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Preparation of Esters Resembling Natural Waxes by Lipase-Catalyzed Reactions

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Esters composed of long chain (C_{16}, C_{18}) and/or very long chain ($\geq C_{20}$) acyl and alkyl moieties, which resemble some naturally occurring waxes of commercial importance, are prepared conveniently in high yield by alcoholysis or esterification reactions catalyzed by lipases. Triacylglycerols of seed oils containing large proportions of very long chain (*n*–9)-(*Z*)-monounsaturated acyl moieties (20:1, 22:1, 24:1) are subjected to lipase-catalyzed alcoholysis with suitable mixtures of long chain and very long chain (*n*–9)-(*Z*)-monounsaturated alcohols (18:1–24:1) to yield esters resembling the waxes from jojoba (*Simmondsia chinensis*) or orange roughy (*Hoplostethus atlanticus*), for example. Similar products are also obtained by lipase-catalyzed esterification of suitable mixtures of (*n*–9)-(*Z*)-monounsaturated fatty acids (18:1–24:1) with those of the corresponding alcohols.

Lipase-catalyzed reactions such as hydrolysis, esterification, and interesterification of lipids have received considerable attention during the past few years in view of their potential biotechnological applications in oils, fats, and oleochemicals industries (Macrae, 1984). In a recent study from this laboratory, it was shown that lipase-catalyzed alcoholysis of triacylglycerols with long chain alcohols yielding wax esters is by far the fastest of the interesterification reactions such as those with fatty acids, methyl esters, triacylglycerols, or glycerol (Schuch and Mukherjee, 1987a,b).

In the present paper we report applications of lipase-catalyzed alcoholysis and esterification reactions in the preparation of esters resembling naturally occurring waxes of commercial interest. The starting materials used in this study are triacylglycerols from seed oils of white mustard, *Sinapis alba* (Mukherjee and Kiewitt, 1984), and honesty, *Lunaria annua* (Mukherjee and Kiewitt, 1986), which are rich in very long chain (*n*–9)-(*Z*)-monounsaturated acyl moieties (gadoleoyl, 20:1; erucoyl, 22:1; nervonoyl, 24:1).

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Such very long chain acyl moieties are major constituents of wax esters of jojoba (*Simmondsia chinensis*) or orange roughy (*Hoplostethus atlanticus*), for example. The alkyl moieties of such wax esters are derived from very long chain (*n*–9)-(*Z*)-monounsaturated alcohols, which, in turn, are conveniently prepared by hydrogenolysis of the above triacylglycerols.

EXPERIMENTAL SECTION

Materials. Triacylglycerols used in various reactions were isolated as described earlier from seeds of *S. alba* (Mukherjee and Kiewitt, 1984) and *L. annua* (Mukherjee and Kiewitt, 1986). The compositions of the major acyl moieties (designated by number of carbon atoms:number of *Z* double bonds) of the triacylglycerols of *S. alba* and *L. annua*, respectively, were as follows: 16:0, 2.5 and 1.0; 16:1, 0.2 and 0.1; 18:0, 0.9 and 0.1; 18:1, 21.4 and 22.6; 18:2, 10.0 and 4.6; 18:3, 8.6 and 0.6; 20:0, 0.7 and 0.1; 20:1, 9.8 and 0.7; 22:0, 0.4 and <0.1; 22:1, 42.5 and 47.1; 24:0, <0.1 and 0.1; 24:1, 2.8 and 24.1. Long chain and very long chain alcohols and other lipids used as standards for chromatography were purchased from Nu-Chek-Prep, Elysian, MN. All reagents and adsorbents were from E. Merck AG, 6100 Darmstadt, Federal Republic of Germany. Column packings for gas chromatography (GC) were supplied by Applied Science Laboratories Inc., State College, PA.

Mixtures of long chain and very long chain fatty acids were obtained by hydrolysis of the triacylglycerols from *S. alba* and *L. annua* according to established procedures (Kates, 1964). Similarly, mixtures of long chain and very long chain alcohols were obtained from the above triacylglycerols by hydrogenolysis (Brown, 1951).

The following immobilized lipase preparations were used: Lipozyme (Novo Industrie GmbH, 6500 Mainz, Federal Republic of Germany) is an *sn*-1,3-specific triacylglycerol lipase from *Mucor miehei* having the activity of 25 batch interesterification units (BIU)/g; 1 BIU corresponds to 1 μmol of palmitic acid incorporated into trioleoylglycerol/min from an equimolar mixture at 40 °C. Lipase G (Amano Pharmaceutical Co. Ltd., Nagoya, Japan), obtained from a selected strain belonging to a *Penicillium* species, preferentially hydrolyzes mono- and diacylglycerols compared to triacylglycerols. The activity of this preparation was given as 55 000 units/g at pH 5.6 with vinyl laurate as substrate.

Lipase-Catalyzed Reactions. Unless otherwise stated, triacylglycerols (1.0 mmol each) from *S. alba* or *L. annua* were subjected to alcoholysis with 2 mmol each of long chain or very long chain alcohols or their mixtures under magnetic stirring in screw-capped tubes at a temperature of 60 °C. Lipase preparations were used in amounts of 1–10% of the weight of the reaction partners.

Lipase-catalyzed esterification reactions were carried out, unless otherwise stated, by reacting 1.0 mmol each of fatty acids or their mixtures, derived from the triacylglycerols by hydrolysis, with 1.0 mmol each of long chain or very long chain alcohols or their mixtures, obtained from the triacylglycerols by hydrogenolysis. Reaction conditions chosen were similar to those used for the alcoholysis reactions given above.

In both alcoholysis and esterification, the reaction products were withdrawn at definite periods and dissolved in hexane, if required, and the immobilized lipase preparations separated by centrifugation.

Analytical Procedures. The reaction products were fractionated by thin-layer chromatography (TLC) on silica gel H into lipid classes: i.e. monoacylglycerols, alcohols plus diacylglycerols, unesterified fatty acids, triacylglycerols, and wax esters. The plates were first developed twice with diethyl ether up to 2 cm and then with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) up to 19 cm. The lipid fractions were recovered by scraping, and all the fractions except the wax esters were converted to methyl esters (Chalvardjian, 1964). Methyl heptadecanoate was added as internal standard to each fraction and the relative proportion of each lipid class determined by GC (Christie et al., 1970). GC of the methyl esters and of the methyl esters plus alcohols (derived from the fraction consisting of alcohols and diacylglycerols) was carried out, as described earlier, on Silar 5CP on Gas-Chrom Q (80–100 mesh) (Schuch and Mukherjee, 1987a). The fraction containing wax esters was eluted with water-saturated diethyl ether, mixed with a known aliquot of methyl heptadecanoate as internal standard, and separated according to carbon numbers by GC on columns containing OV-1 on Gas-Chrom Q (100–120 mesh) as described previously (Schuch and Mukherjee, 1987a). Thus, both the relative proportion of the wax esters in reaction products and their composition could be determined.

Table I. Formation of Wax Esters by Alcoholysis of Triacylglycerols of *L. annua* with Various Alcohols Using Lipozyme

alcohol	lipase, % w/w lipids	water, μL	wax esters formed, μmol·mg^{-1} lipase: 20 min	40 min	1 h	2 h	4 h	6 h
16:0	10	25				10.4	9.8	11.1
18:0	10	25				9.1	9.7	7.9
18:1	10	25				10.4	10.1	9.3
22:1	10	25				7.5	9.3	9.3
16:0	10	50	1.3	2.5	4.8			
16:0	10	none		4.4	5.6	9.8		
16:0	1	50				2.9		
16:0	3	50				5.3		
16:0	10	none				7.6		
16:0	10	20				3.3		

RESULTS AND DISCUSSION

Table I shows the effect of various reaction parameters on the extent of formation of wax esters by alcoholysis of triacylglycerols of *L. annua* with various alcohols with Lipozyme as the biocatalyst. The amount of wax esters formed at 60 °C increases with time up to about 2 h, after which a plateau is reached between 4 and 6 h of reaction. The extent of alcoholysis of the triacylglycerols with the individual alcohols is quite similar. The data given in Table I show furthermore that the extent of formation of wax esters increases with increasing amounts of Lipozyme. Moreover, the amount of wax esters formed is distinctly higher when no water is added to the reaction mixture than in the presence of water.

Table II shows the composition of acyl moieties of the wax esters formed by alcoholysis of the triacylglycerols of *L. annua* with various alcohols. These data exhibit for the wax esters formed with hexadecanol, octadecanol, and (*n*–9)-(*Z*)-octadecenol a close resemblance in the composition of the acyl moieties with those of the triacylglycerols of *L. annua*, indicating that the individual molecular species of the triacylglycerols are utilized to similar extent in the alcoholysis reaction. Composition of the acyl moieties of the wax esters formed with (*n*–9)-(*Z*)-docosenol as compared to the acyl moieties of the triacylglycerols of *L. annua* shows, however, a somewhat preferential utilization in the alcoholysis reaction of triacylglycerols containing C_{18} acyl moieties as compared to those containing C_{22} and C_{24} acyl moieties.

Table III shows the extent of formation of wax esters by esterification of oleic acid with (*n*–9)-(*Z*)-octadecenol

Table II. Composition of Acyl Moieties of Wax Esters Formed by Alcoholysis of Triacylglycerols of *L. annua* with Various Alcohols

	composition (%, w/w) of acyl moieties of				
	wax esters formed with different alcohols[a]				triacylglycerols
acyl moieties	16:0	18:0	18:1	22:1	of *L. annua*
C_{16}	2.2	1.8	1.6	2.0	1.1
C_{18}	28.0	27.8	29.1	34.1	27.9
C_{20}	1.5	0.7	0.8	0.5	0.8
C_{22}	46.2	46.9	46.3	42.8	47.1
C_{24}	22.2	22.9	22.0	20.7	23.1

[a] Alcoholysis was carried out with Lipozyme (10% w/w of lipids) at 60 °C in the presence of water (25 μL) for 6 h as given in Table I.

Table III. Esterification of Oleic Acid with (*Z*)-9-Octadecenol Using Lipases

		wax esters formed, μmol·mg^{-1} lipase		
lipase[a]	water, μL	1 h	2 h	4 h
Lipozyme	15	9.7	12.3	14.4
Lipase G	15	0.1	0.1	0.5
Lipozyme	none		15.0	
Lipozyme	10		13.2	
Lipozyme	50		4.0	
Lipase G	none		0.3	
Lipase G	10		0.2	
Lipase G	50		0.1	

[a] Reactions were carried out with 10% w/w of lipids of the lipase preparations at 60 °C for Lipozyme and 50 °C for Lipase G.

using two different lipase preparations, i.e. Lipozyme and Lipase G. Lipozyme gives higher rates of esterification as compared to Lipase G. The extent of formation of wax esters using Lipozyme approaches a plateau between 2 and 4 h of reaction. Moreover, the data given in Table III show that the addition of water to the reaction mixture distinctly lowers the rate of formation of wax esters as also observed in the alcoholysis reaction (Table I). In general, the rates of formation of wax esters by esterification are higher (Table III) than those observed in alcoholysis reactions (Table I).

With the basic information on the effects of reaction parameters on the formation of wax esters by alcoholysis and esterification (Table I–III), these reactions were applied for the preparation of wax esters resembling commercially important waxes.

Table IV shows the extent of formation of wax esters resembling natural waxes by alcoholysis or esterification catalyzed by Lipozyme. Triacylglycerols from naturally occurring oilseeds, such as *L. annua* and *S. alba*, served as starting materials, from which the mixture of alcohols or fatty acids was prepared by hydrogenolysis or hydrolysis, respectively. The data given in Table IV show that alcoholysis yields distinctly higher amounts of wax esters from triacylglycerols of *L. annua* by reacting with alcohols from *L. annua* or *S. alba* than from the triacylglycerols of *S. alba* with the corresponding alcohols. Analysis of wax esters, fractionated according to carbon numbers, shows close resemblance between all the wax esters obtained by alcoholysis, with the exception of those prepared from triacylglycerols and alcohols from *S. alba* (Table IV).

The data given in Table IV show, in agreement with the results presented in Tables I and III, that esterification of fatty acids with alcohols yields much higher proportions of wax esters than alcoholysis of triacylglycerols with alcohols. Moreover, esterification of fatty acids of *L. annua* with alcohols from *L. annua* yields higher amounts of wax esters than the other esterification reactions (Table IV). Composition of the wax esters formed in various esteri-

Table IV. Formation of Wax Esters Resembling Natural Waxes by Alcoholysis or Esterification Catalyzed by Lipozyme[a]

reaction		wax esters formed, μmol·mg^{-1} lipase	composition (%, w/w) of wax esters								
partner	alcohol		C_{32}	C_{34}	C_{36}	C_{38}	C_{40}	C_{42}	C_{44}	C_{46}	C_{48}
Alcoholysis of Triacylglycerols											
L. annua	*L. annua*	9.1		1.1	13.3	1.7	28.2	13.7	20.4	18.0	3.5
L. annua	*S. alba*	9.0		1.8	14.2	4.7	26.4	14.3	21.3	13.9	3.3
S. alba	*S. alba*	4.0	0.4	4.6	18.6	13.9	31.9	11.8	13.0	5.7	
S. alba	*L. annua*	4.4	0.8	2.7	12.3	9.2	31.7	14.6	18.5	10.2	
Esterification of Fatty Acids											
L. annua	*L. annua*	14.3		0.8	8.0	1.4	25.7	17.9	26.3	17.7	2.2
L. annua	*S. alba*	11.1		1.9	9.6	5.6	28.2	15.6	24.7	14.2	0.3
S. alba	*S. alba*	10.8	0.5	3.6	18.3	11.9	35.2	11.4	16.8	2.3	
S. alba	*L. annua*	9.9	0.7	2.8	13.9	6.4	31.6	14.8	19.1	10.7	
jojoba (*S. chinensis*)				0.1	1.4	6.1	28.2	50.7	11.8	1.7	
orange roughy (*H. atlanticus*)[b]			2.1	11.4	16.7	24.8	23.4	14.8	5.5	1.1	

[a] All reactions were carried out for 4 h with Lipozyme (10% w/w of lipids) with 0.5 mmol each of the reaction partners at 60 °C without water. [b] Data from Buisson (1983).

Table V. Composition of Lipid Classes in Products Formed by Alcoholysis or Esterification Catalyzed by Lipozyme[a]

reaction		composition (%, w/w) of lipid classes					
partner	alcohol	monoacylglycerols	diacylglycerols	alcohols	fatty acids	triacylglycerols	wax esters
Alcoholysis of Triacylglycerols							
L. annua	*L. annua*	1.4	4.4	7.6	0.9	4.6	81.2
L. annua	*S. alba*	1.4	7.4	5.5	1.3	6.6	77.8
S. alba	*S. alba*	2.9	15.1	5.8	4.6	11.1	60.5
S. alba	*L. annua*	2.1	11.9	4.3	4.4	12.5	64.8
Esterification of Fatty Acids							
L. annua	*L. annua*	0.1	0.2	0.5	1.9	0.0	97.2
L. annua	*S. alba*	0.3	0.8	1.3	2.2	0.0	95.4
S. alba	*S. alba*	0.1	1.1	1.0	7.5	0.0	90.5
S. alba	*L. Annua*	0.2	0.2	2.1	1.4	0.0	96.1

[a] Reactions were carried out for 4 h with Lipozyme (10% w/w of lipids) with 0.5 mmol each of the reaction partners at 60 °C without water.

fication reactions is quite similar with the exception of those prepared by esterification of fatty acids with alcohols, both from *S. alba* (Table IV).

It can also be seen from the data given in Table IV that the wax esters prepared by alcoholysis or esterification contain an array of homologues in which the esters having carbon numbers C_{36}, C_{40}, C_{42}, C_{44}, and C_{46} predominate. Composition of some of these wax ester mixtures, especially of those prepared from triacylglycerols of *S. alba* as the only starting material, is intermediary between the composition of wax esters from jojoba and orange roughy (Table IV). A striking feature of the wax esters prepared from triacylglycerols of *L. annua* as one of the starting materials lies in the presence of substantial proportions of C_{46} esters in such products; in contrast, most natural waxes of commercial interest contain very little esters having 46 or more carbon atoms (Table IV).

Finally, Table V shows the composition of lipid classes in the products obtained by alcoholysis and esterification reactions. The wax esters constitute about 80% and 65%, respectively, of the products formed by alcoholysis from triacylglycerols of *L. annua* or *S. alba*. These products contain, in addition to unreacted triacylglycerols and alcohols, the monoacylglycerols, diacylglycerols, and fatty acids, which are formed by hydrolysis of triacylglycerols (Table V).

In contrast to alcoholysis, esterification of fatty acids from both *L. annua* and *S. alba* with the corresponding alcohols yields products almost entirely composed of wax esters (Table V). These products contain, in addition to minor proportions of unreacted fatty acids and alcohols, small amounts of monoacylglycerols and diacylglycerols present as contaminants in the fatty acids used as reaction partners.

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Definition of Functional and Antibody-Binding Sites on Kunitz Soybean Trypsin Inhibitor Isoforms Using Monoclonal Antibodies

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The interaction of monoclonal antibodies with the three Kunitz trypsin inhibitor isoforms designated Ti^a, Ti^b, and Ti^c was studied by ELISA. Antigenic differences among the isoforms and their complexes with bovine trypsin were observed. Some antibodies were selective for one or two of the isoforms, but others bound comparably to all three isoforms. All but one of the antibodies were able to bind equivalently to Ti^a and its complex with trypsin. The results of this study define six epitopes, one of which is either blocked or altered when trypsin binds to the inhibitor. A combination of antibodies can be used to determine which isoforms of Kunitz trypsin inhibitor are present in a sample and whether the inhibitor reactive site is free or occupied. Consideration of the isoform composition of soy products would improve the accuracy of assays for Kunitz trypsin inhibitor in foods. These immunochemical tools could also be used to study the developmental and environmental regulation of KTI expression and its function in the plant.

Kunitz trypsin inhibitor (KTI) from soybeans was the first plant protease inhibitor to be extensively characterized. Its structure and mechanism of action [reviewed by Laskowski and Kato (1980)] and its significance in human and animal health and nutrition [reviewed by Rackis and Gumbmann (1981)] have been extensively studied. We have previously prepared monoclonal antibodies that bind to different epitopes on the Kunitz trypsin inhibitor of soybeans (Brandon et al., 1986, and 1987) and have developed an enzyme-linked immunosorbent assay (ELISA) for KTI in soy products, including infant formulas (Brandon et al., 1988). There are three closely related

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