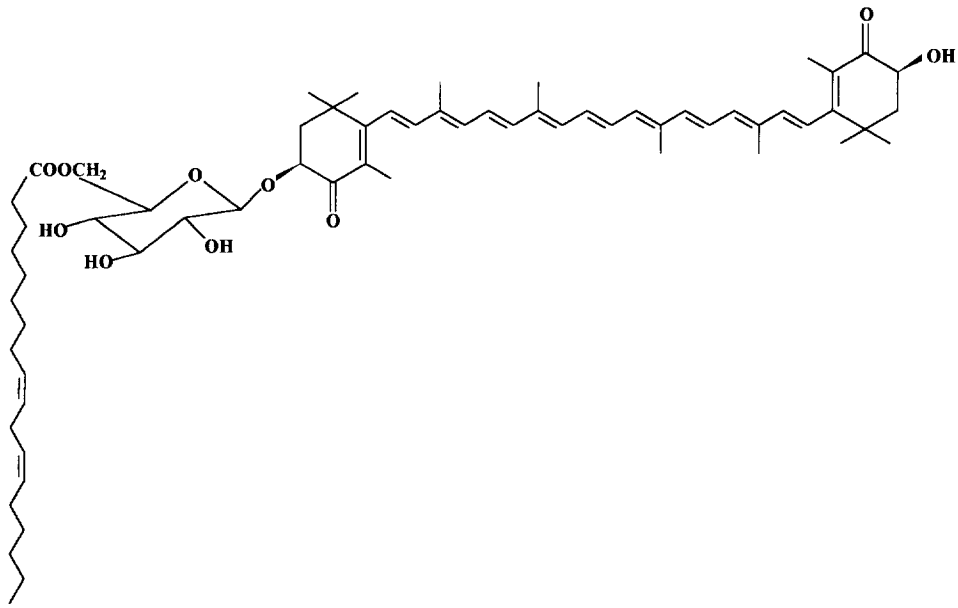

FIG. 1. Astaxanthin glycoside ester, 3-[6-O-(linoleyl-β-D-glyco-pyranosyl)-oxy]-3,3′-dihydroxy-carotene-4,4′-dione was isolated for the first time from the deep-water gammaride *Acanthogammarus (Brachyuropus) grewingkii*

grewingkii astaxanthin-glucoside esters probably forms colourless complexes with protein which makes the members of this species practically invisible in their environment.

We identified astaxanthin glycoside esters from deep-water gammaride A. *grewingkii*. This is the first time that such a diversity of glycoside esters has been found in an animal species.

References

1. Bazikalova, A.Ya. Some data on biology *Acanthogammarus (Brachyuropus) grewingkii* (Dyb.) Trudy Baik. Limnol. Sta. Acad. Sci. USSR, 14:312–326;1954.
2. Cheesman, D.F.; Lee, W.L.; Zagalsky, P.T. Carotenoproteins in invertebrates. Biol. Rev. 42:131–160;1967.
3. Craft, N.E. Carotenoid reversed-phase high-performance liquid chromatography methods: reference compendium. Methods in Enzymol. 213:185–205;1992.
4. Czeczuga, B. Carotenoids in thirteen species of gammaridae from lake Baikal. Comp. Biochem. Physiol. 50B:259–268;1975.
5. Dembitsky, V.M.; Kashin, A.G.; Rezanka, T. Comparative study of the endemic freshwater fauna of lake Baikal. V. Phospholipid and fatty acid composition of the deep-water amphipod crustacean *Acanthogammarus (Brachyuropus) grewingkii*. Comp. Biochem. Physiol. 108B:443–448;1994.
6. Green, P.N.; Bousfield, I.J. Emendation of methylobacterium patt. Int. J. Syst. Bacteriol. 33:875–877;1983.
7. Jackman, L.M.; Sternhell, S. Applications of nuclear magnetic resonance spectrometry in organic chemistry. 2nd ed. Oxford: Pergamon Press; 1969.
8. Jensen, A.; Liaaen-Jensen, S. editors. Carotenoids. Book of abstracts. 10th International Symposium. Tupac, Trondheim, Norway; 1993.
9. Kleinig, H. On the utilization *in vivo* of lycopene and phytoene as precursors for the formation of carotenoid glucoside ester and on the regulation of carotenoid biosynthesis in *Myxococcus fulvus*. Eur. J. Biochem. 57:301–308;1975.
10. Kleinig, H.; Reichenbach, H. A new carotenoid glycoside ester from *Chondromyces apiculatus*. Phytochemistry 12:2483–2485;1973.
11. Kleinig, H.; Reichenbach, H. Carotenoid glycosides and menaquinones from gliding bacterium *Herpetosiphon gigantens*. Arch. Mikrobiol. 112:307–310;1977.
12. Kleinig, H.; Reichenbach, H.; Achenbach, H. Carotenoid pigments of *Sigmatella aurauntiaca* (Myxobacterales). 2. Acylated carotenoid glycosides. Arch. Mikrobiol. 74:223–228; 1970.
13. Kleinig, H.; Reichenbach, H.; Achenbach, H.; Stadler, J. Carotenoid pigments of *Sorangium compositum* (Myxobacterales) including two new carotenoid glycoside esters and two new carotenoid Rhamnosides. Arch. Mikrobiol. 78:224–232;1971.
14. Kleinig, H.; Schmitt, R.; Meister, W.; Englert, G.; Thommen, H. New C_{30} carotenoic acid glycosyl esters from *Pseudomonas rhodos*. Z. Naturforsch., C: Biosci. 34C:181–185;1979.
15. Krinsky, N.I.; Mathews-Roth, M.M.; Taylor, R.F. editors. Carotenoids: Chemistry and biology. New York: Plenum Press; 1990.
16. Koluaev, B.I.; Krekeshyva, T.I. Carotenoid composition of some gammarides of Lake Baikal. J. Evol. Biochem. Physiol., St. Petersburg; 1:144–146;1987.
17. Marshall, J.H.; Wilmoth, G.J. Pigments of *Staphylococcus aureus*, A series of triterpenoid carotenoids. J. Bacteriol. 147: 900–913;1983.
18. Packer, L. editor. Carotenoids. Methods in enzymol. 213:1–432;1992.
19. Pfander, H. editor. Key to carotenoids. Basel: Birkhauser; 1987.
20. Reichenbach, H.; Kleinig, H. Carotenoids of *Myxococcus fulvus* (Myxobacterales). Arch. Mikrobiol. 76:364–369;1971.
21. Rezanka, T. Polyunsaturated and unusual fatty acids from slime moulds. Phytochemistry 33:1441–1444;1993.
22. Rezanka, T.; Dembitsky, V.M. Isoprenoid polyunsaturated fatty acids from freshwater sponges. J. Nat. Prod. 56:1898–1904;1993.
23. Rezanka, T.; Dembitsky, V.M. Identification of unusual cyclopropane monounsaturated fatty acids from the deep-water lake invertebrate *Acanthogammarus grewingkii*. Comp. Biochem. Physiol. 109B:407–413;1994.
24. Rezanka, T.; Dembitsky, V.M. The occurrence and structural identification of long-chain unsaturated ketones in the deep-lake invertebrate *Acanthogammarus grewingkii*. Comp. Biochem. Physiol. 111B:249–255;1995.
25. Schmitz, H.H.; van Breeman, R.B.; Schwartz, S.J. Fast-atom

bombardment and continuous-flow fast-atom bombardment mass spectrometry in carotenoid analysis. Methods in Enzymol. 213:322–336;1992.
26. Straub, O. Key to carotenoids. List of Natural Carotenoids. Basel: Birkhauser Verlag; 1976.
27. Takaichi, S.; Kimihiro, T.; Matsuura, K.; Shimada, K. A monocyclic carotenoid glucoside ester is a major carotenoid in the green filamentous bacterium *Chloroflexus aurantiacus*. Plant Cell Physiol. 36:773–778;1995.
28. Takaichi, S.; Ishidsu, J.I. Carotenoid glycoside ester from *Rhodococcus rhodochrous*. Methods in Enzymol. 213:366–374; 1992.
29. Takaichi, S.; Ishidsu, J.; Seki, T.; Fukuda, S. Carotenoid pigments from *Rhodococcus rhodochrous* RNMS1. 2. Monocyclic carotenoids. A carotenoid monoglycoside and carotenoid glycoside monoesters. Agric. Biol. Chem. 54:1931–1937; 1990.
30. Vacheron, M.J.; Arpin, N.; Michel, G. Isolation of phleixanthophyce of *Nocardia kirovani*. C. R. Hebd. Seances Acad. Sci. Ser. C. 270:881–890;1970.