cyanate to isothiocyanate or isothiocyanate to thiocyanate occurs within the column so that GLC analysis may not represent the exact ratio of these two forms in a sample. Unfortunately, satisfactory resolution was not obtained when operating the column at a temp lower than 115C. This disadvantage is not serious when essential oils are recovered by the conventional atmospheric steam-distillation technique since they would be driven to an equilibrium mixture before analysis.

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Activation and Specificity of *Crambe abyssinica* Seed Lipase

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Abstract

The lipase of *Crambe abyssinica* seed is not active in crushed seeds stored at 5–7% moisture at room temp. Lipase activity is very low even at 10–15% moisture: free acids in the crambe oil increased 1.6% to a total of 4% in 6 weeks. At higher moisture levels the lipase is active, hydrolyzing the oil in 5–7 weeks. Oil in whole seed is resistant to lipolysis when stored air-dry. These results indicate good stability of the oil during seed storage and the usual steps in seed processing.

Crambe lipase hydrolyzes triglycerides in a nearly random fashion. The hydrolysis pattern indicates a small preference for the shorter chain acids (C_{16} and C_{18}), but no specificity for position within the triglyceride is apparent.

Introduction

RECENT INTEREST IN *Crambe abyssinica* Hochst. ex. R. E. Fries as a potential economic plant depends upon the high erucic acid content of its oil-bearing seed (1,2). For the seed to be economically harvested and stored, the oil must be relatively stable to autolysis while still in the seed. Common oilseeds are stable to storage: the oils in air-dry seeds of flax, mustard and rape are stable for 18 months when stored with normal ventilation (3); the oil in castor beans for 5 years (4).

Oil may be stable in whole seeds, but not in cracked or broken seeds. Broken castor beans contain more than 5% free acid in the oil after 5 years of storage compared to 1% free acid in oil from whole beans (4). A more startling example is provided by the seed of the Indian ironweed (*Vernonia anthelmintica*), which contains an easily activated lipase. Whole seeds survive 1–3 years of storage at 6–7% moisture with no significant change in free acid content of the oil (5,6), whereas the oil from coarsely ground seed, stored air-dry for 26 days, contains approx 40% free acid, or more than double its initial free-acid content (6). These figures were obtained by the usual Soxhlet extraction techniques following a 2- and a 26-day storage period after grinding. More recently an initial fatty acid content of 0.5% has been obtained by rapid extraction of freshly ground seed (7).

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Oil in seed at 7–8% moisture of *Dimorphotheca* and *Lesquerella* is stable for many months at room temp. As in ironweed, the oil in crushed seed is hydrolyzed rapidly: oil in crushed *Dimorphotheca* seed contains nearly 10% free acid after 21 days; that in *Lesquerella* seed ca. 15% after only 7 days (8).

Stability of the oil in *Crambe abyssinica* seed was investigated in both crushed and intact seed. Studies of crushed seed held at various moisture levels were undertaken to determine the limits of oil stability during seed processing. Oil in stored seed was analyzed to provide preliminary data on oil stability expected in intact seed from harvest to time of processing.

Several types of positional specificity of lipases are possible: a) cleavage of the triglycerides may be random, as with the lipase of castor beans (9); b) the 2-position may be preferentially split, as is suggested by the 1,3-divernolin present in partially hydrolyzed vernonia oil (6); or c) the 1- and 3-positions may be more easily cleaved, as shown by pancreatic lipase (10). If crambe lipase resembled pancreatic lipase and produced 2-monoglycerides plus the free acids from the 1- and 3-positions, the enzyme might be used to prepare purified erucic acid since it is known that, in the crambe triglycerides, erucic acid occurs exclusively in the 1- and 3-positions (11). However, it was found that crambe lipase catalyzes a nearly random hydrolysis of triglycerides.

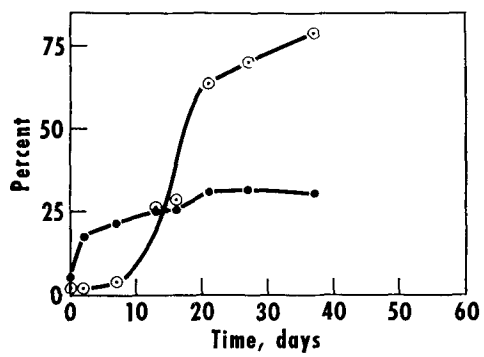

FIG. 1. Lipase activity in flaked crambe seed stored at high-moisture content. ●—● per cent moisture in flakes; ⊙—⊙ per cent hydrolysis of oil.

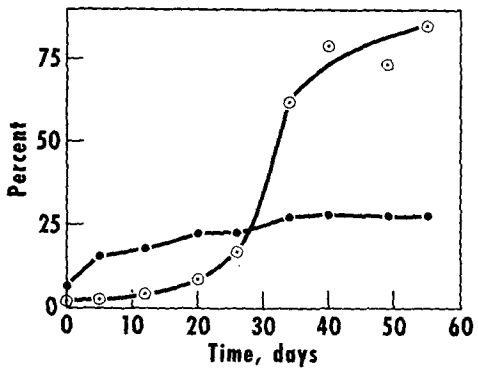

FIG. 2. Lipase activity in flaked crambe seed stored in water-saturated atmosphere. ●——● per cent moisture in flakes; ⊙——⊙ per cent hydrolysis of oil.

Experimental Procedures

Oil Stability in Storage of Flaked Seed. Crambe abyssinica seed was separated from the loosely adherent pericarp by passage through a "Labconco" burr mill followed by aspiration. The seeds were then flaked between smooth rolls set at approx 0.005 in. The flakes were stored in a thin layer (approx 3/8 in. depth) in a desiccator at room temp. The calculated amt of water for the desired moisture level was placed in the bottom of the desiccator; the flakes were uniformly moistened by vacuum infiltration for 48 hr. Before removal of samples, the desiccator was filled with nitrogen and purged with water-saturated nitrogen afterwards.

Moisture content of the flakes was determined by drying overnight at 110C.

At 4–7 day intervals, 0.5–5.0 g of the wet flakes were extracted 6 hr with pentane-hexane according to the AOCS Method Ba 3-38 (12). The oil was dried an hr *in vacuo* at 70C before weighing. The residual meal was further extracted with diethyl ether. In some cases the flakes were extracted only with diethyl ether.

The per cent hydrolysis of the oil samples was determined by titration in alcohol against 0.01*N* base and calculated as per cent methyl ester in triglyceride (number-average mol wt of methyl esters = 328.4). This value is nearly equivalent to per cent free acid in total acids.

Oil Stability in Intact Seed. Immediately upon preparation of flakes from stored seed, the oil was extracted and analyzed for free acid as described above.

Positional Specificity of Lipase. The composition of free acids released by crambe lipase was determined. Crambe seed, flaked as described, was slurried with water to form a thin paste (2½ g flakes plus 25 ml H_2O plus 1 drop 0.05% phenyl mercuric acetate as a preservative) and allowed to incubate at room temp for 0–12 hr. At the end of the incubation, the oil was extracted into ethyl ether (4X) by using centrifugation to separate the phases quickly. After drying, the combined ether extracts were split into two portions:

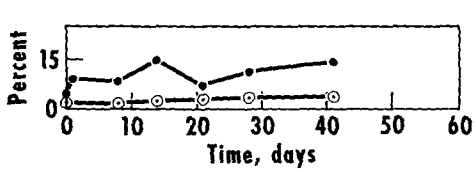

FIG. 3. Lipase activity in flaked crambe seed stored at less than 15% moisture. ●——● per cent moisture in flakes; ⊙——⊙ per cent hydrolysis of oil.

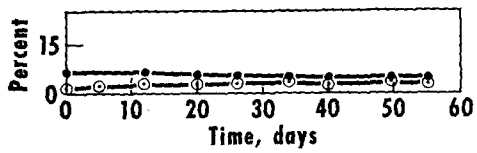

FIG. 4. Lipase activity in flaked crambe seed stored in a dry atmosphere. ●——● per cent moisture in flakes; ⊙——⊙ per cent hydrolysis of oil.

a) for titration of free acids, and b) for separation and identification of free acids.

The liberated fatty acids were separated from the neutral glycerides on Dowex 1-X4 anion-exchange resin by a modification of the procedure devised by Benedict (13). The crambe oil samples containing 0.2–0.4 g free acids were dissolved in 15–25 ml ether-acetone-H_2O (5:5:1) and placed on the ion-exchange column. One hundred fifty ml of the mixed solvent followed by 50 ml 95% ethanol eluted the neutral glycerides from the column. The free acids were eluted with 200–300 ml 10% KOH in 95% ethanol, acidified with HCl, and extracted four times with ether. The ethereal solution was evaporated, and the free acids were weighed. The column was prepared for the next sample by washing with ethanol to neutrality and recycling with HCl after each four samples.

Prior to gas-liquid chromatography (GLC), the acids were converted to methyl esters by refluxing 3 hr with 5% HCl in anhydrous methanol. GLC of isolated methyl esters was on a 20% LAC-2-R 446 2.0-m column at 196C in a Burrell Kromo-Tog Model K-5 equipped with a thermal conductivity detector (14).

Results and Discussion

Lipase Activity in Moist Flakes. At high-moisture contents of crambe flakes, pentane-hexane was not adequate for complete removal of the oil. At 28% mois-

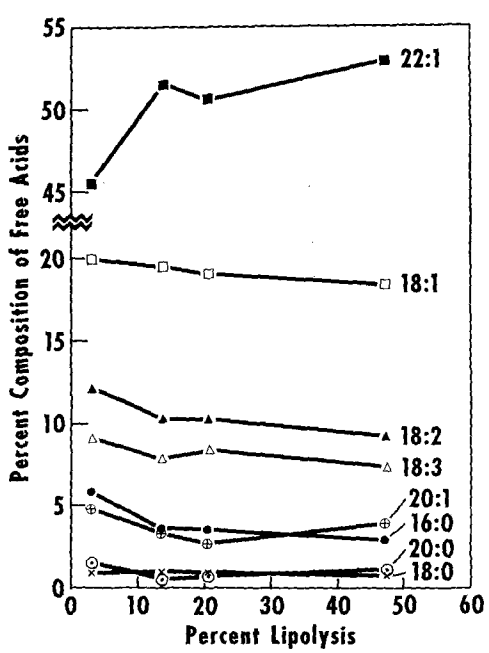

FIG. 5. Composition of free acids released by crambe lipase. Analysis by GLC, per cent methyl esters in total methyl esters. Lipolysis calculated as per cent methyl esters of triglyceride released during experiment. Initial acids in the oil are not included.

TABLE I

Hydrolytic Stability of Crambe Oil Stored in Intact Seed

Lot	Storage condition			Per cent hydrolysis of oil
	Temp	Time, months	Moisture, %	
I, Seed + pericarp	4–5C	36	6.1	1.6
I, Seed + pericarp	Unheated barn	36	4.8	1.7
II, Seed + pericarp	4–5C	36	6.4	1.3
II, Seed + pericarp	Unheated barn	36	5.0	1.4
III, Splits	4–5C	25	5.4	1.2
III, Splits	Unheated barn	25	3.9	1.2
IV, Seed + pericarp	23–27C	Zero	3.7	0.5
IV, Seed + pericarp	23–27C	12	3.7	1.2
V, Cracked seed	23–27C	Zero	....	0.4
V, Cracked seed	23–27C	12	....	1.2

ture, 35.8% of the seed (dry basis) could be extracted with pentane-hexane; an additional 5.2% of the seed (dry basis) could be extracted by subsequent treatment with diethyl ether. It was established that diethyl ether alone extracts as much oil as the two solvents combined. The results reported here represent the combined pentane-hexane and ether extracts, or the ether extract alone.

Figure 1 shows the autolysis of crambe oil when flaked seed is stored under nitrogen at a high-moisture content; the moisture level reaches 30% in ca. 3 weeks. In that time the oil is rapidly hydrolyzed; 50% hydrolysis occurs in approx 17 days.

Figure 2 illustrates autolysis of crambe oil when flakes are stored in a nitrogen atmosphere saturated with water. No vacuum infiltration was employed in this case; the moisture content of the flakes is somewhat lower than in Figure 1. Hydrolysis occurs as before but is delayed; 50% hydrolysis is not reached until 31 days.

At these high-moisture levels mold spoilage readily occurs in an air atmosphere. For this reason nitrogen was substituted. Growth of mold on the flakes can occur at 15% moisture in an atmosphere in which oxygen is not rigidly excluded.

If the moisture content of the flakes is held at 10–15%, hydrolysis of the oil is not appreciable (Fig. 3); nor does it occur if air-dry flakes are stored without additional moisture (Fig. 4). Intact seeds or "splits" and broken seeds may be stored for long periods without accumulation of free acids in the oil. Oil from seed stored 3 years in an unheated barn was less than 2% hydrolyzed (Table I).

Positional Specificity of Crambe Lipase. The composition of fatty acids of completely saponified crambe oil and the composition of the free acids present in the air-dry seed before lipolysis shows in Table II.

The procedure used for separation of the released fatty acids from the remaining mono-, di-, and triglycerides gave good recoveries of acid compared to the titration of free acids (Table III). Recovery of free acid was not calculated at the 46% free-acid level. The possibility of errors caused by fatty acid exchange on the ion-exchange column has been raised by McCarthy et al. (15). The magnitude of such possible errors in this study may be estimated by comparing two methods of analysis. In the first, whole oil is saponified with alkali and converted to methyl esters without using the ion-exchange column. In the second, the oil is saponified with alkali and run through the ion-exchange column before preparation of methyl esters. The GLC analyses of the total methyl esters by these two methods do not differ by more than 1% (Table II).

TABLE II

GLC Analysis of Fatty Methyl Esters Prepared from Various Crambe Oils

Fatty acid	Per cent composition as methyl esters		
	Free acids in original oil[a]	Saponified oil through ion exchange	Saponified oil, no ion exchange
<16	4.5	1.2	0.3
16:0	11.1	2.5	2.1
16:1	1.0	0.3	0.3
18:0	2.2	0.6	0.7
18:1	20.1	17.4	18.2
18:2	13.2	8.9	8.7
18:3	5.1	6.9	6.9
20:0	1.7	0.7	0.5
20:1	4.7	3.5	3.7
22:0	2.6	0.3	0.7
22:1	33.6	55.4	56.4
22:2	<0.1	1.2	0.6
24:0	<0.1	0.6	0.2
24:1	<0.1	0.5	0.7

[a] Oil contained 2.4% free acid.

TABLE III

Recovery by Ion Exhange of Free Acids from Crambe Oil

Free acid in oil by titration (calculated as free acid, MW = 314.4)	Wt oil added to ion exchange	Expected free acid	Found, free acid from column	Recovery of free acids (wt from column cf. titration)
%	g	g	g	%
2.40	2.6575	0.0638	0.0660	103.4
4.98	1.3650	0.0680	0.0691	101.6
15.01	0.7680	0.1153	0.1175	101.9
21.22	0.7625	0.1618	0.1508	93.2

Because it is not known whether the acids initially present are due to lipolysis or whether they are fatty acids which have never been esterified, they have been corrected for. All subsequent free-acid values have been corrected for the contribution of the 2.4% initial free acids present (Table II).

The free acids rapidly approch the composition of the total oil; that is, the lipase splits triglycerides in a nearly random fashion (Fig. 5). The pattern does suggest a small preference for short-chain acids, these appearing in relatively greater abundance early in lipolysis. This abundance is not a positional specificity for the 2-position. In such a case $C_{16:0}$, which is esterified in the 1,3-position (11), should not be relatively abundant early in lipolysis.

The lipase of dormant *Crambe abyssinica* seed thus differs from the positional specificity shown by pancreatic lipase, which preferentially cleaves the moieties esterified on the 1,3-position (10). The lipase also lacks the apparent preference for the β ester that is evidenced by the lipase of *Vernonia anthelmintica* seed (6). It resembles castor bean lipase in that neither appears to possess positional specificity (9).

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