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ALKANES, FATTY ACID METHYL ESTERS, AND FREE FATTY ACIDS IN SURFACE WAX OF *USTILAGO MAYDIS*

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Abstract—The wax fraction from chlamdospores of *Ustilago maydis* (Basidiomycetes) were analyzed by gas chromatography and mass spectrometry combination. The hydrocarbon fraction contained predominantly C_{25}, C_{27}, and C_{29} *n*-alkanes. A second fraction eluted from a silica gel column contained natural methyl esters of both saturated and unsaturated acids with predominant esters being from C_{16} through C_{20}. The methyl esters of C_{18} mono and dienoic fatty acids were present in abundance. The free fatty acid fraction contained fatty acids ranging in carbon number from C_{12} through C_{20}. In both the free acids and natural methyl esters the distribution of carbon skeletons were similar with the predominant compounds having even carbon number chains.

INTRODUCTION

THE EXTERNAL surface of fungal spores is covered by waxy deposit. This deposit is believed to be involved in maintaining water balance and provides a barrier to substances penetrating into the spores. Because the outer surface is continually exposed to the environment, the nature of the waxy layer is an important factor in the survival of fungal spores, yet very little is known about its composition.

Compounds such as fatty acids are normally associated with a wax layer. Tulloch and Ledingham[1–3] extracted the fatty acids from several types of rusts and smut spores, and analyzed them by gas chromatography. They found the predominant saturated fatty acid was palmitic, and the predominant unsaturated fatty acids were oleic and linoleic. For identification, the retention times were compared with known fatty acids, but none of the compounds were fully characterized. A second class of compounds, alkanes, were also reported to be present in the wax fraction of fungal spores.[4]

We have investigated the wax fraction from *Ustilago maydis* using a new combination gas chromatograph–mass spectrometer technique and the hydrocarbons, methyl esters of fatty acids, and free fatty acids characterized are reported here. To our knowledge this data provides the first evidence for the presence of naturally occurring methyl esters of fatty acids in fungal spores, as well as detailed characterization of each major component.

RESULTS

The heptane fraction was found to contain, as determined by gas chromatographic retention times and internal standards, both normal and branched alkanes as shown in Fig. 1. A and B represent two different sources of spores. The normal alkane pattern was

[1] A. P. TULLOCH, B. M. CRAIG and G. A. LEDINGHAM, *Can. J. Microbiol.* **5**, 486 (1959).
[2] A. P. TULLOCH and G. A. LEDINGHAM, *Can. J. Microbiol.* **6**, 425 (1960).
[3] A. P. TULLOCH and G. A. LEDINGHAM, *Can. J. Microbiol.* **8**, 379 (1962).
[4] J. ORÓ, J. L. LASETER and D. J. WEBER, *Science* **154**, 399 (1966).

similar in both cases, but the branched-chain pattern was different. The major components were C_{25}, C_{27}, and C_{29}. The mass spectra confirmed that they were *n*-alkanes having the following respective parent ion peaks: C_{25} (*m/e* of 352), C_{27} (*m/e* of 380), and C_{29} (*m/e* of 408).

The benzene fraction contained a number of components as shown in Fig. 2B with chain lengths ranging from C_{12} through C_{20}. The mass spectra data indicated that these compounds were naturally occurring methyl esters of fatty acids. An intense peak was observed at *m/e* of 74 which has been reported to be characteristic of a methyl ester of a long-chain acid,

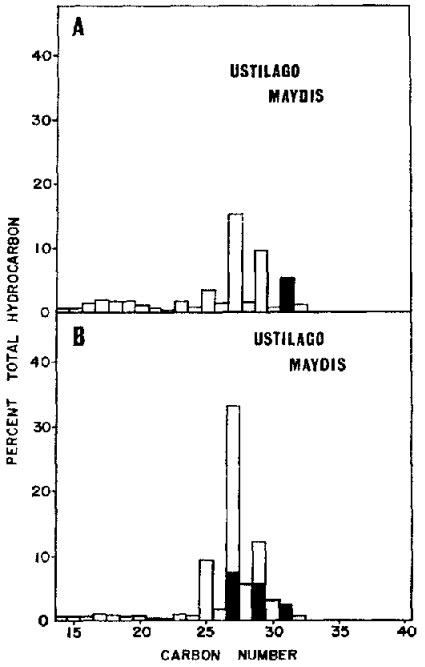

FIG. 1. THE CARBON NUMBER DISTRIBUTION OF NORMAL (OPEN BARS) AND BRANCHED CHAIN (SOLID BARS) ALKANES IN *U. maydis* SPORES FROM TWO SOURCES AS PERCENT TOTAL HYDROCARBONS EXTRACTED. (A from California, collected in 1965 season; B from Illinois, collected in 1966 season.)

unsubstituted at the alpha carbon.[5] In addition there are abundant fragments representing carbon to carbon cleavage which retains the carbomethoxy group (*m/e* 143 and 199). The spectrum of the methyl ester of oleic acid, a monoenoic fatty acid, contained strong intensities at M − 32, M − 74, and M − 116. Hallgren *et al.*[6] reported that the spectra of monounsaturated acids are very similar if the point of unsaturation is beyond the sixth or seventh carbon in the chain. The M − 32 peak corresponds to the loss of methanol. McLafferty[5] suggested that the peak M − 74 is the result of β-cleavage with subsequent rearrangement.

The spectra obtained from the peak corresponding to methyl ester of linoleic acid, a dienoic acid, showed as the most obvious difference, the occurrence of the prominent *m/e* peak equal to M − 31 instead of M − 32 as observed from monoenoic acid esters. As expected, the parent ion shows 2 mass units less than the monoenoic acid ester.

[5] F. W. MCLAFFERTY, *Anal. Chem.* **31**, 82 (1959).
[6] B. HALLGREN, R. RYHAGE and E. STENHAGEN, *Acta Chem. Scand.* **13**, 847 (1959).

The methanol fraction, following the treatment of the free fatty acids to form methyl esters, was also characterized by a gas chromatograph–mass spectrometer combination. The predominant fatty acids detected as shown in Fig. 2A were C_{14}, C_{16}, C_{18}, $C_{18:1}$ and $C_{18:2}$ with smaller quantities of both saturated and unsaturated acids. The mass spectra confirmed

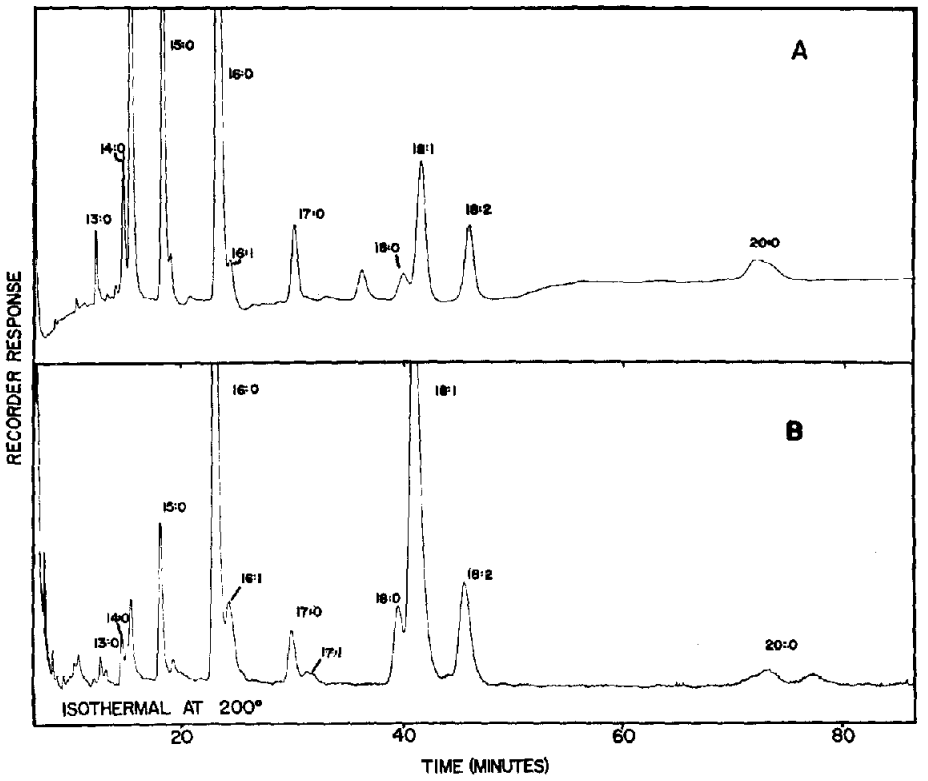

FIG. 2. (A) GAS CHROMATOGRAPHIC SEPARATION OF METHYLATED FREE FATTY ACIDS OF *U. maydis* SPORES ON STAINLESS-STEEL TUBING (200 m × 0·076 cm) COATED WITH IGEPAL CO-880. NITROGEN PRESSURE, 2196 g/cm^2; NO SPLIT. BARBER COLMAN 5000 INSTRUMENT EQUIPPED WITH A FLAME IONIZATION DETECTOR.

Range, 1; attenuation, 10. Temperature held isothermal at 200°. From 2·77 g of spores extracted, $\frac{1}{40}$ of the sample injected. (B) Gas chromatographic separation of natural methyl esters of fatty acids of *U. maydis* spores separated under the same conditions with approximately $\frac{1}{50}$ of the sample injected.

the compounds with the following *m/e*'s for the parent ions observed: myristic acid (*m/e* = 242), stearic acid (*m/e* = 298), palmitic acid (*m/e* = 270), oleic acid (*m/e* = 296), linoleic acid (*m/e* = 294), and arachidic acid (*m/e* = 326). Odd carbon-numbered chains were also present. The results were similar to those reported previously by Tulloch and associates. Table 1 represents a comparison between the free fatty acids and the corresponding free ester by carbon chain number.

TABLE 1. PERCENT DISTRIBUTION OF CARBON CHAINS BETWEEN THE FREE FATTY ACIDS AND THE NATURALLY OCCURRING FATTY ACID METHYL ESTERS IN WAX OF *Ustilago maydis* SPORES

Carbon chain number	Percent free fatty acids (A)	Percent fatty acid methyl esters (B)
13:0	1·75	0·70
14:0	3·74	1·86
14:1	13·80	3·26
15:0	13·90	6·28
15:1	2·92	1·20
16:0	28·90	20·69
16:1	3·92	7·89
17:0	3·86	3·26
17:1	1·21	1·01
18:0	1·29	6·20
18:1	12·40	32·70
18:2	5·65	9·36
18:3		
19:0		
20:0	5·84	4·85

DISCUSSION

While the naturally occurring methyl ester and free fatty acid patterns are similar, some distinct differences can be noted.

In comparing the percentage distribution between the free acids and free esters in Table 1 it becomes apparent that methylation of the C_{18} chains are favored. The greatest increase was noted for $C_{18:1}$, which was over 2·5 times the concentration of the corresponding free acid. Methyl palmitoleate also appeared to be somewhat favored in methylation. The remainder of the free fatty acids appeared to undergo methylation to a lesser degree except the $C_{17's}$ which yielded the same percentage distribution in both fractions. Just what relationship exists between the methyl exter of the fatty acids and the free fatty acids is not clear, but one would anticipate that a metabolic interchange would occur. It is likely that the methyl esters of fatty acids and the free fatty acids are associated in the wax complex similar to the methyl esters of galacturonic acid and free galacturonic acids in pectin.

A recent report[7] suggests that methyl esters of free fatty acids appear to serve as a reserve energy supply in *Euglena*. In considering the abundance of such esters in spores coupled with the fact that an energy supply is required for germination, it is possible that these natural methyl esters may function as a reserve energy source as well as a protective layer.

While considerable research must be completed before we can understand the nature and formation of the waxy layer on spores, we can at least now state that the major components have been characterized as alkanes, methyl esters of fatty acids, and free fatty acids.

METHODS AND MATERIALS

Sample Preparation

Spores of *Ustilago maydis* were obtained from D. Mathre, Montana State University, and from J. Paxton, University of Illinois. The spores were passed through a number 100

[7] A. ROSENBERG, *Science* **157**, 1189 (1967).

sieve (U.S. standard sieve series with 149 μ openings). Microscopic examination was made to verify the purity and type of spores present. All spores were stored at 4°.

Extraction Procedure

The spores were extracted initially with 50 ml of benzene–methanol (3:1) for 30 min at 50°. The mixture was then centrifuged at $1500 \times g$ for 5 min. The residual spores were then re-extracted with 50 ml of *n*-heptane for 30 min at 50°. After centrifugation the two extracts were combined and taken to dryness under N_2. The residue was taken up in *n*-heptane and transferred to the top of a silica gel column (1×20 cm).

A second extraction procedure was used to insure that no esterification occurred during extraction. The wax layer of the spores was extracted with 50 ml of $CHCl_3$–benzene (3:1) for 30 min at 50°. The residual spore material was re-extracted with 50 ml *n*-heptane after the centrifugation of the mixture at $1500 \times g$. The combined extracts were taken to dryness under N_2. The residue was taken up in *n*-heptane as before.

The silica gel had previously been heated for 10 hr at 425° and been washed with three volumes of *n*-heptane immediately before use. After the sample had been placed on the top of the column, the paraffinic hydrocarbons were eluted with 20 ml of *n*-heptane; a second fraction was eluted with 20 ml of benzene, and the final elution of the column was with 20 ml of methanol. Each collected fraction was taken to dryness (N_2).

The free fatty acids in the methanol fraction were methylated by refluxing them in 50 ml of methanol–NaOH (1:1) for 3 hr. The saponified fraction was subsequently taken to dryness under N_2 at 40° following adjustment of the pH to 1·0 with HCl, and then extracted ($3 \times$ *n*-heptane). The residue was taken up in 50 ml of methanol to which 5 per cent 2,2-dimethoxypropane and 0·5 per cent H_2SO_4 had been added by volume. The mixture was refluxed for 2 hr. The reaction was stopped by adding 5 ml of H_2O and then the methyl esters extracted with *n*-heptane.

Each of the fractions was dissolved in approximately 30 μl of benzene from which 1–2 μl portions were injected into a gas chromatograph. The hydrocarbons were separated on 2·7 m $\times$ 3 mm (inner dia.) stainless-steel column coated with SE 30 (a silicone grease purchased from Applied Science Laboratory Incorporated, State College, Pennsylvania) as the stationary phase, and with Chromosorb Q as the support material (also purchased from Applied Science Laboratory Incorporated). The branched-chain hydrocarbons were determined on a 40 m $\times$ 0·025 cm (inner dia.) stainless-steel capillary column coated with apiezon-L (a high-temperature grease purchased from Applied Science Laboratory Incorporated). The methanol fraction following methylation and the benzene fraction were resolved with a 200 m $\times$ 0·076 cm (inner dia.) stainless-steel capillary column coated with Igepal CO-880 (Nonylphenoxy Polyoxyethylene Ethyl Alcohol; General Aniline and Film Corporation, New York, N.Y.). The analyses were made with a Perkin and Elmer 900 and with Barber Colman series 5000 gas chromatographs equipped with dual flame detectors. The individual compounds were compared to authentic samples (purchased from Applied Science Laboratory Incorporated) and verified by analyses with the LKB 9000 gas chromatograph and mass spectrometer combination. Throughout the investigation the procedures were checked by use of suitable solvent blanks.

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