Introduction

The hydrolysis of acyl moieties of triacylglycerols catalyzed by lipases (triacylglycerol acylhydrolases EC 3.1.1.3) may be reversed under certain conditions to yield a variety of esters from glycerol and fatty acids [1]. This possibility has opened a new avenue for biotechnology in the oils and fats industries. One of the reactions that can be carried out by lipase catalysis, with certain advantages over the chemical catalysis, is the interesterification of triacylglycerols. Chemically catalyzed interesterification leads to a randomization in the distribution of acyl moieties in the triacylglycerol molecules, whereas interesterification catalyzed by an sn-1,3 specific lipase ensures the exchange of acyl moieties exclusively in the sn-1 and sn-3 positions, giving rise to products with characteristics that are different from those obtained by chemical interesterification [2]. Lipases have been immobilized on inorganic supports, such as kieselguhr [3, 4], which permits their use in interesterification carried out in continuous flow reactors.

We report here radiochemical methods for monitoring and assaying lipase-catalyzed interesterification of lipids using $^{14}$C-labeled substrates. Using these methods the performance of an immobilized microbial lipase was evaluated. The enzyme employed is an sn-1,3 specific lipase from *Mucor miehei*, supported on a macroporous anion exchange resin (Lipozyme). Triacylglycerols of uchuhua (*Virola surinamensis*) seed, which are mostly composed of medium chain (lauroyl and myristoyl) acyl moieties, were interesterified in hexane at equal molar ratios with methyl [1-$^{14}$C]oleate, [1-$^{14}$C]oleic acid, [carboxyl-$^{14}$C]trioleoylglycerol, [1-$^{14}$C]octadecenyl alcohol, and [U-$^{14}$C]glycerol, each of known specific activity. The reactions were monitored and the rate of interesterification determined by radio thin layer chromatography from the incorporation of radioactivity into acyl moieties of triacylglycerols (from methyl oleate, oleic acid, and trioleoylglycerol), alkyl moieties of wax esters (from octadecenyl alcohol), and into glycerol backbone of monoacylglycerols and diacylglycerols (from glycerol).

Experimental

Materials

Medium chain (C12 plus C14) triacylglycerols were isolated from uchuhua (*Virola surinamensis*) seeds, which were collected in the Amazonic region of Brazil. The composition of the acyl moieties of these triacylglycerols was: lauroyl (12:0) = 14.3%, myristoyl (14:0) = 71.5%, myristoleoyl (14:1) = 2.2%, palmitoyl (16:0) = 5.5%, stearoyl (18:0) = 0.7%, oleoyl (18:1) = 4.2%, and linoleoyl (18:2) = 0.8%. Methyl oleate, oleic acid, glycerol, adsorbents for TLC, and analytical grade reagents were from E. Merck AG, D-6100 Darmstadt. Trioleoylglycerol and octadecenyl alcohol were from Sigma Chemie GmbH, D-8024 Deisenhofen.
Methyl \[1^{14}\text{C}]\text{oleate} was prepared by esterification of \[1^{14}\text{C}]\text{oleic acid}, 59.7 \mu\text{Ci}/\mu\text{mol} (Amersham Buchler, D-3300 Braunschweig), with diazomethane [5]. \[\text{carboxyl-}\text{1}^{14}\text{C}]\text{Trioleoylglycerol}, 55 \mu\text{Ci}/\mu\text{mol}, was from CEA, Gif-sur-yvette, France. \[1^{14}\text{C}]\text{Octadecenyl alcohol} was prepared by reduction of methyl \[1^{14}\text{C}]\text{oleate}, 59.7 \mu\text{Ci}/\mu\text{mol}, with \text{LiAlH}_4 [6]. \[\text{U-}\text{1}^{14}\text{C}]\text{Glycerol}, 152 \mu\text{Ci}/\mu\text{mol}, was from NEN Chemicals, D-6072 Dreieich. Radioactively labeled substances were purified by TLC if required.

Immobilized lipase from \textit{Mucor miehei} (Lipozyme) was a product of Novo Industrie GmbH, D-6500 Mainz.

**Interesterification reactions**

Medium chain triacylglycerols (0.5 mmol) and 0.5 mmol of the reaction partner (methyl oleate, oleic acid, trioleoylglycerol or octadecenyl alcohol) were dissolved in 5 ml hexane, to which were added known amounts (1–2 \mu\text{Ci}) of methyl \[1^{14}\text{C}]\text{oleate}, \[1^{14}\text{C}]\text{oleic acid}, \[\text{carboxyl-}\text{1}^{14}\text{C}]\text{trioleoylglycerol} or \[1^{14}\text{C}]\text{octadecenyl alcohol}, respectively. The solution was then saturated with water at 45 °C. Alternatively, the medium chain triacylglycerols, 0.5 mmol, dissolved in 5 ml hexane, were saturated with water at 45 °C and to this solution was added 0.5 mmol glycerol containing a known amount (2 \mu\text{Ci}) \[\text{U-}\text{1}^{14}\text{C}]\text{glycerol}. Lipozyme (10% of the weight of the reactants) was added to each reaction mixture, which was stirred at 45 °C. Samples were withdrawn from the reaction mixture after 2, 4, and 8 h, water (2 drops) was added, and the aqueous phase containing Lipozyme was removed by centrifugation.

**Analysis of reaction products**

The products of interesterification of medium chain triacylglycerols with each of the following isotopically labeled reaction partners were analyzed as described below.

Methyl \[1^{14}\text{C}]\text{oleate}

The products were fractionated by TLC on Silica Gel H as follows. The thin layer chromatograms were first developed twice with diethyl ether up to a height of 2 cm, then up to 19 cm with hexane : diethyl ether (90:10), and finally up to 19 cm with hexane : diethyl ether : acetic acid (70:30:1). Thin layer chromatograms were assayed by means of a Berthold TLC-Scanner LB 2760 (BF-Vertriebsgesellschaft, D-7547 Wildbad). Alternatively, the lipids were stained with iodine vapor. The fractions corresponding to methyl esters, triacylglycerols, unesterified fatty acids, diacylglycerols, and monoacylglycerols were scraped off, and the radioactivity present in each fraction was determined in a Packard Tri-Carb C 2425 liquid scintillation spectrometer (Packard Instruments Company, Inc., Downers Grove, Ill., USA). A mixture (1:1) of Aquasol-2 (NEN Chemicals GmbH) and Toluene Scintillator (Packard Instruments Company, Inc.) was used as scintillation solution. The amount of methyl oleate, that was exchanged against the medium chain acyl moieties of the triacylglycerols was calculated from the total radioactivity due to \[1^{14}\text{C}]\text{oleoyl moieties incorporated into the triacylglycerols.}

In addition, triacylglycerols were isolated from the reaction mixture by TLC and the positional distribution of labeled acyl moieties was determined by hydrolysis with pancreatic lipase [7] and radio-TLC on Silica Gel H impregnated with boric acid [8].

\[1^{14}\text{C}]\text{Oleic acid}

The products of interesterification were fractionated by TLC, the fractions of unesterified fatty acids, triacylglycerols, diacylglycerols, and monoacylglycerols were scraped off, and the radioactivity in each of the fractions was measured as described above. The amount of oleic acid, that was exchanged against the acyl moieties of the medium chain triacylglycerols, was calculated from the total radioactivity due to \[1^{14}\text{C}]\text{oleoyl moieties incorporated into the triacylglycerol fraction.}

\[\text{Carboxyl-}\text{1}^{14}\text{C}]\text{Trioleoylglycerol}

The triacylglycerols in the interesterification products were fractionated by argentation-TLC on Silica Gel G containing 20% (w/w) silver nitrate. The thin layer chromatograms were developed twice with hexane : diethyl ether : methanol (80:20:0.1). The fractions corresponding to triacylglycerols containing none, 1, 2, and 3 olefinic bonds per molecule were detected under UV light after spraying with 0.2% (w/v) ethanolic 2,7-dichlorofluorescein. The chromatograms were scanned as described, the fractions were scraped off, and the radioactivity present in each fraction was measured using Toluene Scintillator. The amount of trioleoylglycerol interesterified with the medium chain triacylglycerols was calculated from the total radioactivity due to \[1^{14}\text{C}]\text{oleoyl moieties incorporated into the triacylglycerol fraction.}
moieties incorporated into the fractions corresponding to triacylglycerols containing 1 and 2 double bonds per molecule.

\([1-^{14}C]\)Octadecenyl alcohol

The products formed by interesterification were fractionated by TLC on Silica Gel H with hexane:diethyl ether:acetic acid (80:20:1). Thin layer chromatograms were scanned and the fractions of wax esters, long chain alcohol, and those of triacylglycerols, diacylglycerols, and monoacylglycerols, taken together, were scraped off and the radioactivity was determined by liquid scintillation as described above. The amount of octadecenyl alcohol, to which the acyl moieties of medium chain triacylglycerols were transferred, was calculated from the radioactivity due to \([1-^{14}C]\)octadecenyl moieties in the wax ester fraction.

\([U-^{14}C]\)Glycerol

The samples were diluted to a known volume with hexane and radioactivity was directly measured in an aliquot. The amount of glycerol, to which the medium chain acyl moieties of triacylglycerols were transferred, was calculated from the total radioactivity in the aliquots due to the \([U-^{14}C]\)glycerol backbone in monoacylglycerols and diacylglycerols. In addition, the products of interesterification were fractionated by TLC as described for methyl \([1-^{14}C]\)oleate: triacylglycerols, diacylglycerols, and monoacylglycerols were scraped off and the radioactivity in each of these fractions was determined.

**Results and Discussion**

Interesterification reactions of the medium chain triacylglycerols, containing 90% lauroyl and myristoyl moieties, with isotopically labeled reaction partners were carried out in hexane using an sn-1,3 specific lipase from *Mucor miehei*, immobilized on macroporous anion exchange resin. The reactions involved and the expected products are shown in Scheme 1 in a simplified form.

The interesterification of medium chain triacylglycerols with methyl \([1-^{14}C]\)oleate should result in

\[\text{Scheme 1. Simplified scheme of interesterification reactions.}\]
the transfer of $[1^{-14}C]$oleoyl moieties to $sn$-1 and $sn$-3 positions of triacylglycerols and concomitant formation of unlabeled methyl laurate and methyl myristate (Scheme 1). The radio scans of a thin layer chromatogram of the products of interesterification after various reaction periods are shown in Fig. 1. It can be observed that the radioactivity initially present in the methyl ester fraction is gradually transferred to the triacylglycerol fraction. The scans given in Fig. 1 show that radioactivity is also present in the fractions corresponding to unesterified fatty acids and diacylglycerols, which is explained by hydrolysis of the labeled triacylglycerols formed, hydrolysis of labeled methyl oleate, and interesterification of labeled diacylglycerols formed by hydrolysis of triacylglycerols. The rates of interesterification, calculated from the radioactivity due to incorporation of $[1^{-14}C]$oleoyl moieties from methyl oleate are plotted in Fig. 2. The positional specificity of the interesterification reaction is evidenced by pancreatic lipase-catalyzed hydrolysis of the triacylglycerols isolated from the reaction mixture after 2 h of interesterification and analysis of the hydrolysis products by TLC. In the products of hydrolysis most of the radioactivity ($> 90\%$) was found to be located in $sn$-1,2(2,3)-diacylglycerols and fatty acids liberated from $sn$-1 and $sn$-3 positions of triacylglycerols, whereas the $sn$-2-monoacylglycerols contained very little radioactivity.

The lipase-catalyzed interesterification of the medium chain triacylglycerols with $[1^{-14}C]$oleic acid should result in the transfer of $[1^{-14}C]$oleoyl moieties to $sn$-1 and $sn$-3 positions of triacylglycerols and concomitant formation of lauric and myristic acids, as outlined in Scheme 1. The rate of this interesterification, calculated from the incorporation of radioactivity into triacylglycerols, is found to be similar to that of the reaction with methyl $[1^{-14}C]$oleate, as shown in Fig. 2. It can also be observed that the reaction reaches an equilibrium after 4 h. The positional specificity of this interesterification is also evidenced as described above for methyl $[1^{-14}C]$oleate.

Interesterification of triacylglycerols containing saturated medium chain acyl moieties with $[\text{carboxyl-}1^{-14}C]$. 

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**Fig. 1.** Scan of a thin layer chromatogram showing the distribution of radioactivity in the products formed during the interesterification of medium chain triacylglycerols (0.5 mmol) with methyl oleate (0.5 mmol) containing 1 μCi methyl $[1^{-14}C]$oleate. A = Diacylglycerols; B = unesterified fatty acids; C = triacylglycerols; D = methyl esters.

**Fig. 2.** Rates of interesterification of medium chain triacylglycerols with methyl $[1^{-14}C]$oleate (●), $[1^{-14}C]$oleic acid (□), $[\text{carboxyl-}1^{-14}C]$trioleoylglycerol (○), $[1^{-14}C]$octadecenyl alcohol (■), and [U-1$^{14}$C]glycerol (▲).
\(^{14}\text{C}\)trioleoylglycerol should result in an exchange of \(^{14}\text{C}\)oleoyl moieties at the sn-1 and sn-3 positions of the triacylglycerols. Consequently, isotopically labeled triacylglycerols containing 1 and 2 olefinic bonds per molecule, not present in the initial mixture, are formed, as shown in Table I. The rate of this interesterification reaction, determined from the radioactivity due to incorporation of \(^{14}\text{C}\)oleoyl moieties into triacylglycerols containing 1 and 2 olefinic bonds per molecule, is found to be similar to those of the reactions with methyl \(^{14}\text{C}\)oleate and \(^{14}\text{C}\)oleic acid (Fig. 2). The positional specificity of this interesterification is also evidenced by pancreatic lipase-catalyzed hydrolysis of the molecular species of triacylglycerols containing 1 and 2 double bonds, as described above for methyl \(^{14}\text{C}\)oleate.

Table I. Composition (%) of molecular species of \(^{14}\text{C}\)-labeled triacylglycerols formed during interesterification of medium chain triacylglycerols with \([\text{carboxyl-}^{14}\text{C}]\)trioleoylglycerol.

<table>
<thead>
<tr>
<th>Time of reaction (h)</th>
<th>Molecular species of (^{14}\text{C})-labeled triacylglycerols fractionated according to number of double bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 0 0 100</td>
</tr>
<tr>
<td>2</td>
<td>traces 16 32 52</td>
</tr>
<tr>
<td>4</td>
<td>traces 19 41 40</td>
</tr>
<tr>
<td>8</td>
<td>traces 20 44 36</td>
</tr>
</tbody>
</table>

As outlined in Scheme 1, interesterification of medium chain triacylglycerols with \([1-^{14}\text{C}]\)octadecenyl alcohol, catalyzed by the sn-1,3 specific lipase from \(M. miehei\), should give rise to \([1-^{14}\text{C}]\)octadecenyl laurate and \([1-^{14}\text{C}]\)octadecenyl myristate. Concomitantly, nonradioactive sn-1,2-(2,3)-diacylglycerols and sn-2-monoacylglycerols should be formed. The radio scans of a thin layer chromatogram of the products of interesterification (Fig. 3) show a gradual transfer of radioactivity from the long chain alcohol fraction to the wax esters, that are not present in the initial mixture. The rate of this interesterification, determined from the incorporation of radioactivity due to \([1-^{14}\text{C}]\)octadecenyl moieties from octadecenyl alcohol into the wax esters, is found to be the highest among all the reactions investigated (Fig. 2). Similar to other reactions, an equilibrium is reached after 4 h of interesterification. Positional specificity of this interesterification has been established in separate experiments by comparing the composition of the acyl moieties of wax esters formed with that of the acyl moieties at the sn-1-(3)-positions of the medium chain triacylglycerols (J. Agric. Food Chem., in press).

Interesterification of medium chain triacylglycerols with \([U-^{14}\text{C}]\)glycerol, catalyzed by the sn-1,3 specific lipase from \(M. miehei\), yields a mixture of \(^{14}\text{C}\)-labeled sn-1-(3)-monoacylglycerols and sn-1,3-diacylglycerols and unlabeled sn-1,2-(2,3)-diacylglycerols as well as sn-2-monoacylglycerols. Radio TLC of the products on layers of Silica Gel H containing boric acid [8] confirmed the presence of radioactivity almost exclusively in sn-1,3-diacylglycerols and sn-1-(3)-monoacylglycerols, which shows that the transfer of acyl moieties occurs specifically to the sn-1 and sn-3 positions of glycerol. The rate of interesterification, determined from the radioactivity due to the \([U-^{14}\text{C}]\)glycerol backbone in monoacylglycerols and diacylglycerols, is found to be the lowest of all the reactions investigated (Fig. 2).

Fig. 3. Scan of a thin layer chromatogram showing the distribution of radioactivity in the products formed during the interesterification of medium chain triacylglycerols (0.5 mmol) with octadecenyl alcohol (0.5 mmol) containing 2 nCi \([1-^{14}\text{C}]\)octadecenyl alcohol. A = Long chain alcohol; B = wax esters.
The radiochemical methods described in the present study should be widely applicable to the assay of lipase-catalyzed reactions for biotechnological processes, such as interesterification, esterification, and hydrolysis of lipids. The methods involving TLC in conjunction with gas chromatography as well as those using high performance liquid chromatography are generally used for assay of the rates and products of interesterification reactions. Although these methods enable a detailed characterization of the products of interesterification as compared to radiochemical methods described here, the latter, according to our experience are quite simple, rapid, and accurate techniques for determining the overall rates of interesterification. A major advantage of the radiochemical methods is the accuracy in the determination of positional specificities of lipase-catalyzed reactions.

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