Changes in the Lipid Content of Fermented Peanuts

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Full-fat peanuts were fermented with *Neurospora sitophila*, *Aspergillus oryzae*, *A. niger*, *Rhizopus oligosporus*, and *R. delemar*. Total lipids, total fatty acids, and free fatty acids (FFA) were measured at incubation times ranging up to 116 hr. With the exception of *R. oligosporus*, which showed a slight reduction in total lipid, none of the fungi appeared to utilize lipid during fermentation. The extent of lipolytic activity varied among the fungi, as evidenced by final FFA content which ranged from 3.9% for *N. sitophila* cultured at 21° to 54.1% for *R. delemar* cultured at 28°. The FFA fraction of fermented peanuts was lower in linoleic and higher in saturated acids than the total lipid of control and fermented peanuts, thus indicating lipase activity specific for the 1,3 positions of triglycerides. Preferential utilization of FFA during fermentation was not detected.

Ontjom is an Indonesian food made by the fermentation of peanut press cake (Hesseltine, 1965). In the preparation of ontjom, oil is first extracted from peanuts and, after washing and steaming, the press cake is carefully mixed with ontjom from an earlier batch and packed in banana or other suitable leaves so that the packages can be opened for aeration and/or cooling if necessary. *Neurospora sitophila* is generally used to produce red ontjom; however, the tempeh mold, *Rhizopus oligosporus*, may be used to produce white ontjom (van Veen *et al.*, 1968; van Veen and Steinkraus, 1970). The homemade product contains considerably more oil than that made from technical grade press cake.

Changes in lipids which take place during the fermentation of peanuts, as well as the nutritive quality of the finished product, have been of interest for many years but have not been fully described. A series of experiments was therefore designed to gain added information pertaining to biochemical changes which occur during the fermentation of peanuts with selected fungi. This paper reports changes in total and free fatty acid content of peanuts during pure-culture fermentation with the ontjom molds, *N. sitophila* and *R. oligosporus*. In addition, *Aspergillus niger*, *Aspergillus oryzae*, and *Rhizopus delemar*, also used in commercial fermentations, were included in the study.

EXPERIMENTAL PROCEDURES

Peanut Fermentation. Florunner variety peanuts (No. 1 grade) were blanched at 210° for 6 min and skins were removed by abrasion. Nuts were then chopped in a Toledo food chopper to produce a coarse meal. Citric acid (1.25% by weight), tapioca (1.0%), and sodium chloride (0.63%) were added to the peanut meal and the mixture was combined with tap water (peanut meal mixture–water, 4:5, w/v). After occasional stirring during 30 min at room temperature, the peanut slurry was sterilized in shallow pans by heating at 121° for 15 min.

N. sitophila NRRL 2884, *A. oryzae* NRRL 1988, *A. niger* NRRL 3122, *R. oligosporus* NRRL 2710, and *R. delemar* NRRL 1472 were cultured on potato dextrose agar slants at 28° for 5 days. Spore suspensions of the fungi were prepared by washing the surfaces of the cultures with sterile 0.001% Tween 81. Ten milliliters of each of the suspensions was added individually to 1000-g portions of sterile peanut substrate and mixed thoroughly. The inoculated lots were then subdivided and spread in layers approximately 1 cm deep in sterile 4 × 15 cm petri dishes. The dishes were covered with four layers of cheesecloth and incubated under an atmospheric relative humidity of 70% at 28° for lengths of time ranging to 99 hr. Fermentation of peanuts by *N. sitophila* was also studied at 21° for up to 116 hr. Controls consisted of autoclaved, uninoculated peanut mixtures which were (1) not incubated, (2) incubated for 99 hr at 28°, and (3) incubated for 116 hr at 21°.

Samples were removed from incubation at selected times, treated 5 min with live steam, frozen at −40°, and freeze dried. All samples were passed through a 20-mesh screen prior to lipid analyses.

Lipid Analyses. Total lipids were determined in duplicate by a 16-hr diethyl ether extraction using a Goldfisch extractor. Lipid samples were dried in a vacuum oven at 65° prior to quantitation.

Percentages of free fatty acids (FFA) contained in extracted lipid samples were determined both by titration with standard alkali (American Oil Chemists' Society, 1949) and by the addition of an internal standard (Ray, 1954) prior to gas–liquid chromatography (glc). Values obtained by titration were calculated as oleic acid (18:1) and expressed as per cent FFA. In the internal standard procedure, known quantities of heptadecanoic acid were added to weighed samples of extracted lipid and the FFA fraction was subsequently isolated on 0.5-mm silica gel G thin layer plates. After developing in a solvent system of hexane–diethyl ether–acetic acid (90:10:1, v/v), the plates were sprayed with a dye solution containing Rhodamine B and 2,7-dichlorofluorescein (Jones *et al.*, 1966) and observed under ultraviolet light. The fatty acid fractions were removed and converted to methyl esters prior to glc as described below.

Fatty acid methyl esters were prepared from extracted lipid samples representing the entire lipid fraction of control and fermented peanuts as well as from the FFA fraction of peanut lipid isolated by thin layer chromatography (tlc). Esterification was accomplished with a 2:1 mixture of methanol–benzene containing 3% sulfuric acid as previously described (Worthington *et al.*, 1972).

Methyl esters were analyzed using a MicroTek Model 220 gas chromatograph equipped with a dual flame ionization detector and an Infotronics electronic integrator. Glass columns, 1.85 m × 4 mm i.d., were packed with 80–100 mesh Chromosorb W (acid washed, dimethylchlorosilane treated) coated with 20% (w/w) phosphorylated ethylene glycol succinate. Separation of fatty acid methyl esters (0.1 μl) was carried out at 190° with a helium flow rate of 100 ml/min. Peak identifications were made by comparing retention times obtained with samples and standard fatty acid esters under identical conditions of operation.

Percentage fatty acid compositions of the entire lipid extract of control and fermented peanuts as well as the

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FFA fractions isolated by tlc were determined by digital integration and normalization of peak areas; the values reported are, therefore, relative proportions of total fatty acids. In addition, the percentage of FFA in the extracted lipid was obtained by relating total area (integrator count) for all methyl esters of fatty acids isolated by tlc to the area produced by the added internal standard, methyl heptadecanoate.

RESULTS AND DISCUSSION

Fermentation. The consistency of the inoculated peanuts as fermentation was initiated could be described as "thick porridge." Preliminary experiments revealed that tapioca starch and citric acid, which lowered the acidity to pH 5.1, enhanced the growth of fungi tested in this study. Tapioca is often used by Indonesians in the preparation of ontjom and was reported to promote growth of *N. sitophila* on acidified peanut press cake (van Veen *et al.*, 1968). Without tapioca, extremely poor growth was obtained. Hesseltine *et al.* (1967) also noted poor growth of *Rhizopus* spp. on peanuts which did not contain added tapioca. In addition to reinforcing these findings, added sodium chloride was judged to elicit maximal growth of the five strains studied. Salt may exert a beneficial osmotic or ionic effect on the fungal mycelium, whereby extracellular enzymes are more readily freed to act upon substrate constituents. *Mucor hiemalis,* a mold used in Chinese cheese (sufu) fermentation, was previously reported to have increased proteinase activity when sodium chloride was added to a soybean substrate (Wang, 1967). This author also theorized that increased proteinase activity was due to sodium chloride.

Moisture content of the semisolid peanut substrate was initially 63%. The substrate exhibited some of the problems associated with solid state fermentations as enumerated by Hesseltine (1972). It cannot be stated that optimal spore inocula were used, that substrate size and vessel shape were most satisfactory, or that aeration was adequate to provide the best condition for studying lipid hydrolysis during peanut fermentation, since these variables were not extensively studied. Lack of oxygen within the substrate, which resulted from autoclaving, undoubtedly had an inhibitory effect on mycelium development. This was evidenced by growth occurring only on the surface of the substrate in the early stages of fermentation. However, extended fermentation times resulted in penetration of fungal mycelium throughout the substrate. The data reported from lipid analyses of fermented peanuts are representative of the entire substrate at each incubation time examined.

Lipid Analyses. Acid values of control and fermented peanuts as determined by alkali titration are shown in Figure 1. These values were calculated as oleic acid and are expressed as milligrams/gram oil. It should be noted that the values shown in Figure 1 would be expected to include, in addition to FFA, other acidic metabolites extracted by diethyl ether. FFA did not exceed 3.2 mg/g (0.32%) of oil in controls, thus indicating minimal autolytic activity resulting from heat treatment and incubation at 21 and 28°. Lipolytic activity of *R. delemar* and *R. oligosporus* was essentially linear throughout fermentation, while initial lag periods were required before FFA were noted in ferments inoculated with *A. niger, A. oryzae,* and *N. sitophila.* Although luxuriant growth was noted after 116-hr incubation for *N. sitophila* cultured at 21°, hydrolysis rates of triglycerides were about 20% of the 28° culture for comparable incubation times. Induced formation and specificity of lipases by fungi are dependent upon the nature of the triglyceride (Tsujisaka *et al.*, 1972, 1973; Hoover *et al.*, 1973; Iwai *et al.*, 1973). Moisture (Dorworth and Christensen, 1968) and temperature (Alford and Pierce, 1961) also influence fungal lipase production and activity on oilseeds. Whether nutrient and environmental

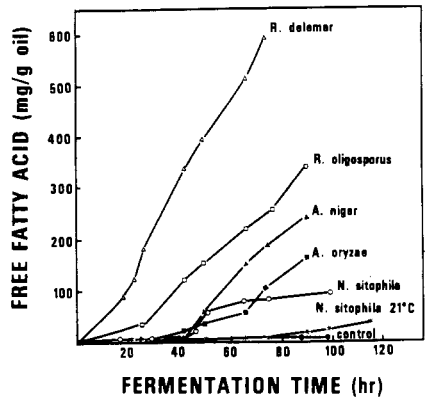

Figure 1. Free fatty acid content of control and fermented peanuts. Values were determined by standard alkali titration of extracted oil and expressed as milligrams of oleic acid per gram of oil. The incubation temperature was 28° except where indicated.

conditions established in this study were optimum for lipid hydrolysis is not known; however, all fungi studied displayed considerable lipolytic activity in full-fat peanuts.

Table I shows the lipid content of control and fermented peanuts at the maximal times of fermentation by the five fungi included in this study. With the exception of *R. oligosporus,* percentage lipid appears to have increased as a result of fermentation. Increases in per cent ether-extractable material may reflect either the selective utilization of nonlipid materials during fermentation or the production of lipids. In addition, low molecular weight acids such as acetic, lactic, succinic, gluconic, and oxalic, which may be produced during fungal fermentation (Shibasaki and Hesseltine, 1962; Hesseltine, 1965), would be extracted with diethyl ether. Extracted fat from peanuts fermented with *R. delemar* and *R. oligosporus* also contained insoluble material in late stages of fermentation which may have contributed to inaccurate measurement of true lipid content. van Veen and Schaefer (1950) also reported insoluble materials in ether extracts of tempeh. Even with the inclusion of these insoluble particles, however, ferments from *R. oligosporus* contained somewhat less ether-extractable material when compared to the controls. This would indicate that *R. oligosporus* possibly utilizes peanut lipid as a partial source of energy during fermentation. These results are in agreement with Sorenson and Hesseltine (1966), who reported that cottonseed, soybean, and rapeseed oils, in addition to peanut oil, served as excellent sole sources of carbon for growth of *R. oligosporus.*

Distribution of fatty acids in control and fermented peanuts is also shown in Table I. Fatty acids were measured after various times of fermentation, but since values did not fluctuate significantly over the incubation periods studied, only percentages for extended fermentation times are presented. With the exception of slightly lower percentages of palmitic acid (16:0) and slightly higher percentages of oleic and linoleic acids calculated for *R. oligosporus* and *R. delemar,* the remaining fatty acids [stearic (18:0), arachidic (20:0), eicosenoic (20:1), behenic (22:0), and lignoceric (24:0)] in fermented peanut lipid were essentially unchanged from the controls.

Table II shows the percentage of FFA in the total lipid portion of fermented peanuts. Percentages of individual fatty acids in the FFA fraction of fermented peanuts at the time fermentations were terminated are also shown. Percentages are representative of those measured at selected times during fermentation. Total FFA in the controls was extremely low and not considered to contribute

Table I. Lipid and Fatty Acid Composition of Control and Fermented Peanuts

Fungus	Temp, °C	Time, hr	Lipid, %[a]	Fatty acid, %[b]							
				16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0
Control		0	50.8	10.6	2.7	51.2	28.3	1.4	1.3	2.8	1.6
Control	28	99	50.5	10.6	2.8	50.9	28.5	1.4	1.4	2.8	1.6
Control	21	116	50.5	10.7	2.8	51.1	28.2	1.4	1.3	2.9	1.4
N. sitophila	28	99	52.1	10.3	2.7	50.3	28.3	1.4	1.4	3.0	1.6
N. sitophila	21	116	51.6	10.5	2.7	50.7	28.2	1.3	1.3	2.7	1.6
A. oryzae	28	90	53.3	10.3	2.3	50.9	28.4	1.4	1.3	2.8	1.6
A. niger	28	90	52.5	10.1	2.7	50.7	28.4	1.4	1.4	2.8	1.7
R. oligosporus	28	90	48.3	9.4	2.5	51.6	28.7	1.4	1.3	2.8	1.7
R. delemar	28	74	51.4	9.8	2.4	52.1	29.1	1.2	1.2	2.4	1.4

[a] Percentage of peanut, dry weight basis; average of two determinations. [b] Percentage of total fatty acid in peanut lipid; average of four determinations.

Table II. Extent of Lipid Hydrolysis and Fatty Acid Composition of FFA in Fermented Peanuts

Fungus	Temp, °C	Time, hr	FFA, %[a]	Fatty acid, %[b]							
				16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0
N. sitophila	28	99	9.4	11.5	3.6	53.4	22.9	1.5	1.4	3.5	2.2
N. sitophila	21	116	3.9	13.8	4.4	51.6	22.8	1.3	1.3	2.9	1.9
A. oryzae	28	90	16.3	11.6	3.8	51.3	23.8	1.8	1.4	4.1	2.0
A. niger	28	90	24.9	10.3	3.2	50.3	24.8	1.7	1.4	5.4	2.8
R. oligosporus	28	90	31.5	10.2	3.2	52.6	22.4	1.9	1.8	4.1	3.6
R. delemar	28	74	54.1	11.7	3.3	54.0	24.5	1.4	1.5	2.9	1.7

[a] Percentage of total lipid; average of four determinations. [b] Percentage of total FFA; average of four determinations.

significantly to the FFA profiles of fermented peanuts; these data are not presented. The FFA fraction contained significantly higher levels of saturated fatty acids, particularly palmitic and stearic acid, and lower levels of linoleic acid than total lipid extracts (Table I). Oleic acid was essentially unchanged. These differences in FFA distribution are those which would be expected from the action of 1,3-lipases, since saturated acids are located primarily in the 1,3 position and linoleic acid is in the 2 position of peanut triglycerides (Brockerhoff and Yurkowski, 1966). Oleic acid is approximately equally distributed among the three positions. Thus, the lipase activity of all organisms investigated in this study shows 1,3 specificity. Of particular interest in this respect is the apparent 1,3 specificity of lipase from *A. oryzae* and *A. niger*. Alford *et al.* (1964) reported approximately equal rates of hydrolysis of fatty acids in the 1, 3, and 2 positions of triglycerides by another species of this genus, *A. flavus*.

We found no evidence of preferential utilization of any of the free fatty acids by any of the organisms. This is in agreement with Wagenknecht *et al.* (1961), who reported that *R. oryzae* did not preferentially utilize those fatty acids of soybean which are in common with peanuts. The organism did, however, utilize 40% of the linolenic acid, a fatty acid not found in significant quantity in peanuts (Worthington and Holley, 1967). Murata *et al.* (1967) likewise reported decreases in linolenic acid content of tempeh cultured with *R. oligosporus*. However, the latter authors also reported increases in percentage of palmitic and oleic acids and decreases in stearic and linoleic acids. Hansen *et al.* (1973) reported that *A. niger* tended to utilize lauric, myristic, linoleic, and arachidic acids in pure-culture fermentation of cacao beans. Increases in percentage of stearic and palmitic acids and decreases in oleic and linoleic acids were noted in naturally fermented cacao beans (Kavanagh *et al.*, 1970). The latter report is not always consistent with data of Hansen *et al.* (1973), which indicated that lipolysis and fatty acid utilization were dependent upon bean variety and indigenous microflora. These reports, as well as the data presented from this study involving fermented peanuts, serve to further stress the dependence of the extent of lipolysis and utilization on triglyceride structure, environmental conditions, and, most importantly, the fungus under examination.

The percentages of total FFA in samples representing the longest fermentation times for each fungus were calculated by the internal standard procedure. Values for *N. sitophila*, *A. oryzae*, and *A. niger* are nearly identical with corresponding acid values determined by alkali titration (Figure 1). Values for *R. oligosporus* and *R. delemar* were 31.5 and 54.1%, respectively, somewhat lower than the 34.2 and 59.4%, calculated by titration. Chromatographic data shown in Table II do not reflect the presence of acidic metabolites other than long-chain fatty acids produced during fermentation. These compounds would contribute to higher acid values when total acidity is measured by titration and may account for the observed differences in FFA values obtained by alkali titration and the internal standard procedures.

Only major and minor fatty acids normally found in peanut oil were quantitated in this study. Fatty acid methyl esters in combined concentrations of not greater than 1% of the extract appeared on some gas–liquid chromatograms. These methyl esters were thought to be formed from fatty acids synthesized by the fungi during growth. Experimental design did not permit distinguishing between fatty acids originating from peanuts and fungal mycelia, although major fatty acids in mycelia (Shaw, 1966) are similar to those in peanuts.

Nutritional upgrading through the appearance of γ-linolenic acid (18:3ω6) resulting from fermentation of cassava flour with *Rhizopus arrhizus* was suggested by Harris (1970). γ-Linolenic acid is found in mycelia of genera belonging to the class Phycomycetes (Shaw, 1966) and is an intermediate in arachidonic acid (20:4) biosynthesis in animals. γ-Linolenic acid may therefore be considered as an "essential fatty acid." This acid was not noted to accumulate during fermentation of peanuts with fungi included in this study. At the same time, relative quantities of saturated and unsaturated fatty acids were essentially unchanged after fermentation, thus resulting in products no less desirable than unfermented peanuts with respect to the nutritional value of fatty acids based on the degree of saturation. It is possible that liberation of fatty acids

might, in fact, increase the digestibility of peanuts and thereby increase their nutritional value. Investigations are in progress to explore these possibilities.

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Aflatoxin Q_1. A Newly Identified Major Metabolite of Aflatoxin B_1 in Monkey Liver

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Incubation of aflatoxin B_1 with monkey liver microsomal preparations in phosphate buffer (pH 7.4), in the presence of an NADPH regenerating system, resulted in the formation of two main metabolites. One was aflatoxin M_1 (1-3% of the substrate) and the other was a novel metabolite (16-52% of the substrate) which we isolated and identified as an isomer of aflatoxin M_1, with the hydroxyl on the carbon atom β to the carbonyl of the cyclopentenone ring. The name aflatoxin Q_1 was proposed for this newly identified metabolite.

The potent fungal hepatotoxin and hepatocarcinogen aflatoxin B_1 requires metabolic activation to become the immediate carcinogenic agent. This activation occurs with liver microsomal fractions (Goodall and Butler, 1969; Garner *et al.*, 1972; Moule and Frayssinet, 1972; Ames *et al.*, 1973). The difference in susceptibility to aflatoxin B_1 carcinogenesis varies among species and may be related to variation in metabolism (Patterson, 1973). Although susceptibility of man is not clearly known, exposure to aflatoxin appears associated with increased incidence of liver cancer in certain populations (Shank and Wogan, 1972). Comparative metabolic studies in different species including primates are thus relevant to the question of susceptibility in man.

Aflatoxin M_1 is recognized as a major *in vivo* metabolite in mammals (de Iongh *et al.*, 1964; Allcroft *et al.*, 1966; Holzapfel *et al.*, 1966; Masri *et al.*, 1967). Conversion of aflatoxin B_1 to M_1 (1-3%) by rat liver microsomal fractions has been shown (Portman *et al.*, 1968; Masri *et al.*, 1969; Patterson and Allcroft, 1970; Patterson and Roberts, 1971). More recently, aflatoxin P_1, an O-demethylated product of aflatoxin B_1, was shown to be a major *in vivo* metabolite in monkeys given aflatoxin B_1 by intraperitoneal injection (Dalezios *et al.*, 1971). Aflatoxin P_1 appeared in the urine mainly in conjugated form (glucuronide and sulfate) and represented about 20% of the dose, whereas aflatoxin M_1, which was also formed, accounted for only 2.3% of the dose.

Aflatoxin M_1 does not appear to be the immediately active carcinogenic metabolite in a rat microsome mediated bacterial test system for carcinogens (mutagens) (Garner *et al.*, 1972; Ames, 1973). Aflatoxin P_1 was not toxic to the chicken embryo at levels that produce significant mortality when aflatoxin B_1 is used (Stoloff *et al.*, 1972).

We reported the conversion of aflatoxin B_1 by rat and monkey liver postmitochondrial preparations to two main metabolites: aflatoxin M_1 and a newly identified metabolite which we referred to as aflatoxin Q_1 (Masri *et al.*, 1973). We present here evidence of the structure of aflatoxin Q_1 as an isomer of aflatoxin M_1 with the hydroxyl group on the carbon atom β to the carbonyl of the cyclopentenone ring.

EXPERIMENTAL SECTION

Aflatoxin B_1. This was extracted from cultures of *Aspergillus parasiticus* NRRL 2999. It was purified by col-

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