Triglycerides in Wheat Germ as Chemical Stimuli Eliciting Aggregation of the Confused Flour Beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae)

Yoshio Tamaki,[1] Samuel R. Loschiavo, and Arthur J. McGinnis*

1-Palmito-2,3-diolein, 2-linoleo-1,3-dipalmitin, and 1-palmito-2-linoleo-3-olein were identified as major triglycerides in three active fractions from wheat germ that elicit aggregation of the confused flour beetle. These triglycerides were synthesized and their biological activities verified. This appears to be the second time that naturally occurring triglycerides have been shown to elicit aggregation of insects.

Natural products that affect insect behavior have received much attention in recent years. Chemical characterization of these components and elucidation of their effects on insect behavior may form the basis of alternative methods of insect control in the future. Highly active substances eliciting aggregation and feeding by the confused flour beetle, *Tribolium confusum* Jacquelin Du Val, have been demonstrated in wheat germ (Loschiavo, 1965b). This insect is an economically important pest of stored products and is found most frequently in cereal stocks high in wheat germ (Loschiavo, 1952; Smallman and Loschiavo, 1951). It was of interest, therefore, to isolate and characterize the biologically active components. This paper shows that certain triglycerides are the substances in wheat germ that elicit aggregation of the confused flour beetle.

MATERIALS AND METHODS

Extraction of Active Substances. One-hundred grams of wheat germ was thoroughly homogenized in 10 times its volume of hexane (*n*-hexane) and filtered. The residue was similarly treated with acetone, and then with methanol. Each filtrate was concentrated in a rotary evaporator at a temperature below 40° C.

Column Chromatography. Florisil (60- to 100-mesh) was used as the adsorbent for separation of lipid classes in the hexane extract (Carrol, 1961). Less than 500 mg of lipid materials was added to 30 g of Florisil packed in a glass column of 17 mm in diameter. Seven fractions were collected by using stepwise elution with the following solvents: hexane, 50 ml; hexane-ether (diethyl ether) (95 to 5), 120 ml; hexane-ether (85 to 15), 150 ml; hexane-ether (75 to 25), 150 ml; hexane-ether (50 to 50), 150 ml; ether-methanol (98 to 2), 150 ml and ether-glacial acetic acid (96 to 4) 150 ml. The major lipid classes in the seven fractions eluted by these solvents were tentatively identified by thin-layer chromatography as follows: hydrocarbons; nonglycerol esters such as sterol esters and waxes; triglycerides; sterols; diglycerides; monoglycerides; and free fatty acids. There was no overlap of lipid classes in adjacent fractions.

Silicic acid impregnated with silver nitrate was used in column chromatography for preliminary separation of triglycerides according to the degree of unsaturation. Silicic acid, 160 g (Mallinckrodt, 100-mesh) was mixed with 40 g of $AgNO_3$ dissolved in 160 to 170 ml of water, and activated at 120° C for 24 hr. The $AgNO_3$-silicic acid was mixed with half its weight of Celite-545, and packed in a glass column as a slurry with hexane. Mixtures of ether and hexane were used for elution in which the ether concentration was changed stepwise from 5 to 100%. The elution pattern was monitored by thin-layer chromatography on $AgNO_3$-Kieselgel plates.

Thin-Layer Chromatography. Thin-layer plates 20 cm square and 0.25 mm thick prepared with Kieselgel (Camag, DO-5) were used for checking separation of each fraction in column chromatography, for identifying lipid classes, and for purifying monoglycerides produced by lipase-hydrolysis of triglycerides. The solvent systems employed were hexane-ether-glacial acetic acid (70:30:1) for separation of lipid classes, and ether-methanol-glacial acetic acid (98:2:1) for purification of monoglycerides.

Thin-layer plates impregnated with silver nitrate, prepared by using a slurry composed of 25 g of Kieselgel and 50 ml of 15% aqueous $AgNO_3$ solution were used for analytical and preparative separation of triglycerides, according to the number of double bonds in the triglyceride molecule. The solvent for development consisted of chloroform-methanol (99.2 to 0.8) for low unsaturates and chloroform with 2 to 3% methanol for high unsaturates (Prevett *et al.*, 1965).

Lipids on thin-layer plates were detected as yellowish-brown spots which appeared in the presence of iodine vapor, or as yellow spots under uv light after spraying with 0.05% Rhodamin 6G in ethanol. The latter method was always used for detection of bands in preparative thin-layer chromatography.

Gas-Liquid Chromatography. Fatty acids derived from the glycerides were determined by gas-liquid chromatography. A Varian Aerograph, Series 1700, equipped with hydrogen flame ionization detector was used. Fifteen parts of ethyleneglycoladipate polyester on 100 parts of HMDS-treated Chromosorb W (Johns-Manville, 60–80 mesh) was packed in a glass spiral column 6 ft long and 2 mm i.d. Operating temperatures were 185°, 195°, and 200° C for column oven, injection ports, and detector oven, respectively. A strip chart recorder (Varian Model 20) was used at a chart speed of 0.5 in. per min. Flow rates of carrier gas (N_2), hydrogen, and air were 30, 30, and 250 ml per min, respectively.

The fatty acids were analyzed as their methyl esters which were prepared by treating the glycerides with 0.5 *N* methanolic potash and 14% BF_3-methanol (Metcalfe *et al.*, 1966). Gas chromatograph peak areas were determined by multiplying peak height by peak width at half-height.

Lipase Hydrolysis. Pancreatic lipase was used to hydrolyze the triglycerides to determine distribution of fatty acids in the molecules. About 5 mg of triglyceride mixture was emulsified with bile salts (Bile salts No. 3, Difco Lab, Detroit, Mich.) in

Research Station, Canada Department of Agriculture, Winnipeg 19, Manitoba, Canada.

[1] Present address: National Institute of Agricultural Sciences, Nishigahara, Tokyo, Japan.

Table I. Aggregation Response of the Confused Flour Beetle to Solvent Extracts of Wheat Germ[a]

Extract	Dry matter % of wheat germ	Response index
Hexane extract	9.6	22.3
Acetone extract	0.4	5.7
Methanol extract	12.3	10.0

[a] For assay each extract made to concentration equivalent of 1 g wheat germ/ml was used at rate of 30 μl/disk.

1.0 *M* Tris buffer and incubated for 20 min at 40° C with the lipase steapsin (Nutritional Biochemicals Corp., Cleveland, Ohio) according to the method of Mattson and Volpenhein (1961). Triglycerides were completely hydrolyzed to 2-monoglycerides and free fatty acids within 20 min. Five ml of *ca.* 5 *N* hydrochloric acid and 15 ml of ethanol were added to the mixture after hydrolysis was complete, and free fatty acids and monoglycerides were then extracted with 30 ml of ether. Monoglycerides were separated from free fatty acids by the preparative thin-layer chromatography procedure described.

Synthesis of Triglycerides Microscale syntheses of triglycerides were conducted according to Mattson and Volpenhein (1961). 1-Monopalmitin, 1,3-dipalmitin, 1-palmitoyl-3-oleoyl glycerol, oleoyl chloride, and linoleoyl chloride were of 99+% purity (Analabs, North Haven, Conn.). Twenty-five mg of mono- or diglyceride were dissolved in 1 ml of anhydrous chloroform. About 1.5 mol equivalent of the appropriate acyl chloride and two drops of anhydrous pyridine were added to the solution, and the mixture was allowed to stand for 3 days at room temperature in the dark. The reaction mixture was extracted with 25 ml of ether and washed successively with water, 1% hydrochloric acid, and water. The ether was dried with anhydrous sodium sulfate, and the triglycerides were purified by passing them through a column containing 12 g of Florisil. The developing solvents were hexane-ether (95 to 5), 50 ml, and hexane-ether (85 to 15) 75 ml. The triglyceride was eluted by the latter solvent, and the fractions yielded a single spot on thin-layer chromatograms of Kieselgel impregnated with $AgNO_3$. The net yield of each triglyceride was above 75% of theoretical.

Bioassay. Bioassay methods for aggregation and feeding by the confused flour beetle on various fractions or synthesized compounds were those of Loschiavo (1965a). Most of the samples were applied as hexane solutions on elder pith disks at 30 μl per disk. After drying, one test disk (treated with sample) and one control disk (treated with solvent alone) were placed diametrically opposite each other and about 35 mm apart in a 90 mm Petri dish. The bottom of each dish was filled with paraffin covered with a sheet of Whatman No. 1 filter paper. Two small pins driven through each disk and into the paraffin prevented movement of the disks during a test. Twenty-five beetles of the same age were introduced into the center of each dish, and the whole was placed in the dark at 29 ± 1° C. The number of beetles on each disk was recorded at 15 min intervals during the first hour of the test; the amount of feeding was measured after 24 hr. Each test was conducted in triplicate.

Aggregation activity was expressed as a response index, 100(T-C)/N, similar to that of McEnroe and Dronka (1966), where T and C are total number of beetles on treated and control disks, respectively. N is the number of beetles used per test (25), times the number of observations per test (4), times the number of replications (3). Thus, the index can be simply expressed as (T-C)/3.

RESULTS AND DISCUSSION

Successive extraction of wheat germ with hexane, acetone, and methanol revealed that substances eliciting aggregation of the confused flour beetle are extractable with hexane (Table I). The hexane extract at a concentration equivalent to 1 g of wheat germ per ml elicited a strong aggregation response from the beetles but induced no feeding. The methanol extracts elicited limited aggregation but strong feeding. The acetone extract elicited neither aggregation nor feeding. These results indicated that the major components responsible for aggregation and for feeding are different. A chloroform-methanol (2 to 1) extract of wheat germ prepared according to Folch's method (Folch *et al.*, 1957) for extraction of total lipids from fresh tissues did not evoke aggregation. Purification of this crude extract on a silicic acid column, however, showed that the neutral lipid fraction eluted by chloroform was active. This result suggests the presence of a masking substance derived from wheat germ or produced during the extraction procedures. The free fatty acid fraction, unsaponifiable matter, saponifiable matter of the hexane extract, and the methyl esters prepared from the saponifiable matter were all inactive. Similarly, the polar lipid fraction eluted from a silicic acid column with methanol evoked no aggregation response. These preliminary trials suggested that the substances which stimulate aggregation were neutral lipids with hydrolyzable structures.

The hexane extract was separated into seven fractions by Florisil column chromatography. Seven lipid classes, one in each fraction, were tentatively identified by comparing the R_f values with those of authentic lipids after thin-layer chromatography. The percentage concentration in the hexane extract of each lipid class and their response indices are shown in Table II. The glyceride fraction constituted 80% of the total and was the only fraction that elicited an aggregation response. The triglyceride fraction also contained traces of substances which seemed to be *n*-alcohols, as shown by thin-layer chromatography. Saponification of components in this fraction, however, completely destroyed biological activity, and the separated unsaponifiable fraction was inactive. Triglycerides, therefore, appear to be the active substances in wheat germ that elicit aggregation of the confused flour beetle.

The optimum concentration of triglycerides eliciting aggregation response was 200 mg per ml of hexane, *i.e.*, 6 mg of triglycerides per disk (Table III). Aggregation of the beetles was detected with a triglyceride concentration as low as 10 mg per ml (300 μg/disk). It is noteworthy that hardly any feeding occurred during the standard 24-hr interval at any of the concentrations tested.

The wheat germ triglycerides were further fractionated on columns of silicic acid impregnated with silver nitrate. The fractions eluted with 5 to 15% ether in hexane were combined and are referred to as fraction L (low unsaturates); the others eluted with 20 to 100% ether were combined and referred to as fraction H (high unsaturates). Thin-layer chromatography of fractions L and H on $AgNO_3$-Kieselgel showed four and three spots, respectively. Fractions L and H were then fractionated by preparative thin-layer chromatography on $AgNO_3$-Kieselgel. The four bands of fraction L (L1 to L4) and the three bands of fraction H (H1 to H3) were scraped from the plates, and the triglycerides extracted with ether-methanol (95 to 5). Each extracted triglyceride band yielded a single spot after thin-layer chromatography on $AgNO_3$-Kieselgel, indicating that fractionation was complete. The triglycerides in each fraction were quantitatively determined by measuring fatty acids by gas-liquid chromatography with methyl penta-

decanoate as an internal standard. The triglycerides in each fraction were also hydrolyzed with lipase; the 2-monoglycerides were separated and the esterified fatty acids were quantitatively determined by gas-liquid chromatography. By comparing fatty acid compositions of the intact triglycerides and the 2-monoglycerides, structures of triglycerides in each fraction were determined (Table IV). We found that the major triglycerides of wheat germ comprising 57% of the total are 1-palmito-2-linoleo-3-olein, 1-palmito-2,3-dilinolein, and trilinolein. The structures of the triglycerides in fraction H3, which comprised 11.6% of the total, were not established, but a number of them had more than five double bonds per molecule.

Biological activity was distributed in three fractions, L2, L3, and L4 (Table V) constituting by weight 4.3, 7.8, and 15.9%, respectively, of the total wheat germ triglycerides (Table IV). These three fractions were also mixtures of triglycerides. The major components of L2, L3, and L4 were 1-palmito-2,3-diolein (POO) 94%, 2-linoleo-1,3-dipalmitin (PLP) 85%, and 1-palmito-2-linoleo-3-olein (PLO) 73%, respectively (Table IV).

The three triglycerides, POO, PLP, and PLO tentatively identified as the major components of the active fractions, were synthesized from corresponding mono- or diglycerides, and biological activities of the synthesized triglycerides were examined. As shown in Table VI, synthesized POO, PLP, and PLO elicited strong aggregation of the confused flour beetle at concentrations ranging from 2.5 to 20 mg per ml (75–600 μg/disk). These results showing that naturally-occurring triglycerides elicit aggregation of *T. confusum* confirm those of Starratt and Loschiavo (1971).

The biological activities of the synthesized triglycerides are higher than expected, based on results with the natural wheat germ fractions shown in Table V. For instance, the response index of fraction L3, which contains 85% PLP, is 13.3 at 10 mg per ml (Table V), whereas it is 13.7 for synthesized PLP at 2.5 mg per ml (Table VI). The biological activity of wheat germ triglycerides (Table III) in low concentrations may be explained by the presence of POO, PLP, and PLO. At high concentrations, however, the activity of the triglycerides extracted from wheat germ (Table III) was much lower than that observed with synthesized triglycerides (Table VI). For example, the response index for wheat germ triglycerides was 16.3 at 10 mg per ml, and 39.3 at 100 mg per ml. In contrast, the three active triglycerides, POO, PLO, and PLP, which rep-

Table II. Aggregation Response of the Confused Flour Beetle to Lipids Extracted from Wheat Germ with Hexane

Class	% of Total Extracted Lipid	Response index: 1 g wheat germ equiv./ml	Response index: 100 mg lipid/ml
Hydrocarbons	0.3	−3.0	−7.7[a]
Esters	5.3	4.2	−5.3
Triglycerides	80.0	33.7	39.3
Sterols	2.9	3.3	7.0[b]
Diglycerides	4.5	7.5	2.0
Monoglycerides	0.7	5.7	−6.3
Free fatty acids	6.4	9.6	0.7

[a] 50 mg/ml. [b] 75 mg/ml.

Table III. Effect of Concentration of Wheat Germ Triglycerides on Aggregation Response of the Confused Flour Beetle

Triglyceride concentration mg/ml	Response index
1.0	3.0
3.3	9.0
10.0	16.3
20.0	24.3
33.3	32.7
100.0	39.3
200.0	62.0
500.0	13.7

Table IV. Composition of Triglycerides in Hexane Extract of Wheat Germ

Fraction	Triglyceride	Shorthand Designation	% in Fraction	% of Total Triglycerides
L1	2-Oleo-1,3-dipalmitin	POP	88	1.8
	1-Stearo-2-oleo-3-palmitin	SOP	12	0.2
	2-Oleo-1,3-distearin	SOS		
L2	1-Palmito-2,3-diolein	POO	94	4.0
	1-Stearo-2,3-diolein	SOO	6	0.3
L3	2-Linoleo-1,3-dipalmitin	PLP	85	6.6
	Triolein	OOO	7	0.6
	1-Stearo-2-linoleo-3-palmitin	SLP	8	0.6
	2-Linoleo-1,3-distearin	SLS		
L4	1-Palmito-2-linoleo-3-olein	PLO	73	11.6
	1-Palmito-2-oleo-3-linolein	POL	24	3.8
	1-Stearo-2-linoleo-3-olein	SLO	2	0.4
	1-Stearo-2-oleo-3-linolein	SOL	1	0.2
H1	1-Palmito-2,3-dilinolein	PLL	74	29.3
	1-Oleo-2,3-dilinolein	OLL	15	5.9
	2-Oleo-1,3-dilinolein	LOL	5	3.2
	1-Stearo-2,3-dilinolein	SLL	3	1.2
H2	Trilinolein	LLL	86	16.2
	1-Palmito-2-linoleno-3-olein	PLnO	8	1.5
	1-Palmito-2-oleo-3-linolenin	POLn	6	1.1
H3	Higher unsaturates of more than five double bonds		100	11.6

Table V. Concentrations of Triglyceride Fractions Separated by Silver Nitrate-Impregnated Silicic Acid Chromatography and Aggregation Responses of the Confused Flour Beetle

Fraction (10 mg/ml)	Percent in total	Response index
L1	1.9	−5.3
L2	4.3	18.7
L3	7.8	13.3
L4	15.9	15.7
H1	39.6	1.7
H2	18.8	2.7
H3	11.6	2.7

Table VI. Aggregation Responses of the Confused Flour Beetle to Synthesized Triglycerides

Triglyceride	Response index: 2.5 mg/ml	5.0 mg/ml	10 mg/ml	20 mg/ml
1-Palmito-2,3-diolein (POO)	12.3	29.0	35.0	60.7
1-Palmito-2-linoleo-3-olein (PLO)	21.0	20.3	49.7	57.7
2-Linoleo-1,3-dipalmitin (PLP)	13.7	37.3	56.0	70.0
Mean	15.7	28.9	46.9	62.8

resent 22% of the total wheat germ triglycerides, gave a mean response index of 15.7 at 2.5 mg per ml and 62.8 at 20 mg per ml (Table VI). Thus it appears that the biological activity of a particular triglyceride is greater alone than as part of a mixture. It is possible, therefore, that active triglycerides, other than the three mentioned above occur in fractions L1, H1, H2, and H3.

Triglycerides are major components in the fats and oils of various foods eaten by insects, and can be utilized by most insects for energy. Therefore, the role of triglycerides in stimulating aggregation of the confused flour beetle is of much interest, perhaps comparable to that of carbohydrates as feeding stimulants (Dethier, 1953; Thorsteinson, 1960). A similar phenomenon has been observed with the housefly, *Musca domestica* L., which is attracted to the fruiting bodies of mushrooms belonging to the families Tricholomataceae and Amanitaceae (Muto and Sugawara, 1965). One active principle responsible for this attraction was isolated from *Amanita muscaria* (L.) and identified as 1,3-diolein (Muto *et al.*, 1968).

The bioassay method used in this study did not show whether the triglycerides were attractants or arrestants as defined by Dethier (1960). Modified bioassay methods and detailed observations on the aggregation behavior of the beetles are necessary to elucidate the effect of triglycerides on the feeding behavior of the confused flour beetle.

ACKNOWLEDGMENT

The authors thank Boris Salamon for his technical assistance.

LITERATURE CITED

Carrol, K. K., *J. Lipid Res.* **2,** 135 (1961).
Dethier, V. G., "Chemoreception," In *Insect Physiology*, pp. 568–571, K. Roeder, Ed., Wiley, New York, 1953.
Dethier, V. G., *J. Econ. Entomol.* **53,** 134 (1960).
Folch, J., Lees, M., Sloane-Stanely, G. H., *J. Biol. Chem.* **226,** 497 (1957).
Loschiavo, S. R., *Ann. Entomol. Soc. Amer.* **58,** 383 (1965a).
Loschiavo, S. R., *Ann. Entomol. Soc. Amer.* **58,** 576 (1965b).
Loschiavo, S. R., *Cereal Chem.* **29,** 91 (1952).
Mattson, F. H., Volpenhein, R. A., *J. Lipid Res.* **2,** 58 (1961).
McEnroe, W. D., Dronka, K., *Science* **154,** 782 (1966).
Metcalfe, L. D., Schmitz, A. A., Pelka, J. R., *Anal. Chem.* **38,** 514 (1966).
Muto, T., Sugawara, R., *Agr. Biol. Chem.* **29,** 949 (1965).
Muto, T., Sugawara, R., Mizoguchi, K., *Agr. Biol. Chem.* **32,** 624 (1968).
Prevett, O. S., Blanc, M. L., Codding, D. W., Nickell, E. C., *J. Amer. Oil Chem. Soc.* **42,** 381 (1965).
Smallman, B. N., Loschiavo, S. R., *Cereal Chem.* **29,** 431 (1952).
Starratt, A. N., Loschiavo, S. R., *J. Insect Physiol.* in press (1971).
Thorsteinson, A. J., *Ann. Rev. Entomol.* **5,** 193 (1960).

Received for review September 28, 1970. Accepted November 23, 1970. Contribution No. 450 of the Canada Agriculture Research Station, Winnipeg 19, Manitoba. The first author was supported in this work on a Post-Doctorate Fellowship from the National Research Council of Canada.