ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 4, pp. 451–454. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © T.V. Kafanova, N.G. Busarova, Yu.V. Khudyakova, S.V. Isai, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 4, pp. 508–511.

EXPERIMENTAL ARTICLES

Marine Fungus *Stilbella aciculosa* as a Potential Producer of Prostaglandins

T. V. Kafanova, N. G. Busarova, Yu. V. Khudyakova, and S. V. Isai[1]

Pacific Institute of Bioorganic Chemistry, Russian Academy of Sciences, Far East Division, Vladivostok, 690022 Russia

Received June 18, 2007; in final form, December 25, 2007

Abstract—The amount and composition of fatty acids in the fungus *Stilbella aciculosa* associated with the marine macroorganism *Apostichopus japonica* (trepang) were determined by gas-liquid chromatography and gas chromatography–mass spectrometry. In the culture liquid of *S. aciculosa*, prostaglandins (PG) of groups E and F were revealed by UV spectroscopy. This finding was confirmed by the presence of direct precursors of PG, polyunsaturated eicosapentaenoic and docosahexaenoic acids, in the culture liquid. The biomass of this fungus contained PG of group B.

Key words: marine fungus *Stilbella aciculosa*, fatty acids, prostaglandins.

DOI: 10.1134/S0026261708040115

Marine fungi (MF) are infrequently studied microorganisms; the literature contains mainly descriptions of terrestrial ecoforms of these fungi. A strain of the fungus *Mortierella alpina* is known as a producer of arachidonic acid (20 : 4) [1]; mycelium of the yeast-like fungus *Candida rugosa* contains up to 40% of arachidonic acid, a direct precursor of prostaglandins (PG) [2]; long-chain hydroxy fatty acids were revealed in fungi [3]. *Aspergillus fischerii* contains terrein, a PG synthon, with the absolute configuration required for the PG synthesis [4]. A strain of soil fungus *Fusarium sambucinum* was shown to produce PG E_2 and PG $F_2\alpha$ [5]. From the fungus *Aspergillus nidulans*, genes of oxylipin responsible for sexual and asexual reproduction were isolated [6]. Along with *Aspergillus* sp. and *Mortierella* sp., other fungi were also tested for their capacity for PG synthesis [7]. A new trend of investigations in biology, microbial endocrinology, is directly connected with fungal metabolism: the reproduction of fungi was stimulated by oleic (18 : 1), linolenic (18 : 3), and arachidonic (20 : 4) fatty acids [8].

The aim of this work was the search for an active producer of PG and characterization of its lipid composition among the fungi associated with marine macroorganisms exhibiting high PG activity. According to our knowledge, such macroorganisms include the holothurians *Apostichopus japonica* (trepang) and *Cucumaria japonica* (cucumaria) [9].

MATERIALS AND METHODS

The study was carried out with strains of marine fungi isolated from the surfaces of trepang and cucumaria. The fungi were grown in a medium containing wort (20 ml) and sea water (80 ml); pH 7.8 [10, 11]. The glucose content of the wort was 0.044 g/l [11]; the medium contained no fatty acids. Cultivation was carried out in 250-ml Erlenmeyer flasks with 100 ml of the medium at 22°C for 7 days.

Strains isolated from *Apostichopus japonica* belonged to the following fungal species: *Cladosporium atroseptum* Pidopl., *Cladosporium brevicompactum* Pidopl. et Deniak, *Trichoderma viride* Pers., *Aspergillus fumigatus* Fresen., *Alternaria alternate* (Fr.) Keissl., and *Stilbella aciculosa* (Ellis et Everh.) Seifert. Strains isolated from *Cucumaria japonica* belonged to the species *Cladosporium brevicompactum* Pidopl. et Deniak.

Both the biomass and the culture liquid of the mentioned strains were used in the study.

Extracts of fungal biomass were prepared as described earlier [12]. An aliquot of biomass (20 g) separated from the culture liquid by centrifugation was fixed with a mixture of 100 ml of ethanol and 0.5 ml of 40% acetic acid; then, the biomass was separated by centrifugation and extracted with diethyl ether. The upper phase was separated, evaporated, and shaken with a mixture of hexane and 70% aqueous solution of ethyl alcohol (1 : 1, vol/vol). The lower phase was separated, evaporated, acidified, and extracted with diethyl ether; the obtained extract was evaporated, diluted with a chloroform–methanol mixture (1 : 1), blown with argon, and stored at –4°C. The culture liquid obtained after biomass separation was evaporated to minimal volume and treated as described above.

UV spectroscopy. Ethanol and methanol extracts of the biomass and the culture liquid before and after alka-

[1] Corresponding author; e-mail: isai@piboc.dvo.ru

line treatment were analyzed by UV spectroscopy at 200–280 nm on a Cecil CE 7200 spectrophotometer (Aquarius, United Kingdom) in a 1-cm quartz cuvette with a volume of 1 ml.

Thin-layer chromatography (TLC) of the extracts was performed on Sorbfil PGSKh-AF-A plates developed in the following systems: benzene–ethyl acetate–formic acid (75 : 15 : 1), chloroform–ethyl acetate–ethanol–acetic acid (20 : 20 : 4 : 1), and petroleum ether–diethyl ether–acetic acid (82 : 18 : 1). The latter system was used for separation of the mixture of PG and phospholipids from neutral lipids. PG spots were visualized with the use of 1% vanillin in 15% solution of phosphoric acid in ethanol and 10% solution of sulfuric acid in ethanol.

GLC analysis of fatty acids. Methyl esters of fatty acids were obtained by the method of Hartman et al. [13] and analyzed on an Agilent 6850 chromatograph (Germany) equipped with a capillary column (30 m × 250 μm × 0.25 μm) packed with an HP-5-MS-5% (phenylmethylsiloxane) phase under isothermal regime (220°C); the carrier gas was helium.

Gas chromatography–mass spectrometry (GC–MS) of fatty acids was performed on a Hewlett Packard 6890 device (United States).

RESULTS AND DISCUSSION

Extracts of fungal biomass and culture liquid were analyzed by UV spectroscopy to determine the presence of cyclopentane ring, which is a typical structural component of all PG [14]. It is known that three groups of PG, PG E_2, PG A_1, and PG B_1, are characterized by absorption at 205, 217, and 278 nm, respectively (ε is 4400, 13400, and 10950, respectively). It is known that under alkaline or acidic conditions, PG of groups A and E are transferred into PG of group B [15].

Some species of microorganisms are capable of synthesis and excretion into the medium of significant amounts of lipids, which differ in composition from the intracellular lipids [16]. That is why both fungal biomass and culture liquid were analyzed. Since the most clear spectra were revealed in*S. aciculosa* this strain was used in further experiments. In the extract of the culture liquid before alkaline treatment, peaks at 207, 252, and 274 nm were revealed, whereas after the treatment, a clear peak at 280 nm appeared, corresponding to PG of group B, and a peak at 213 nm. UV spectra of the extract from *S. aciculosa* biomass before alkaline treatment had a peak at 213 nm and a diffused peak at 278 nm; after the treatment, the peak at 213 nm remained, and the peak at 278 nm became clear. Therefore, it can be assumed that untreated biomass of *S. aciculosa* contained PG of group B, which were retained after the alkaline treatment.

The occurrence of PG in the biomass and culture liquid of *S. aciculosa* was confirmed by TLC with the use of specific reagents for PG visualization. Chromatograms of the extract of the culture liquid treated with 1% solution of vanillin in ethanol showed a pronounced spot with R_f corresponding to PG E_2. Then neutral lipids were removed from the extract by preparative TLC; the area containing the total fraction of PG and phospholipids (PL) was scraped off the start of the chromatogram and analyzed by qualitative TLC. The treatment of the chromatogram with 1% solution of vanillin in 15% solution of phosphoric acid in ethanol revealed a yellow–green spot with R_f corresponding to PG E_2. After subsequent spraying of this plate with 10% solution of sulfuric acid in ethanol, two additional spots appeared, one of which had R_f corresponding to PG $F_2\alpha$. Additionally, the total fraction of PG and PL was analyzed by TLC developed in a chloroform–ethyl acetate–ethanol–acetic acid (20 : 20 : 4 : 1) system. The treatment of the chromatogram with 1% solution of vanillin in 15% phosphoric acid in ethanol revealed three spots, two of which according to their R_f values can be referred to PG $F_2\alpha$ and PG E_2; the third yellow spot can be assigned either to PG E_2 esters or to PG B_2. The total fraction of PG and PL isolated by preparative TLC was analyzed by UV spectroscopy. Alkaline treatment of an aliquot of this fraction resulted in the appearance of a clear peak at 280 nm.

Thus, preliminary results indicate that culture liquids of *S. aciculosa* from *A. japonica* contained PG E_2 and PG $F_2\alpha$. According to TLC data, extract of *S. aciculosa* biomass contained PG B.

Fatty acid composition of the extracts from *S. aciculosa* biomass and culture liquid is presented in the table. Prior to GLC, methyl esters of fatty acids were purified by TLC on KSK Silica Gel plates developed in benzene. Both biomass and culture liquid contained mainly unsaturated fatty acids; however, the content of individual fatty acids in these samples differed considerably. The family of C18 acids predominated in the biomass; polyunsaturated fatty acids (PUFA) were absent. In the culture liquid, eicosapentaenoic (20 : 5) and docosahexaenoic (22 : 6) acids were revealed, which are direct precursors of PG. We cannot compare our results with literature data, since there is no information concerning the fungus *S. aciculosa*. There are data that the fungus *Mortierella alpina* is a producer of arachidonic acid [1, 17]. Kawashima et al. [17] studied the effect of cultivation time (two and seven days), temperature (from 12 to 28°C), and glucose concentration (0–3%) on fatty acid composition of *M. alpina.* It was shown that the predominant fatty acids consisted of 18 : 1 (14.5–20.6%); 18 : 2 11.2–15.4%), and 20 : 1 (20.0–35.9%); the amount of 20 : 4 acid varied from 4.4 to 12.0%, depending on the cultivation conditions. Eroshin et al. [1] selected the strain *M. alpina* LPM-30, which contained 20 : 4 acid (up to 46–60% of the sum of fatty acids); at the same time, the content of the family of C18 acids was also significant. In *Aspergillus nidulans*, the acids of the 16 : 0 and the C18-families prevailed [6]. According to our data, the amount of 16 : 0 acid in *S. aciculosa* biomass reached 25.6%. In addition

The content and composition of fatty acids in the fungus *S. aciculosa* (% of the sum of the fatty acid methyl esters)

Fatty acid (FA)	Fungal biomass	Culture liquid
14 : 0	0.55	4.00
15 : 0	0.70	
16 : 0	25.60	15.20
16 : 0i	1.20	
Tetramethyl-16 : 0		2.25
16 : 1n7	1.45	
16 : 1n9		10.50
17 : 0	0.75	
17 : 1n9	0.70	
18 : 0	3.66	2.50
18 : 1n8		7.70
18 : 1n9	32.30	18.00
18 : 2n6	29.70	
18 : 4n3		1.60
19 : 1n9	1.80	
19 : 2n6	1.35	
20 : 1n9		6.20
20 : 1n15		6.80
20 : 5n3		7.90
22 : 1n9	2.10	
22 : 1n11	8.90	
22 : 6		6.30
The sum of saturated FAs	32.50	24.00
The sum of unsaturated FAs	67.30	75.94
The sum of monoenoic FAs	36.25	60.10
The sum of polyunsaturated FAs		15.90

to the fatty acids listed in the table, we revealed by GC–MS analysis the presence of cholesterol and ergosterol hormones, which are known to regulate sexual reproduction in fungi [8]. In the culture liquid of *S. aciculosa*, unusual polybranched phytanic acid (3,7,11,15-tetramethylhexadecanoic) was identified (up to 2% of the sum of fatty acids). The presence of this acid was shown to be associated with a rare hereditary disease of the peripheral nervous system [18].

Thus, it was revealed that the extract of fungus *S. aciculosa* contained up to 22 saturated and unsaturated fatty acids. The amount and composition of fatty acids in the biomass and culture liquid differed considerably. In the culture liquid, 20 : 5 and 22 : 6 PUFA were identified, which are direct precursors of PG. By using UV spectroscopy, E and F groups of PG were revealed in the culture liquid, and PG of group B was identified in the biomass of *S. aciculosa*.

REFERENCES

1. Eroshin, V.K., Dedyukhini, E.G., Satrutdinov, A.D., and Chistyakova, T.I., Growth-Coupled Lipid Synthesis in *Mortierella alpina* LPM 301, a Producer of Arachidonic Acid, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 200–204 [*Microbiology* (Engl. Transl.), vol. 71, no. 2, pp. 169–172].
2. Vali, S.R., Sheng, H.Y., and Ju, Yi-H., An Efficient Method for the Purification of Arachidonic Acid from Fungal Single-Cell Oil (ARASCO), *J. Am. Oil Chem. Soc.*, 2003, vol. 80, no. 7, pp. 725–730.
3. Van Dyk, M.S., Kock, J.-L.F., and Botha, A., Hydroxy Long-Chain Fatty Acids in Fungi, *J. Microbiol. Biotechnol.*, 1994, vol. 10, no. 5, pp. 495–504.
4. Mitscher, L.A., Clark, G.W., and Hudson, P.B., Terrein, An Optically Active Prostaglandin Synthon of Fungal Origin. II. Chemical Conversion To 4(*R*)-Acetoxy-2-Cyclopentenone, *Tetrahedron Lett.*, 1978, no. 29, pp. 2553–2556.
5. Zeleneva, R.N., Ustynyuk, T.K., Bragintseva, L.M., Polin, A.N., and Kovalenko, V.A., Fungal strain *Fusarium sambucinum*, a Prostaglandin Producer, RF Patent No. 1446928, *Byull. Izobret.*, 1994, no. 24.
6. Tsitsigiannis, D.I., Kowieski, T.M., Zarnovski, R., and Keller, N.P., Three Putative Oxylipin Biosynthetic Genes Integrate Sexual and Asexual Development in *Aspergillus nidulans, Microbiology (UK)*, 2005, vol. 151, pp. 1809–1821.
7. Lamačka, M., Šajbidor, J., The Occurrence of Prostaglandins and Related Compounds in Lower Organisms, *Prostagl. Leukotr. Essent. Fatty Acids*, 1995, vol. 52, pp. 357–364.
8. Feofilova, E.P., Heterothallism of Mucoraceous Fungi: A Review of Biological Implications and Uses in Biotechnology, *Prikl. Biokhim. Mikrobiol.*, 2006, vol. 42, no. 5, pp. 501–519 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 42, no., 5, pp. 439–454].
9. Mischenko, T.Ya., Korotchenko, O.D., and Isai, S.V., Search for Compounds of Prostaglandine-Type Action in Marine Organisms, *Byull. Eksperim. Biologii I Meditsiny,* 1982, vol. 94, no. 11, pp. 94–96.
10. Bilai, V.I., *Metody eksperimental'noi mikologii. Spravochnik*: (Handbook on Methods in Experimental Mycology), Kiev: Naukova dumka, 1982.
11. Semenov, S.M., *Laboratornye sredy dlya aktinomitsetov i gribov. Spravochnik* (Handbook on Laboratory Media for Actinomycetes and Fungi), Moscow: Agropromizdat, 1990.
12. Korotchenko, O.D., Mischenko, T.Ya., and Isay, S.V., Prostaglandins of Japan Sea Invertebrates in Echinoderms, *Comp. Biochem. Physiol.*, 1983, vol. 74C, no. 1, pp. 85–88.
13. Hartman, L., Lago, C.A., and Regino, N., The Quick Method of Preparation of Methyl Ester Fatty Acids from Lipids, *Lab. Pract.*, 1973, vol. 22, pp. 475–476.
14. Weisberger, A., *Elucidation of Organic Structures by Physical and Chemical Methods*, Bentley, K.W., Ed., New York: Wiley, 1963 [Rus. Transl. Moscow: Khimiya, 1967, vol. 1].
15. Perera, S.K. and Fedor, L.R., Acid- and Base-Catalyzed Dehydration of Prostaglandin E_2 To Prostaglandin A_2

and General-Base-Catalyzed Isomerization of Prostaglandin A_2 To Prostaglandin B_2, *J. Am. Chem. Soc.,* 1979, vol. 101, no. 24, pp. 7390–7393.

16. Ruban, E.L., *Mikrobnye lipidy i lipazy* (Microbial Lipids and Lipases), Moscow: Nauka, 1977.
17. Kawashima, H., Kamada, N., Sakuradani, E., Jareonkitmongkol, S., Akimoto, K., and Shimizu, S., Production of 8,11,14,17-*cis*-Eicosatetraenoic Acid by Δ5 Desaturase-Defective Mutants of an Arachidonic Acid-Producing Fungus, *Mortierella alpina, J. Am. Oil Chem. Soc.*, 1997, vol. 74, no. 4, pp. 455–459.
18. Wierzbicki, A.S., Lloyd, M.D., Schofield, C.J., Feher, M.D., and Gibberd, F.B., Refsum's Disease: a Peroxisomal Disorder Affecting Phytanic Acid α-Oxidation, *J. Neurochem.,* 2002, vol. 80, no. 5, pp. 727–735.