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Fatty Acid Analysis from Leech Skin

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Abstract: The lipids of a Korean native blood-sucking leech (*Hirudo nipponia*) were isolated and analyzed. Cholesterol, fatty acids, triglycerides, glycerylether diester and cholesteryl esters were found from epidermal extracts. The major fatty acids in the leech skin were $C_{16:0}$, $C_{18:0}$, $C_{20:1}$, $C_{20:3}$ components. These components were derived from cholesteryl esters, glycerides, fatty acids and other polar lipids. After 60 days fasting, cholesterol, fatty acids and triglycerides in the skin were reduced in number and the total fatty acid composition was changed slightly. The data of total fatty acids in fasted and fed leech showed that medium-chain fatty acids were more affected than long-chain fatty acids. Other trace fatty acids appeared to be decreased by fasting.

Key Words: epidermal lipid, fasting, fatty acid, leech.

The hydrocarbon constituents, including lipids, found in the surface of animals vary from species to species as well as among the environments which animals inhabit. The epidermal organ of the leech, that is covered with mucus, constitutes an effective barrier against desiccation, penetration of light and parasites. The regions of skin serve transepithelial exchange of gases, ions and sensory functions. The epidermal barrier is still pliable enough to undergo stretching and contraction with maintenance of ultrastructural organization. At the same time, the surface is important for such functions as respiration, osmoregulation, aestivation, excretion, species recognition and protection from physical attacks (Sawyer, 1986).

Lipids are the major components of the epidermal organ and their various lipid classes are sterol esters, cholesterol, wax, diesters, triglycerides, ceramide, squalene, free fatty acids, and other minor complex lipids. Many of these lipids contain fatty acid molecules that are necessary for maintaining physical and chemical barriers for the organism in addition to having biochemical importance (Nishimaki *et al.*, 1988; Jacob and Hoppe, 1991; Stewart *et al.*, 1989).

Previous observation in this laboratory revealed that leech skin surface is covered by a mucus, made of mucin, carbohydrates and various lipids (Hong *et al.*, 1993). The present study was put emphasis on fatty acid composition to present an epidermal lipid profile, including a comparison of natural habitat and laboratory environment. This work may also reveal the metabolic relationships to fatty acid compositions of skin with designated dietary intakes.

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Materials and Methods

Sample collection

The laboratory analyses for leech epidermal lipids were carried out by TLC and GC from two groups; for the first group, fatty acid measurements were immediately performed upon collection at the natural habitat, and for the second, leeches were kept in fasting condition in a laboratory culture system. The leeches were collected from a numerous ponds and rice paddies in the southwestern region of the country and raised in fish tanks in the laboratory, being fed by a porcine bladder filled with blood (Hong *et al.*, 1993). The fasting group was maintained for at least 60 days in clean natural water before use.

Lipid extraction

The lipids were extracted in chloroform-methanol with an antioxidant, 2,6-di-tertbutyl-4-methylphenol added to the chloroform, after the leech was cleaned with a dry cloth (Bligh and Dyer, 1959). The lipid layer was separated by adding an equal amount of distilled water to chloroform-methanol in a glass tube. Impurities in the extract were sometimes removed by a Folch wash ($CHCl_3$: MeOH : 0.1 M KCl = 3 : 48 : 47) (Folch *et al.*, 1957). The lipid phase of the extract was concen-

trated under nitrogen gas and brought to a known volume in chloroform for analyses.

TLC of lipids

Thin layers (0.25 mm) of Silica gel G were prepared on 20×20×0.2 cm glass plates, and the absorbent was activated for 30 min at 100~105℃ before use. Oxidizable impurities contained in the silica gel were removed by complete development of the blank plate with methanol prior to using them for the separation of lipids. The glycerides, free fatty acids and other lipids were separated by development of the plates for about 1½ h with 50 ml of a mixture of petroleum ether : diethyl ether : acetic acid=80 : 20 : 1 (Masami and Privett, 1969). The developed plates were air dried at room temperature and placed in an iodine vapor for lipid identification.

Transmethylation

Methylation procedures were carried out by the method described by Christies (1970) and Metealf and Wang (1981). The lipid spots, glycerides and free fatty acids were scrapped from TLC in separate tubes and the lipid was reextracted the lipid in a similar method as above. The reextracted lipids and an aliquot of the total extract were dried under nitrogen gas and combined with 1 ml of 5% HCl in methanol in the test tubes. The tubes were flushed with nitrogen gas and capped at 80℃ for 2 h, cooled and added to 2 ml of distilled water. Methylated fatty acids were extracted with 1 ml of hexane three times and dried under nitrogen to a known amount to prepare for GC.

Gas chromatography

The methyl esters of fatty acids were separated with a Hewlett Packard HP5890 Series II (Palo Alto, USA) column equipped with HP-1 (0.53 mm×30 m×264 μm), at temperature programmed from 180℃ to 300℃ along a 3℃/min gradient. Identification of fatty acid methyl esters was made by comparing their relative retention times with known standard samples (palmitoleic, stearic, oleic, linoleic, linolenic, nonadecanoic, arachidic, 11-eicosenoic erucic acid methylester) purchased from Supelco (Bellefonte, USA) as well as Sigma Co. (St. Louis, USA).

Results and Discussion

The weight of leeches collected from the natural habitat in the spring ranged from 200~600 mg, excluding the juveniles. In the laboratory, regular feeding and maintenance increased them to the full size. Their weights were slowly decreased by fasting and reached 2/3 of their original size after 60 days.

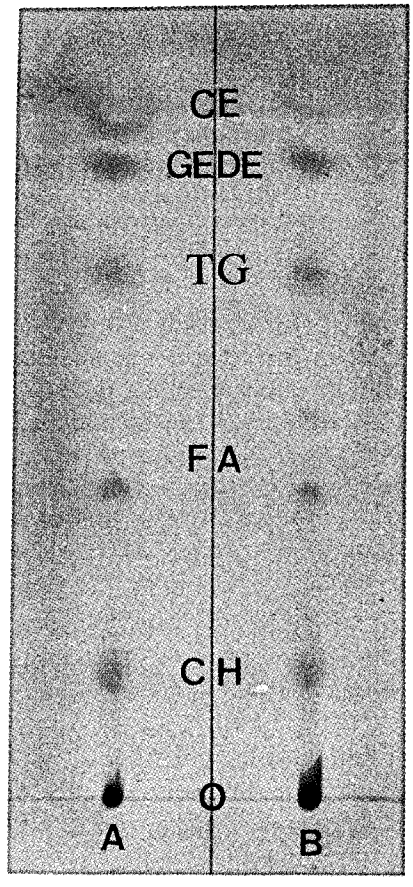

Fig. 1. Thin layer chromatography separation of *Hirudo nipponia* epidermal lipid. The separation was made with solvent mixture of petroleum ether : diethyl ether : acetic acid=80 : 20 : 1 (CE : cholesteryl ester, GEDE : glycerylether diester, TG : triglycerides, FA : fatty acids, CH : cholesterol, O : origin). (A) Neutral Lipid that were extracted immediately after collection from the natural habitats. (B) Lipids from leeches fasted for 60 days in the laboratory.

Fig. 1 shows a typical separation of the lipid by TLC. The major spots are polar lipids, cholesterol, fatty acids, triglycerides, glycerylether diesters and cholesteryl esters. The identification was made from Rf value compared to standards. These lipid patterns are similar to those of other epidermal lipids reported (Helmy and Hack, 1967). From the TLC analysis a great deal of the lipids remained in the original spots containing polar lipids. It would be interesting to study the composition of the various cell layers of the epidermis and of the structure of peculiarities of different lipids in detail.

The lipids from epidermal extraction of leeches fasted for 60 days in the laboratory were separated by TLC (Fig. 1B). The lipid pattern indicated some changes due to the starvation. The visible changes noticed were fatty acids, cholesterol esters and triglycerides. From fasted animals, we have found a new spot, apparently composed

Table 1. Fatty acid composition of total lipid extracts from leeches collected from the natural habitats and leeches fasted at least 60 days in the laboratory.

Fatty acid	Leech skin fatty acid	
	Starved (%)	Fed (%)
$C_{14:0}$	5.23± 0.498	3.53± 1.401
$C_{16:0}$	5.98± 1.598	10.05± 2.073
$C_{18:0}$	5.79± 0.843	6.41± 1.107
$C_{18:2}$	4.77± 0.278	8.18± 2.351
$C_{18:3}$	5.16± 1.171	7.54± 2.095
$C_{20:0}$	1.62± 0.445	2.05± 0.903
$C_{20:1}$	10.84± 0.487	11.47± 1.923
$C_{20:2}$	4.15± 0.432	7.80± 3.080
$C_{20:3}$	16.93± 0.824	16.50± 1.005
$C_{22:0}$	1.98± 0.663	1.91± 0.675
$C_{24:0}$	4.12± 1.450	6.58± 2.968
Other trace fatty acids	33.43%	17.98%
Total	100%	100%

of different types of fatty acids. From the GC analysis major differences were formed from medium chain and other traceable fatty acids (Table 1).

Table 1 shows the fatty acid compositions of total lipid extract from the animals collected at natural habitats and animals fasted in the laboratory. Percents are means and standard deviations measured from 6 individuals in separate extractions and fatty acid analyses. The data are presented for the components that appeared in substantial amounts in the chromatography. There were other medium-chain fatty acids C_{10}, through C_{14}, but we were not able to identify them in detail, except for the carbon number at the present time. For comparison, the fatty acid profile from fasted leech skin shows a similar picture to leeches from the natural habitat except as stated above.

Fig. 2 shows the separation of total fatty acids derived from leech skin. Each fatty acid was identified by comparison to a standard and its quantity was measured by integration of each peak. To explain the composition and mode action of fatty acids in the epidermal barrier under aqueous conditions, we have determined fatty acids from several sources of lipids in the skin. The lipids, as well as fatty acids, were found to be subjected to major interaction among the lipid types, which makes the interpretation very difficult. Some fatty acids are essential dietary requirements for animals and the absence of these from the diet results in a general deterioration and specifically, in the development of a scaly condition in the higher organisms (Castledine and Buckley, 1980; Bauer and Ransone, 1983). The nutrition of laboratory leeches is from a single source,

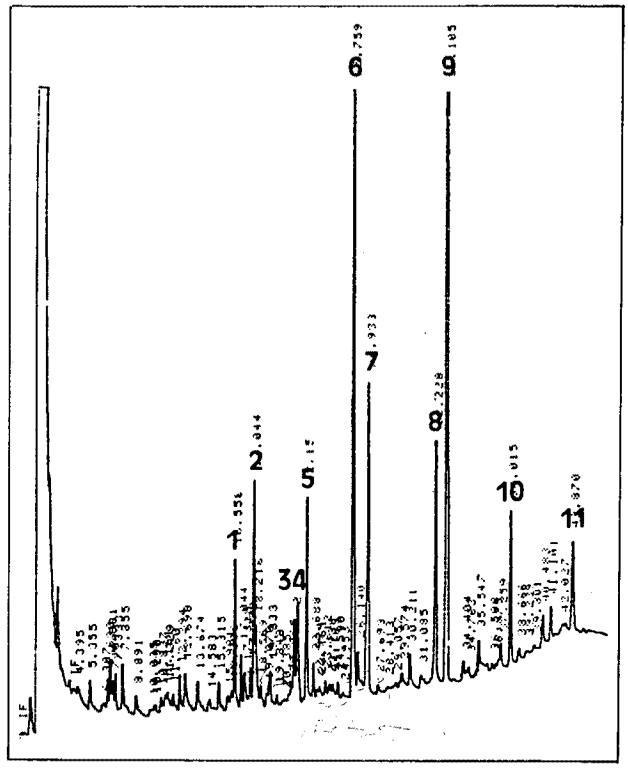

Fig. 2. Total fatty acid profile of the epidermal lipid. Gas chromatography was done on HP5890 with HP-1 capillary column (0.53 mm×30 m×264 μm). Temperature program: 180℃ (1 min)→300℃ (5 min), 3℃/min. Peak 1, $C_{16:0}$; 2, branched C_{16}; 3, $C_{18:3}$; 4, $C_{18:2}$; 5, $C_{18:0}$; 6, $C_{20:3}$; 7, $C_{20:1}$; 8, $C_{22:1}$; 9, $C_{22:0}$; 10, $C_{24:0}$; 11, $C_{26:0}$.

supplied from porcine blood. It would be quite interesting to study the metabolic states of lipids to compared by sources and designated types. The leech's digestive system is rather simple and dependent upon a symbiosis with microorganisms in the guts. The hypothesis is that the lipids in the epidermal region of leeches are a metabolic product from the blood sources made by the symbiotic organism. Since we could determine the lipid composition of the dietary blood and trace the products from the host organism, we could understand the mechanism of action of an essential portion of the skin. From the fatty acid analyses, the major fatty acids were C(16:0; 18:0; 20:1; 20:3) components. Changes following fasting occurred relatively short chain fatty acids, C_{14} through C_{18}. During fasting there was a selective retention of eicosatrienoic acid in the lipid pool, but noticeable decreases in the net amounts of each major fatty acid were found to occur in leech skin.

We have further determined fatty acids from the free fatty acids and the fatty acids of triglyceride spots separated by TLC. The fatty acids of these classes are the major portion of the total amount recovered from the TLC separation. It is surprising to find that the medium

carbon chain (C_6-C_{12}) fatty acids from these classes are rather a great quantity. We are pursuing this finding at present time. These may play a major role in energy metabolism, as sources of energy through the release of fatty acids from triglycerides. However, it is not yet determined whether the fatty acid chain in triglycerides would be modified during transportation from the inner tissue to the surface.

Polyunsaturated fatty acids are necessary for maintaining the proper condition of barrier in the skin for many animal (Hansen and Jensen, 1985; Schneeberger *et al.*, 1988; Ziboh, 1992; Sardesai, 1992). It is quite clear that the leech epidermal cells contain various species of lipids with numerous fatty acids that help the structural as well as the physical function of skin. Some of these fatty acids are synthesized from symbiotic processes and the leech's natural metabolic processes, and others, that are known as essential fatty acids must be supplied from the diet. The specific types of these acids have not yet been all defined, but further studies are necessary to clarify the interaction of various lipid classes, including fatty acids.

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