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LIPIDS OF MICROALGAE.

I. LIPIDS AND A PROTEIN HYDROLYSATE FROM *Monochrysis lutheri*

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The class composition and the total fatty acid composition of the readily- and difficultly-extractable lipids and the amino acid composition of a protein hydrolysate of the dry biomass from the golden-yellow microalga Monochrysis lutheri *have been determined for the first time. It has been established that the difficultly-extractable lipids differ sharply from the readily extractable lipids in the composition of the neutral and glycolipid components and also in that of the fatty acids; 27% of the weight of the fatty acids of the latter is composed of the 20:4 and 20:5 species. The protein hydrolysate of* M. lutheri *contains almost the whole set of essential fatty acids.*

Algae form a very large part of flora but one which has been inadequately studied in comparison with higher plants. At the present time, they are assigned to 12 independent divisions of photosynthesizing living organisms, a considerable part of which are of microscopic dimensions. Microalgae are the main subject of the new biotechnological direction of algology — phycotechnology, dealing with their cultivation and processing for industrial purposes. The lipids of microalgae have been less studied than those of higher plants, but the available information and structure of this class of compounds in microalgae is far more complex and diverse [1].

Microalgae of the class Chrysophyceae (golden yellow microalgae) have scarcely been investigated. Isolated investigations have been devoted to the choice of conditions for the cultivation [2] and for the use in mariculture [3] of one of the species of this class promising for mass industrial cultivation — *Monochrysis lutheri*. *Monochrysis* accumulates a considerable amount of lipid enriched with polyunsaturated fatty acids (PUFAs) of the C_{20} series [4], including arachidonic (20:4), eicosapentaenoic (20:5), and dicosahexaenoic (22:6) acids, which, as is known, are precursors of the prostaglandins, leukotrienes, and thromboxanes and are of independent interest as agents for the treatment and prophylaxis of oncological and cardiovascular diseases and of thrombotic and arteriosclerotic pathologies [5, 6]. There is no information on the proteins of *Monochrysis* in the literature.

We have investigated the lipids and a protohydrolysate of a freeze-dried biomass of *Monochrysis*. The possibility of using an enzymatic-chemical method of isolating lipids in combination with preliminary extraction by Folch's method for the quantitative and qualitative estimation of the readily-extractable free and bound lipids (RLs) and the difficultly-extractable native strongly bound lipids (DLs) of the reserve tissues of plants have been shown previously for the case of the seeds of industrial plants [7, 8]. We used this method since there is no information in the literature on the composition of the DLs of photosynthetic tissues. The RLs were isolated from the biomass of *Monochrysis* by extraction with a mixture of chloroform and methanol.

To separate the nonlipid impurities from the lipids, the chloroform–methanolic extract was treated with an aqueous solution of NaCl and the amount of total protein (protein fraction) in the wash-waters and its amino acid composition were determined. The DLs and a water-soluble protein hydrolysate were isolated by proteolysis with a protosubtilin having its optimum action in alkaline media from the meal remaining after the extraction of the RLs. The solid residue after proteolysis was subjected to severe alkaline hydrolysis, and the remaining components were isolated.

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TABLE 1. Yields of Lipids and of the Protein Hydrolysate from the Freeze-Dried Biomass of *Monchrysis*

Lipids, proteins and accompanying compounds	Yield, % of the biomass (% of the weight of the lipids)
Readily-extractable	
a) lipids	35,80 (98,41)
b) nonlipid substances,	7,40
including the protein fraction, % by weight	4,20 (By Lowry's method)
	2,70 (From the amino acid composition)
Difficultly-extractable	
a) lipids	0,58 (1,59)
b) nonlipid substances	Tr.
Protein hydrolysate total protein,	20,2
% on the weight of the hydrolysate	37,0 (By Lowry's method)
	30,1 (From the amino acid composition)
Residual substances	0,09
Total yield of lipids	36,38 (100)
Total yield of extractive substances	64,07
Residual meal	35,9

The yields of lipids, of the compounds accompanying them, and of the protein hydrolysate are given in Table 1. In the *Monochrysis* cells the bulk of the lipids (the RLs) was bound to the protein by weak hydrogen bonds, and only an insignificant part was strongly bound with the proteins by covalent bonds (RLs). A comparison of the results that we have obtained with the literature [7] shows that the enzymatic-chemical method permits the extraction of the lipids from *Monochrysis* more completely than in the case of the reserve tissues of higher plants and, consequently, the alkaline protease cleaves the lipid-protein complex of the freeze-dried biomass of this microalga practically completely.

The qualitative compositions of the RLs, the DLs, and the residual lypophilic substances were determined by TLC in solvent systems 1-5. The results of the analysis show that they differed sharply. Thus, in the RLs 12 classes of neutral lipids were detected: hydrocarbons, carotenoids, wax esters, fatty acid methyl esters, triacylglycerols, free fatty acids (FFAs), chlorophylls, triterpenols, sterols, xanthophylls, and two unidentified classes. The neutral lipids of the DLs consisted of only four classes: FFAs, sterols, and two unidentified classes with R_f values coinciding with those of the triacylglycerols and fatty acid methyl esters but not reacting with alkali. A quantitative predominance of FFAs was common for the compositions of the RLs and the DLs.

Two components were detected in the residual lipophilic substances, with R_f values coinciding with those of the FFAs while one of them on treatment with H_2SO_4, followed by heating assumed a bright red color. In view of the fact that these compounds were not methylated by diazomethane and had a sharp unpleasant smell not characteristic for lipids and similar to the smell of skatole [9], they were assigned to substances of nonlipid nature and were not studied further. The composition of the residual substances confirmed the complete quantitative isolation of the lipids from the dry biomass of *Monochrysis*.

In a study of the polar lipids of *Monochrysis* by TLC in systems 4 and 5, it was found that the RLs and the DLs had identical qualitative compositions of the phosholipids, namely: phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, lyso-phosphatidylcholine, and phosphatidic acid. However, they differed sharply with respect to the composition of their glycolipids: in the RLs mono- and digalactosyldiacylglycerols, steryl glycosides and their esters were found, while in the DLs only digalactosyldiacylglycerols were detected. In addition, both the RLs and the DLs gave a positive qualitative reaction with tungstosilicic acid and the TLC of these acids in system 4 revealed considerable amounts of a compound with R_f 0.82 reacting positively with the Dragendorff reagent but not interacting with the Vas'kovskii reagent, which permitted its assignment to the alkaloids [10].

The quantitative amounts of unsaponifiables and fatty acids in the lipids of *Monochrysis* were also determined (mg/g of lipids):

	RLS	DLD
Unsaponifiables	88,4	147,5
Fatty acids	621,5	361,0

As we see, the DLs of *Monochrysis* contained 1.7 times more unsaponifiables and 1.7 times less fatty acids than the DLs.

TABLE 2. Composition of the Fatty Acids from the Freeze-Dried Biomass of *Monochrysis*

Acid	Amount, % on the weight of the acids		Acid	Amount, % on the weight of the acids	
	RLS	DLD		RLS	DLD
10:0	0,3	1,1	17:0	Tr.	Tr
11:0	Tr.	0,9	18:0	1,2	7,6
12:0	0,3	1,1	18:1	8,4	9,0
13:0	0,2	0,7	18:2	3,8	2,4
14:0	4,0	6,9	18:3	0,4	0,7
14:1	0,3	4,7	20:0	0,8	1,8
15:0	0,6	2,7	20:4	8,1	—
14:3	0,2	1,6	20:5	18,9	—
16:0	23,0	46,5	PUFAs *	33,0	7,8
16:1	28,0	9,2	Σ sat	31,4	69,3
16:2	1,1	2,3	Σ unsat	68,6	30,7
16:3	0,5	0,8			

*PUFAs) sum of the dienic and more highly unsaturated fatty acids.

The fatty acid composition of the *Monochrysis* lipids determined by a combination of GLC analysis and mass spectrometry is given in Table 2. Appreciable differences are observed with respect to the composition and amounts of the fatty acids of the RLs and DLs and, above all, with respect to the polyunsaturated fatty acids (PUFAs). Thus, the RLs contained 4.2 times more PUFAs than the DLs, and the 20:4 and 20:5 acids were not detected in the latter even in trace amounts. In the RLs, there was three times as much of another acid with a high biological activity - the 16:1 acid - and this in an amount comparable with that of the acids of the C_{20} series.

The DLs were characterized by a larger set of saturated acids. In addition to the acids shown in Table 2, the mass spectrum of the methyl esters of fatty acids of the DLs revealed the 21:0, 22:0, 23:0, and 24:0 species, which corresponded to the peaks of the molecular ions M^+ in the high-mass-number region of 340, 354, 368, and 382, and also the fragments $[M-31]^+$ with m/z 309, 323, 337, and 351 and $[M - 43]^+$ 297, 311, 325, and 339. Consequently, the DLs contained almost the whole homologous series of fatty acids from 10:0 to 24:0, the amount of which was 2.2 times more than in the RLs. Furthermore, in the microalgae a tendency detected in the tissues of higher plants is observed: with a rise in the degree of binding of the lipids with the protein the degree of saturation of the faty acids increases [7]. It must be mentioned that docosahexaenoic acid, 22:6, detected previously in a moist paste of *Monochrysis* [4], was absent from the freeze-dried biomass.

The compositions of the amino acids of the protein fraction (I) and of a protein hydrolysate (II) are given in Table 3. The *Monochrysis* biomass contained 15 amino acids, among which glutamic acid predominated. Almost the whole set of the essential amino acids was detected, with the exception of tryptophan, which was not determined in this sample. The amount of essential amino acids in the protein hydrolysate of the *Monochrysis* biomass was comparable with that of animal proteins having a high nutritional value [11].

Thus, the freeze-dried biomass of *Monochrysis* can serve as a source of lipids (37%) with a high content of PUFAs, of a protein hydrolysate (20%) with essential amino acids, and of a meal (36%) with potential fodder properties.

EXPERIMENTAL

The mass spectrum was taken on a 1310 instrument with direct insertion of the sample at an ionizing voltage of 70 V a collector current of 80 μA, a temperature of the ionization chamber of 130-150°C, and a temperature of the evaporation ampul of 130-140°C.

The *Monochrysis* biomass was supplied L. V. Spektorova of the Institute of Fishing and Oceanography (Moscow). The algae were grown in 1991 near Anapa in glass-tube cultivators in sea water and were then freeze-dried. The RLs were extracted four times with chloroform–methanol (2:1, v/v) for 18 h at 40-50°C with periodic stirring. Proteolysis, the isolation of the DLs and the residual components, the saponification of the lipids, and the GLC of the fatty acid methyl esters were carried

TABLE 3. Amino Acid Composition of the Protein Fraction (I) and of a Protein Hydrolysate (II) of the Freeze-Dried Biomass of Monochrysis

Amino acid,* g per 100 g of protein	I	II
Lysine	0,28	6,44
Arginine	—	3,08
Histidine	0,28	3,92
Aspartic acid	0,28	7,84
Threonine	0,28	4,76
Serine	0,28	3,64
Glutamic acid	2,24	18,48
Proline	1,68	4,48
Glycine	0,28	—
Alanine	0,87	5,32
Valine	0,28	5,32
Methionine	—	1,4
Isoleucine	0,08	3,92
Leucine	0,28	7,0
Tyrosine	0,28	3,9
Phenylalanine	0,28	4,8

*Tryptophan was not determined.

out as described in [7]. Nonlipid substances were separated from the concentrated extract by two treatments of the lipids with a 1% aqueous solution of NaCl.

The analytical TLC of the lipids was performed on type L5/40 silica gel (Czechoslovakia) with the addition of 10% of $CaSO_4$ on Silufol (Czechoslovakia) in the following systems: hexane–diethyl ether: 1) (9:1); 2) (7:3); 3) (7:8); 4) chloroform–methanol–25% ammonia (65:25:5); 5) direction I: chloroform–methanol–25% ammonia (8:3:1); direction II: chloroform–methanol–acetic acid–water (8:3:1:1). The chromatograms were revealed as described in [12].

The lipids were identified by comparison with standard individual compounds isolated previously from various plant materials and with literature figures [12]. Total proteins were determined by Lowry's method [13].

To determine the amino acid composition, a weighed sample (10 mg) of the protein was dissolved in 8 ml of 6 N HCl. Hydrolysis was performed in vacuum-sealed tubes, at 110°C for 24 h. The hydrolysate was evaporated with the addition of fresh portions of water and was then dried in a vacuum desiccator over alkali. The composition of the amino acids was determined on a T-339 amino acid analyzer (Czechoslovakia).

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