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Effect of Oat Constituents on Aggregation Behavior of *Oryzaephilus surinamensis* (L.)

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Components isolated from pentane extracts of rolled oats that stimulate aggregation of *Oryzaephilus surinamensis* (L.) (saw-toothed grain beetle) adults have been identified. These materials include mixtures of fatty acids consisting mainly of palmitic, oleic, and linoleic acids and triglycerides with acyl substituents made up of similar mixtures of these same fatty acids. Food-grade coconut, corn, and sunflower oils also induce aggregation when administered in the bioassay. Triglycerides from rolled oat extract containing 9,10-epoxyoctadecanoic and 9,10-epoxy-*cis*-12-octadecenoic acids showed no activity. Appreciable amounts of C_{18} hydroxydiene acids were identified in triglyceride fractions that induced a distinct avoidance response in the insects.

The sawtoothed grain beetle [*Oryzaephilus surinamensis* (L.)] is an economically important stored-product pest of worldwide distribution. Although numerous investigations have dealt with food attractants for *Tribolium*, *Trogoderma*, and *Sitophilus* species [see Nara et al. (1981) for a summary], only recently have two reports appeared concerning oat constituents that elicit chemosensory responses in *O. surinamensis* adults (Pierce et al., 1981; Freedman et al., 1982). Freedman et al. (1982) presented results indicating that crude mixtures of essentially nonvolatile triglyceride materials from pentane extracts of rolled oats stimulate aggregation of the saw-toothed grain beetle; however, both groups of workers also suggested that volatile chemical stimuli are also present in oats and oat extracts and are responsible for the actual olfactory attraction that was observed in pitfall and olfactometer bioassays.

Since none of the oat constituents had been identified, we conducted this investigation to extend the work of Freedman et al. (1982) by isolating and identifying those constituents responsible for the observed insect behavior.

MATERIALS AND METHODS

Insect Rearing and Bioassay. *O. surinamensis* rearing, Petri dish bioassays, and statistical treatment of data were all done as previously described (Freedman et al., 1982). Test beetles were reared on a diet of rolled oats containing 5% of brewers yeast, were 3–5 weeks old, and had been starved from 5 to 7 days prior to being used in a bioassay. Petri dishes with two filter paper disks were used as test arenas. Percent response data shown in Tables

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Table I. Aggregation Response of *O. surinamensis* to Oat Extract Fractions

sample designation		sub-frac-tions	type of material	recovery from extract, %	% response[b,c]
new	previous[a]				
A	2		triglyceride	76.4	67**[a]
B	3-3		triglyceride	0.57	42**[a]
C	4-3	1	triglyceride	0.28	11
		2	triglyceride	0.44	6
		3	triglyceride	0.59	-9
D	5-1, 5-2, 5-3		free fatty acids	0.79	61**
E	5-5	1	triglyceride	0.14	-56**
		2	triglyceride	0.27	-30**
		3	free fatty acids	0.04	20*
F	5-6	1	mixture	0.48	3
		2a	triglyceride	0.04	d
		2b	triglyceride	0.03	d
		2c	di- + monoglyceride	0.16	20*[e]
		2d	di- + monoglyceride	0.91	23*[e]
		3	mixture	0.93	3
		4	free fatty acids	0.04	52**
G	6-2, 6-3, 6-4		free fatty acids	0.55	24**[f]

[a] From Freedman et al. (1982), Table 6.   [b] Based on 4 replicates, 25 beetles per replicate, and 2500 μg of material/test.   [c] (*) and (**) are significant at $P < 0.05$ and $P < 0.01$, respectively, based on x^2 analysis.   [d] Insufficient quantity for reliable bioassay.   [e] 0.025 μg/test; eight replicates.   [f] 2.5 μg/test.

I and II are calculated by the formula $100(T - C)/N$, where T and C are the number of beetles aggregating at the treated and control disks, respectively, and N is the total number of beetles used. Reference bioassays using pentane extract of rolled oats (Quaker Old Fashioned) at 5×10^4 and 1×10^4 μg/test were conducted with each set of four replicates. All solvents were redistilled before use.

Table II. Aggregation Response of *O. surinamensis* to Various Lipid Materials

test material	amount per test, $\mu g \times 10^{-3}$	% response[a,b]
triglycerides		
fraction A	10	29**
fraction A, base-washed	10	20*
fraction A, vacuum-treated	10	39**
fraction A, vacuum-treated, based-washed	10	24**
rolled oat extract	10	35**
rolled oat extract	2	45**
coconut oil,[c] base-washed	25	38**
coconut oil,[c] base-washed	2	29**[d]
corn oil[c]	10	31**
corn oil[c]	2	39**[d]
sunflower oil[c]	25	41**
sunflower oil[c]	2	33**[d]
others		
FFA[e] from oat extract	2.5	18*
FFA[e] from coconut oil	0.5	-50**
FFA[e] from fraction A	2.5	41**
FFA[e] from fraction C	2.5	3
unsaponifiables from oat extract	2.5	-55**

[a] Based on 4 replicates of 25 beetles each. [b] (*) and (**) indicate significance at $P < 0.05$ and $P < 0.01$, respectively, based on χ^2 analysis. [c] Commercial food grade—alkali-refined, bleached, and deodorized. [d] Based on 8 replicates of 25 beetles each. [e] Free fatty acids obtained by saponification.

Column Chromatography. Crude extract obtained by Soxhlet extraction of rolled oats with pentane was fractionated on silica gel as described by Freedman et al. (1982).

Thin-Layer Chromatography. Analytical TLC was done on precoated Merck silica gel 60 F-254 plates of 0.25-mm thickness with one or more of the following solvent systems: 4% diethyl ether in dichloromethane, 15% and 35% diethyl ether in hexane, and 0.9%, 2%, 10%, and 25% methanol in chloroform. Spots were visualized by staining with iodine vapor or by charring the plate after spraying with 50% aqueous sulfuric acid.

Preparative TLC separations were performed on either 0.25- or 2.0-mm layers depending on the degree of resolution required and on sample size. Component bands were located by exposing about a 1-cm edge of the developed plate to iodine vapor or by spraying the plates with dichlorofluorescein solution and observing them under ultraviolet light.

Fraction C (Table I) was resolved on 0.25-mm silica layers at the rate of 4 mg/20 × 20 cm plate with 4% diethyl ether in methylene chloride. Subfractions E3 and unresolved E1 and E2 were obtained from 2-mm silica plates after development with 1.75% methanol in chloroform. E1 and E2 were purified on 0.25-mm plates at 3 mg/plate with the same solvent system. Finally, fraction F was separated into F1, F2, F3, and F4 on 2-mm plates with 2.5% methanol in chloroform. Resolution of F2 was accomplished on 0.25-mm plates with 1.75% methanol in chloroform. (Note: When using solvent systems containing up to about 5% methanol in chloroform, one must take into account any ethanol added to the chloroform as a stabilizer, since it can drastically alter R_f values.)

Gas Chromatography. Glyceride samples were analyzed on a 91.5 cm × 2 mm i.d. glass column containing 3% OV-1 packing by using helium at a flow rate of 25 mL/min. The column temperature was programmed from 200 to 350 °C at 4 °C/min. GC analyses of methyl esters

were done on two columns; one was 122 cm × 4 mm i.d. glass containing 5% Apiezon L (Ap.L) packing (helium, 30 mL/min) and the second was 305 cm × 4 mm i.d. glass containing 5% LAC-2-R 446 (R 446) packing (helium, 30 mL/min). Both columns were operated isothermally at 185 °C. Area percentages and equivalent chain lengths (ECL's) were computer calculated.

Gas Chromatography–Mass Spectrometry. The GC–MS system included a 91.5 cm × 2 mm i.d. glass column packed with 3% OV-1 and operated isothermally at 160 °C. It was coupled with an all-glass interface to a Kratos MS 30 mass spectrometer; spectra were obtained at 70 eV.

Saponification and Esterification. Triglyceride materials from oats and coconut were saponified by refluxing 2 h with 2 N NaOH in 80% aqueous ethanol. Unsaponifiables were recovered (from oat extract only) by diethyl ether extraction of the basic solution. After acidification, the solution was again extracted with ether to obtain the free fatty acid mixtures. Isolation of reference free fatty acid mixtures from *Vernonia pauciflora* and *Castalis nudicaulis* seed oils containing epoxy and hydroxydiene acids, respectively, was done as described by Binder et al. (1964) to minimize possible side reactions. Methyl ester preparation from both glyceride materials and free fatty acids was accomplished by using the BF_3 procedure described by Metcalfe et al. (1966).

Removal of Volatiles and Free Fatty Acids. A 0.75-g sample of the major triglyceride fraction (fraction A, Table I) was heated intermittently at 150–160 °C under reduced pressure (2–4 mmHg) in a 250-mL round-bottomed flask for a total of 15 h over a 3-day period. The material was bioassayed immediately after dismantling the apparatus. For removal of any free acids from fraction A, from vacuum-treated fraction A, and from coconut oil, the samples were dissolved in diethyl ether, and the solutions were washed 3 times with 5% K_2CO_3 solution and then 3 times with water and dried over $MgSO_4$. Ether was removed from the triglycerides under vacuum at room temperature.

RESULTS AND DISCUSSION

The pentane extract of rolled oats had previously been fractionated by column chromatography into 25 unidentified fractions [Freedman et al. (1982), Table 6]. A number of these fractions evoked no aggregation response from the beetles and others were obtained in amounts inadequate for further fractionation; all of these were eliminated from the current investigation. The remaining fractions were redesignated in the interest of clarity and are listed in the first column of Table I. Fractions C, E, and F were subfractionated as indicated. No attempt was made to further resolve fractions A, B, D, and G since each gave only one spot on TLC.

Certain oat triglycride fractions (A, B) stimulated aggregation of *O. surinamensis*, others (C1, C2, C3) had no effect, and still others (E1, E2) inhibited, deterred, or prevented the insects from aggregating on the treated disks. Since all of these fractions were triglycerides it was desirable to ascertain existing differences in their fatty acid compositions. GC analyses of methyl esters (Table III) derived from neutral fractions C1, C2, and C3 demonstrated the presence of relatively large amounts of hydroxymethoxy C_{18} esters. The ECL's of these esters matched those reported by Kleiman et al. (1969). Fragmentation patterns observed by GC–MS analysis were identical with those obtained for hydroxymethoxy esters derived from methyl 9,10-epoxystearate and methyl 9,10-epoxy-*cis*-12-octadecenoate by treatment with BF_3–methanol (Kleiman and Spencer, 1973). Bioassays con-

Table III. Gas Chromatographic Analyses of Methyl Esters from Oat Extract Fractions

	components, %										
methyl esters of	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	conjugated $C_{18:3}$[a]	methoxy $C_{18:2}$[b]	hydroxy-methoxy $C_{18:0}$	hydroxy-methoxy $C_{18:1}$	unknown	others[c]
A	18.3	1.7	40.4	37.2	1.1						1.3
B	12.7	1.4	29.3	47.5	1.2	3.7	3.3				0.9
C1	9.5	1.6	29.3	28.0			1.8	4.4	24.2		1.2
C2	13.1	1.7	28.6	25.9				17.8	11.9		1.0
C3	12.9	1.3	30.6	24.5	0.6			25.5	3.5		1.1
D	29.8	1.9	33.7	33.2	0.9						0.5
E1	18.0	3.0	35.4	19.8	0.3	4.7	8.1	3.9	1.7	4.1	1.0
E2	16.3	1.8	28.6	21.9	0.4	8.3	15.3	0.8	0.9	3.4	2.3
E3	27.4	1.6	34.1	35.4	0.3						1.2
F2a	18.4	3.0	35.3	25.0		1.9	5.9	0.9	1.0	6.1	2.5
F2b	14.8	2.0	39.0	27.8		2.7	4.0	0.8	0.7	5.4	2.8
F2c	19.2	2.9	37.8	32.8		0.7	2.1	1.1	0.7	1.0	1.7
F2d	12.9	3.3	34.6	22.4			1.0	1.7	2.2	1.9	3.0
F4	30.1	0.6	32.8	34.9	0.5						1.1
G	30.2	1.5	18.4	44.5	2.0						3.4
rolled oat extract	17.0	1.7	38.6	38.9	1.5						2.3

[a] ECL's on Ap.L (R 446) = 19.0–19.6 (22.2–22.6). Derived from hydroxy- and methoxydienes. [b] ECL's on Ap.L (R 446) = 19.4 (22.7). Derived from hydroxydienes. [c] Includes $C_{14:0}$, $C_{16:1}$, C_{20}, and C_{22} fatty acids and minor unidentified components.

ducted on a fatty acid mixture derived from the seed oil of *Vernonia pauciflora*, containing over 50% of 9,10-epoxy-*cis*-12-octadecenoic acid, showed that this mixture significantly deterred aggregation at a 2500-μg dose.

Methyl esters prepared from triglyceride fractions E1 and E2 contained appreciable amounts of C_{18} hydroxydienes and methoxydienes, as well as an unidentified short-chain component of ECL 10.7 (Ap.L) and 15.6 (R 446) that was probably an oxidative cleavage product. A reference bioassay sample was prepared by saponifying *C. nudicaulis* seed oil, which contains more than 50% of 9-hydroxy-*trans,trans*-10,12-octadecadienoic acid, and recovering the fatty acid mixture. This mixture elicited a strong avoidance response when tested at 250–2500-μg levels; test amounts of 25 μg or less gave a neutral response. What effect that short-chain component has on the observed insect response is not known. Although bioassays were not done on fractions F2a and F2b, one might infer that these would also cause the insects to avoid the treated disk since their fatty acid compositions are similar to those of E1 and E2.

The hydroxydienes in oat extract are derivable from linoleic acid moieties by autoxidation or enzymic activity (Heimann and Schreier, 1971); subsequent treatment with BF_3-methanol would at least partially convert them to methoxydienes (Powell et al., 1967). These hydroxydienes, and to a lesser extent (under our conditions) methoxydienes, are converted to C_{18} conjugated trienes during GC analysis. GC–MS of the methoxydiene esters gave the following ions (fragment identity and relative intensity in parentheses): 324 (M+, 20), 292 (M – CH_3OH, 15), 253 [M – $CH_3(CH_2)_4$–, 36], and 167 [M – $CH_3CO_2(CH_2)_7$–, 100]. These data are consistent with a mixture of methyl 9-methoxy-10,12-octadecadienoate and methyl 13-methoxy-9,11-octadecadienoate. Presumably, the precursor dienols have their funtionalities located at the same positions.

These oxygenated fatty acid moieties have not previously been reported in oat extracts (Sahasrabudhe, 1979); however, the small amounts present in a crude extract would make identification by GC parameters tenuous. We detected some very small (0.1–0.2%) peaks having appropriate ECL's in the Ap.L GC analysis of rolled oat

extract methyl esters, but in the absence of R 446 ECL values these components are included in the "others" column of Table III.

It appears that, in general, only those triglycerides that contain little or no oxygenated acyl groups stimulate aggregation. Fraction A, the major triglyceride fraction (76%) of oat extract, is devoid of oxygenated materials and provides the strongest stimulation observed (Table I). Fraction B, however, presents an apparent anomaly in that it contains 7% of oxygenated acid moieties yet induces a strong aggregation response.

Partial glyceride fractions F2c and F2d had no significant effect on the insects when applied to the test arena in amounts ranging from 2500 μg down to 0.25 μg. However, at 0.025 μg/disk a marginal positive response was observed. Both fractions consisted mainly of diglycerides; F2c contained about 10% monoglyceride and F2d contained about 30%. Reference bioassays using mono- and dilinolein (Nu-Chek-Prep, Inc.) at 250–2500 μg/disk produced neutral responses. When applied at levels of 0.25–25 μg, these two materials stimulated aggregation if the test solutions were bioassayed immediately after being prepared. However, if an overnight delay occurred, presumably allowing the onset of autoxidation, only neutral responses were observed.

Mixtures of free fatty acids (D, E3, F4) isolated from oat extract all stimulated aggregation although the response to E3 was marginal. Fraction G, also a free fatty acid mixture, evoked a significant response at 2.5 μg/disk but not at larger amounts. However, G contained considerably more linoleic acid than the other three fatty acid fractions and therefore would be prone to more rapid accumulation of oxygenated products; we have indicated that these materials tend to elicit negative responses. As the test amount is decreased, the aggregation-stimulating effect of the nonoxygenated acids present can predominate. Cohen et al. (1974) observed numerous differences in response to various fatty acids by *Tribolium castaneum* and *Trogoderma granarium*, but the question of whether or not oxygenated components might be responsible for the variations did not arise.

Table II lists bioassay results for some additional materials that were tested in attempts to provide further

insight into the behavior patterns observed. Fraction A, the major triglyceride component of rolled oat extract, was shown by all analytical techniques to contain only ordinary triglycerides, yet a faint odor of rolled oats was detectable. Prolonged heating of this material under a vacuum produced an odorless oil that still stimulated aggregation of the beetles. Similarly, base washing of fraction A to remove possible free acid contaminants did not significantly alter its activity. Samples of commercial food-grade coconut, corn, and sunflower oils all elicited significant aggregation responses at various dose levels. The fact that oat, corn, and sunflower oils (all containing virtually 100% palmitic, oleic, and linoleic acids) are aggregation inducing is not surprising, since triglycerides from various sources reportedly stimulate this behavior in other adult stored-product insects such as *Trogoderma*, *Tribolium*, and *Sitophilus* spp. (Tamaki et al., 1971a,b; Nara et al., 1981). However, it seems odd that coconut oil, containing 80% of C_6–C_{14} fatty acids and not more than 20% of palmitic, oleic, and linoleic acids, should also demonstrate aggregation-inducing ability.

Free fatty acid mixtures from saponification of the crude oat extract and from fraction A were both stimulative (Table II), although those from the crude extract were significantly less so than those from fraction A. The crude extract fatty acid mixture contained all acids present (including oxygenated ones) in any form in the extract, whereas the fatty acid mixture derived from highly purified fraction A contained only oridinary acids; this might explain the difference observed in beetle responses to the two fractions.

The fatty acid mixtures obtained by saponification of coconut oil and those of fraction C prior to subfractionation were the only fatty acid fractions isolated in this work that did not stimulate aggregation. Since the three subfractions of C (Table I) gave a neutral response, it is not surprising that fatty acids derived by saponification of these glycerides also were inactive. The volatile short chain fatty acids of coconut oil are probably responsible for this mixture being strongly repellent (Cohen et al., 1974) at the 500-μg level. It became less repellent in smaller doses but did not stimulate aggregation at any level tested.

Finally, the unsaponifiable materials from crude oat extract strongly inhibited aggregation of the saw-toothed grain beetles.

To summarize, it appears that the stimulated aggregation observed when adult *O. surinamensis* are presented a crude pentane extract of rolled oats is the net result of a number of interacting factors. Ordinary (nonoxygenated) free fatty acid mixtures containing varying amounts of palmitic, oleic, and linoleic acids as major components, and triglycerides (Tamaki et al., 1971a,b) composed of such mixtures, seem to contribute most to the observed response. Partial glycerides present in the extract elicited marginally significant aggregation at low concentrations, but this result may be clouded somewhat because the two fractions assayed were mixtures of mono- and diglycerides and also contained some oxygenated constituents.

An opposing effect is produced by triglycerides containing large amounts of oxygenated fatty acid moieties. The unsaponifiable fraction of oat extract, which caused an avoidance response, may contain constituents that were not present in the original extract; therefore, it is inappropriate to consider possible contributions made by other unsaponifiable components to the observed response to the crude extract.

The volatile olfactory stimuli in oats that attract *O. surinamensis* mentioned by Freedman et al. (1982) are being investigated, and results will be communicated at a later date.

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