Rapid Synthesis of Fatty Acid Esters for Use as Potential Food Flavors

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ABSTRACT: Lipase-catalyzed esterification has been performed in hexane to generate novel mixtures of fatty acid esters from specially chosen combinations of fatty acids and alcohols. By varying the alcohol and enzyme compositions in the enzymatic reactions, different ester mixtures were produced, which were further purified and analyzed for ester composition by gas chromatography–mass spectrometry. Depending on the combination of alcohols and enzymes used, the final ester mixture exhibited significant compositional variation. These mixtures could be manipulated at the synthesis step, thereby enabling a high degree of product control. Such manipulation over enzyme-catalyzed ester synthesis in mixtures may be useful in the preparation of ester flavors for use in the food industry.
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KEY WORDS: Flavor ester synthesis, GC–MS analysis, lipase-catalyzed esterification, rapid synthesis and identification, *sn*-1 and *sn*-3 milk fatty acids.

The rapid growth of the flavor and fragrance industry has fueled demand for the generation of new flavors from synthetic or natural sources (1). Esters are common flavoring agents in, for example, fruit-flavored products (e.g., beverages, candies, jellies, and jams), baked goods, wines, and diary products (e.g., cultured butter, sour cream, yogurt, and cheese) (2). Ester extraction from natural sources has therefore become particularly appealing. However, ester isolation from these sources is often expensive and time consuming. Ester flavorings may alternatively be synthesized enzymatically utilizing inexpensive natural raw materials such as fatty acids and alcohols.

The use of enzymes for producing flavor esters is well-known (3–5), with synthesis usually directed to individual ester products. While this methodology is suitable for simple flavors, complex food materials often contain mixtures of flavor esters whose content and composition impart the desired flavor characteristics. Such flavor complexity is evident in many dairy products as a result of the milk fermentation process (2,6). Unfortunately, the complicated nature of the fermentation process precludes the consistent production of certain desirable flavors.

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In the present study, we describe a general methodology that can be used to generate fatty acid ester mixtures of controlled number and composition through the use of enzymatic catalysis in organic solvents. Specific emphasis is placed on the rapid generation and identification of ester mixtures that mimic flavor profiles found in dairy products; however, this approach may also find application for other food products.

EXPERIMENTAL PROCEDURES

Materials. Reagent-grade chemicals were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI) while food-grade chemicals (including alcohols) were purchased from Aldrich Flavors & Fragrances (Milwaukee, WI). Hexane was dried over 3Å molecular sieves for at least 3 h prior to use. Food-approved lipases were obtained from commercial suppliers. The lipases were divided into three categories, each of which was used as a catalytic mixture. Category I consisted of the microbial lipases R10 (*Penicillium roqueforti*) and AY30 (*Candida rugosa*) from Amano Enzyme (Lombard, IL), and lipase 5000 (*Aspergillus niger*) from Enzyme Development Corp. (New York, NY). Category II consisted of the mammalian lipases Italase C (calf) and Capalase K (kid) from SBI (Waukesha, WI). Category III consisted of Lipozyme 10,000 L (*Mucor miehei*) and Palatase M 1000 L (*Rhizomucor miehei*) from Novo Nordisk A/S (Bagsvaerd, Denmark). Lipozyme and Palatase (which are liquid preparations) were dialyzed in 20-mM bis-Tris propane buffer (pH 7.0), ultrafiltered in 20-mM sodium phosphate buffer (pH 7.0), and lyophilized before use. Category I and II enzymes were used as received.

Gas chromatography–mass spectrometry (GC–MS) analysis. GC–MS (Shimadzu GC Model 17A, MS Model QP-5000, Columbia, MD) was used for compositional analysis and to check the purity of the ester mixtures prepared. The gas chromatograph–mass spectrometer was equipped with a 25-m × 0.32 mm × 0.52 μm film thickness HP-5 column (Hewlett-Packard, Wilmington, DE); the carrier gas was He flowing at 2.6 mL per min following a 20-fold split ratio. Elution was performed with an initial oven temperature of 100°C for 1 min followed by a temperature increase to 300°C at a rate of 10°C per min terminating with a 1 min hold at 300°C. The detector and injector temperatures were 300°C. Identification of the es-

TABLE 1
Alcohols Employed in This Study Along with Their Organoleptic Properties

#	Alcohols[a]	Organoleptic properties[b]	Conversion after 20 h (%)[c]
1	3-Methyl-2-buten-1-ol	Fresh, fruity, green, lavender	66
2	4-Hexen-1-ol		53
3	Geraniol	Sweet, floral, rose, fruity	84
4	3-Heptanol	Herbaceous	12
5	1-Carveol	Caraway, spearmint	10
6	1*R*-Myrtenol	Camphoraceous, minty	94
7	3-Methyl-1-pentanol	Pungent, wine-like, cocoa	87
8	Farnesol	Delicate, floral, oily	84
9	Benzyl alcohol	Sharp, burning taste	47
10	1-Propanol	Alcohol, sweet	31
11	1-Butanol		64
12	3-Hexanol	Alcoholic, ethereal, medicinal	17
13	dl-3-Methyl-2-butanol		16
14	*trans*-2-Hexen-1-ol	Leafy, green, wine-like, fruity	58
15	dl-Menthol	Minty, woody	21
16	Fenchyl alcohol	Lemon	0
17	Para-anisyl alcohol	Floral, mild, sweet	0
18	Vanillyl alcohol	Balsamic, sweet	0
19	Cinnamyl alcohol	Sweet, balsamic, hyacinth	0
20	Propylene glycol	Virtually odorless	0
21	*cis*-3-Hexen-1-ol	Fresh, green grass	27
22	Nonyl alcohol	Rose, citrus	93
23	3,7-Dimethyl-1-octanol	Sweet, rose	68
24	α-Methylbenzyl alcohol	Mild hyacinth	35
25	Phenethyl alcohol	Rose, honey, fragrant, floral	64
26	3-Phenyl-1-propanol	Sweet, balsamic, floral	62
27	2-Ethyl-1-hexanol	Mild, oily, sweet, slight rose	90
28	1-Octanol	Sharp, fatty, waxy, citrus	81
29	S-Perillyl alcohol	Green, pungent, fatty	39
30	Amyl alcohol	Strong, somewhat sweet, balsamic	78
31	1-Hexanol		56
32	2-Octanol	Fatty, oily, earthy	11
33	2,6-Dimethyl-4-heptanol		20
34	1-Penten-3-ol	Butter, mild green	0
35	Furfuryl alcohol	Low odor, cooked sugar taste	17

[a]All alcohols have an associated FEMA number (Flavor and Extract Manufacturers' Association of the United States) and are commercially available in food-grade.
[b]Organoleptic properties are from the Aldrich Flavors & Fragrances (Milwaukee, WI) catalog.
[c]Conversion after 20 h was calculated based on the disappearance of octanoic acid. Enzyme category I was used in this reactivity screen. All the reactions were performed in hexane except alcohol numbers 17–20, which were performed in acetone due to poor solubility of these alcohols in hexane.

ters by GC–MS enabled rapid compositional analysis of the product mixtures.

Screen for alcohol reactivity. Alcohol reactivity was measured initially using octanoic acid as a representative fatty acid (Table 1). The esterification mixture contained 10 mM octanoic acid, 20 mM of a given alcohol, and 2 mg/mL of each category I enzyme suspended in hexane (2 mL), except for reactions involving alcohols 17–20 (Table 1), which were performed in acetone due to the limited solubility of these alcohols in hexane. Reactions were initiated by reagent addition to the enzyme suspension, followed by incubation at 30°C and orbital shaking at 250 rpm. Conversions after 20 h were measured by GC.

Ester production. Preparative ester synthesis used a synthetic mixture of fatty acids (Table 2) at a total fatty acid concentration of 1 M together with an equimolar alcohol mixture at 0.5 M total concentration. The reactions were initiated by addition of reagents to the hexane-suspended enzyme (categories I and III, 60 mg/mL of each enzyme in the mixture; and category II, 300 mg/mL of each enzyme in the mixture), yielding a total volume of 20 mL. The twofold excess of fatty acids to alcohols facilitated ester purification by promoting high conversion of the alcohol substrates. The enzymatic esterification reaction was terminated after 4 d and the enzymes were removed by centrifugation. The supernatant was analyzed by GC–MS for fatty acid, alcohol, and ester composition. Esters were purified from the residual fatty acids and alcohols by silica gel flash chromatography [40 μm/60 Å silica gels (J. T. Baker, Phillipburg, NJ) with a mobile phase of 20% (vol/vol) ethyl acetate in hexane].

RESULTS AND DISCUSSION

Short-chain fatty acids (C_4–C_{12}) typically play a significant role in determining the flavors of dairy products, while long-

TABLE 2
Synthetic Fatty Acid Mixture Used to Mimic Natural Composition Found in Milk Fat Triacylglycerols[a]

Fatty acid	FA in *sn*-1 + *sn*-3 positions on milk fat triacylglycerols (wt%)	Relative amount of FA for 5-component mixture[b] (wt%)
4:0 (Butyric)	3.3	34.0
6:0 (Caproic)	1.5	15.5
8:0 (Caprylic)	1.1	11.3
10:0 (Capric)	2.2	22.7
12:0 (Lauric)	1.6	16.5
Total	9.7	100.0

[a]See Reference 7.
[b]Relative amount of fatty acid (FA) = (100/9.7) × (% FA in *sn*-1 + *sn*-3 positions)

chain fatty acids contribute little to the flavor (6). During fermentation of milk, free fatty acids are continuously produced by the hydrolysis of milk fat triacylglycerols, mostly at the *sn*-1 and *sn*-3 positions (7). As fermentation proceeds, the released fatty acids undergo esterification, yielding the characteristic flavor esters of dairy products. We therefore prepared a synthetic fatty acid mixture according to Christie (7), based on the natural composition of fatty acids at the *sn*-1 and *sn*-3 positions of milk fat triacylglycerols (Table 2). A total of 35 alcohols were selected for this study based on their organoleptic properties. This combination of fatty acids and alcohols should lead to the synthesis of 175 unique esters. Individual synthesis of all these esters, and subsequent blending to prepare mixtures of varied composition, would represent a time-intensive and laborious task. Alternatively, direct generation of ester mixtures allowed for accelerated development.

Prior to the production of ester mixtures, it was first necessary to ascertain the reactivity of the selected alcohols and eliminate those for which our enzymes exhibited particularly low activity. To that end, octanoic acid was used as a representative fatty acid, along with the mixture of enzymes from enzyme category I. This category was chosen because its enzymes exhibited the broadest reactivity against a subset of 10 structurally diverse alcohols (data not shown). The yield of octanoic esters ranged from zero to 94% following 20 h of incubation in hexane or acetone (which was used for alcohols 17–20). Six of the 35 alcohols were unreactive (Table 1), thereby demonstrating that lipase mixture I had broad alcohol selectivity for ester synthesis in hexane. The least reactive alcohols were branched and/or secondary, which are known to be relatively poor substrates for lipases (8). The reactive alcohols were then grouped into six representative categories based on their organoleptic properties: fruity (category A: alcohols 1, 2, 3), minty or herbaceous (B: 4, 5, 6), mixed (C: 7, 8, 9), sweet or green (D: 21, 22, 23), floral (E: 24, 25, 26), and oily or fatty (F: 27, 28, 29). These six alcohol categories represented the full spectrum of organoleptic properties listed in Table 1.

Esterification of the synthetic fatty acid mixture was performed using the three categories of enzyme and the six mixtures of alcohols to give 18 separate reaction mixtures. Following the enzymatic reaction, the resulting ester mixtures were purified from unreacted fatty acids and alcohols. Figure 1 shows the GC ester profile for the reaction of alcohol group F with the fatty acid mixture using enzyme categories I–III. The relative reactivity values are only approximate and are based on peak amplitudes, with the maximum ester peak representing 100%

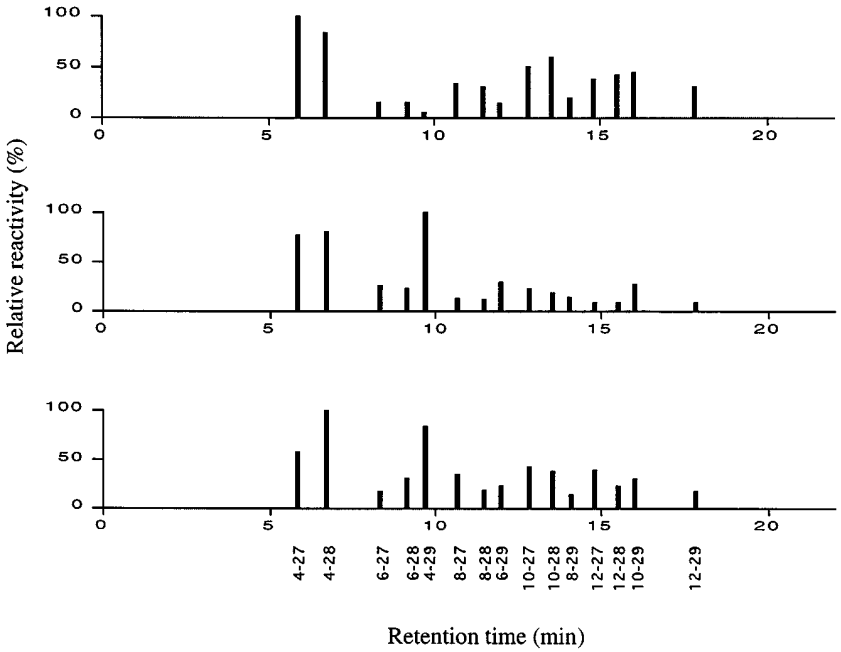

FIG. 1. Compositional analysis (gas chromatographic ester profiles) of purified ester mixtures resulting from alcohol category F and the three enzyme categories: I (top), II (middle), and III (bottom). The two-number code identifies each ester (e.g., 4–27 is the 2-ethyl-1-hexyl ester of butyric acid). In all cases, the total alcohol conversion was >90% and the reported relative reactivity is normalized such that the most abundant ester product equals 100%.

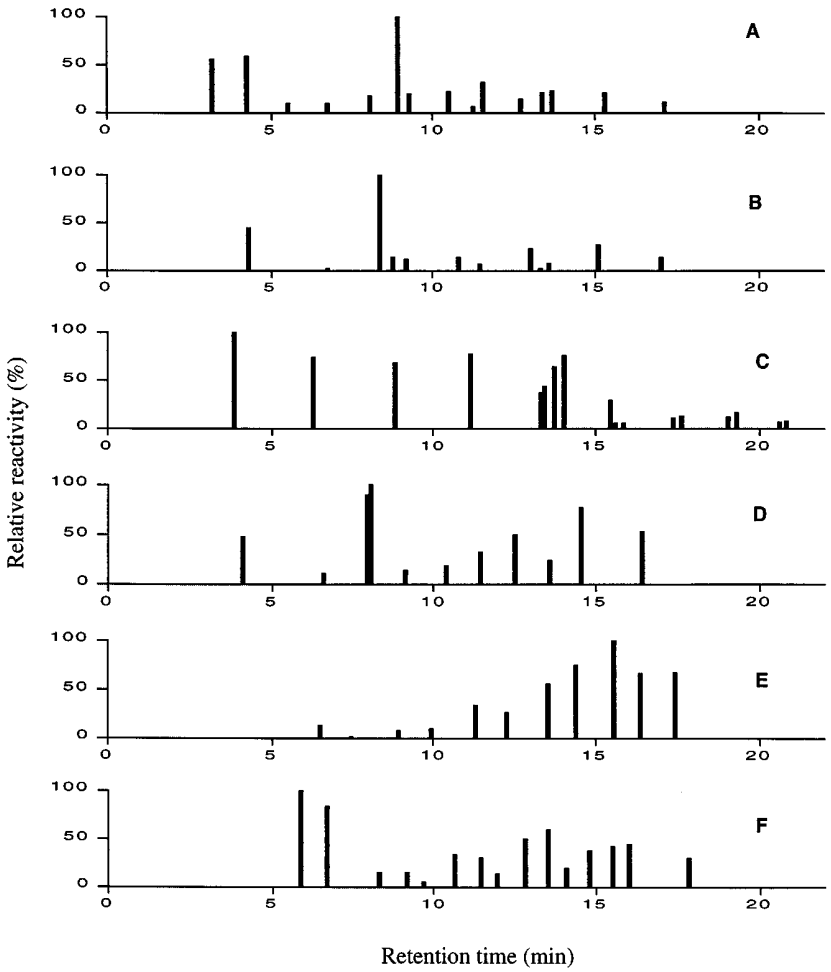

FIG. 2. Compositional analysis (GC ester profiles) of products from the combination of enzyme category I and the six alcohol categories (A to F, from top to bottom). In all cases, the total alcohol conversion was >90% and the reported relative reactivity is normalized such that the most abundant ester product equals 100%. See Figure 1 for abbreviation.

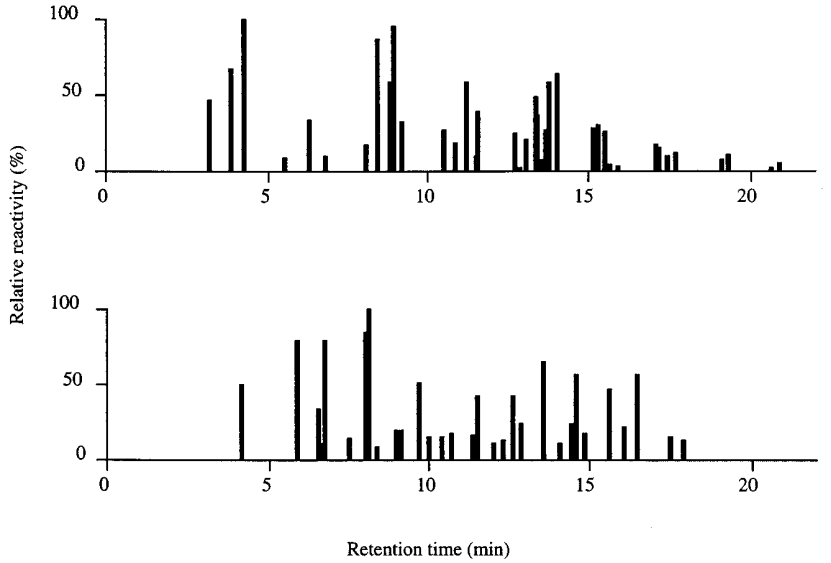

FIG. 3. Compositional analysis (GC ester profiles) of purified ester mixtures resulting from enzyme category I and each combined alcohol category: AC (top, alcohol categories A, B, and C), and DF (bottom, alcohol categories D, E, and F). Relative reactivity is normalized with respect to the most abundant ester product. For abbreviation see Figure 1.

reactivity in each case. Each ester is identified by an x-y designation, where x is the fatty acid chain length and y is the alcohol number from Table 1. The identification of individual esters was done by GC–MS. These representative results demonstrate the compositional variation introduced by the different enzyme mixtures used. Similarly, Figure 2 shows six ester mixtures obtained from the reactions of acid mixture with the six categories of alcohols using enzyme category I. Both figures provide fingerprints of ester syntheses, denoting both identification of the esters in a given mixture and the relative abundance of each ester. Hence, depending on the combination of alcohols and enzymes, the final ester mixture can show significant compositional variation that may ultimately lead to differences in flavor.

This rapid approach to generate potential dairy flavors was further used to increase the size of the synthetic mixtures. For example, we regrouped the 18 alcohols into two groups: group AC which contained alcohol categories A, B, and C; and group DF which contained alcohol categories D, E, and F. As shown in Figure 3, the combination of enzyme category I and each of the two new groups of alcohols (AC and DF) produced very different ester mixtures compared to those highlighted in Figure 1. Thus, not only were larger ester mixtures synthesized but also the relative abundance of each ester in the mixture was altered.

The results presented in this work demonstrate that different combinations of enzymes, alcohols, and fatty acids can be used to generate diverse mixtures of esters. These mixtures can be manipulated at the synthesis step, thus enabling a high degree of product control. Enzyme-catalyzed synthesis of ester mixtures may therefore prove useful in the preparation of ester flavors in dairy products, as well as other products in which complex mixtures of esters determine flavor.

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