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Monitoring of lipase-catalyzed cleavage of acylglycerols by high-temperature gas chromatography

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Abstract

High-temperature gas chromatography was applied to assay the activity and selectivity of lipases, and to test various reaction conditions. This technique is capable to analyze mixtures of fatty acids and acylglycerols simultaneously and, therefore, to monitor the hydrolysis of acylglycerols. Lipases can catalyze the hydrolysis of acylglycerols in aqueous solution. The reaction conditions employed are milder than those of conventional chemical hydrolysis. In practice, this method was used in the sample preparation procedure for the quantitative analysis of γ-linolenic acid in triacylglycerols.

1. Introduction

Modification of fats and oils is a special area of enzyme technology which can be used for analytical purposes. Esters can be synthesized, rearranged and hydrolyzed by lipase enzymes (E.C. 3.1.1.3). Sometimes it is sufficient to observe the appearance or disappearance of free fatty acids (FAs) in order to monitor the course of these reactions. However, if complex mixtures have to be transformed, it is advisable to profile the complete lipid pattern. Unwanted or changed substrate specificities of the enzymes could cause problems in the case of non-quantitative transformations. The results of the experiments are strongly influenced by the way reactions are carried out.

Various analytical methods have been developed to follow, e.g., the lipase-catalyzed hydrolysis of wax esters [1], esterification and interesterification of lipids by TLC [2], hydrolysis of triacylglycerols by HPLC [3,4], enantioselective transesterification with allylic alcohols [5] and interesterification of cholesteryl esters by capillary GC [6]. Zhang and Wainer [7] developed an enzyme reactor with immobilized lipase coupled on-line to an HPLC-column. Berg et al. [8] also used an immobilized lipase for the on-line supercritical fluid extraction–supercritical fluid chromatography approach.

In our investigations, high-temperature GC was used to monitor the hydrolysis of acylglycerols by lipases. Control of the conversion rates was carried out by analysis of FAs and acylglycerols in a single GC run following methylation of the acids by diazomethane.

According to the IUPAC method [9] FA methyl esters of $(n-3)$ and $(n-6)$ oils are prepared by alkali-catalyzed transesterification of the triacylglycerols with methanol. The deficiencies of this method are the following: (i) free FAs are saponified but not esterified, and will not be detected in the subsequent analysis; (ii) reaction conditions for the transesterification

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need to be anhydrous because water would lead to saponification and, therefore, to a loss in recovery of FAs; (iii) the basic reaction conditions applied may result in isomerisation of the double bonds of unsaturated FAs [10,11]. Other ways to produce FA methyl esters, including the boron trifluoride method which is also accepted as a standard procedure [12,13], are discussed by Shantha and Napolitano [10].

For quantitative determination of γ-linolenic acid (γLn) in triacylglycerols of borage oil and a corresponding water-in-oil emulsion (produced as a galenical preparation) the lipase from *Candida cyclindracea* was applied in the sample preparation procedure. By using standard GC equipment and commercially available capillary columns, this method is applicable in routine laboratories. The method demonstrates its benefits especially in the analysis of samples containing water.

2. Experimental

2.1. Chemicals

Buffer components

Sodium hydroxide (0.1 *M*) and 0.1 *M* hydrochloric acid, Titrisol, were obtained from Merck (Darmstadt, Germany); calcium chloride ($CaCl_2$) and disodium EDTA (purum) and Tris (BioChemika) from Fluka (Buchs, Switzerland); deionized water from own deionization plant.

Derivatizing reagents

1-Methyl-1-nitroso-3-nitroguanidine, 97%, was obtained from Aldrich (Steinheim, Germany); N-methyl-N-nitroso-*p*-toluenesulfonamide (Diazald) from Merck; N-nitroso-N-methylurea synthesized according to [14]. Ethereal diazomethane was prepared from these reagents (see Section 3.2).

Enzymes

Lipase (E.C. 3.1.1.3) type VII (from *Candida cyclindracea*) was obtained from Sigma (Deisenhofen, Germany); lipase from *Candida cyclindracea*, 22%, lipase from *Candida cyclindracea* My, 13%, and lipase from *Candida cyclindracea*, immobilised on Gulsenit, from Chemie Linz (Linz, Austria).

Reference substances

Triheptadecanoin, triolein and γLn, 99%, were obtained from Sigma; heptadecanoic acid, 99%, from Fluka.

Solvents

Hexane and diethyl ether, analytical-reagent grade, were obtained from Loba (Fischamend, Austria).

2.2. Sample preparation

To the lipid sample [10 mg of the water-in-oil emulsion or 6 mg of the borage oil; for the preparation of stock solutions the emulsion is dissolved in dichloromethane–hexane–methanol (2:2:1), the oil as well as the I.S. in *n*-hexane] and the I.S. (1.5 mg of triheptadecanoin) were added the lipase (approximately 2 mg), *n*-hexane (0.1 ml), and the buffer (1 ml of a 10 m*M* Tris buffer —adjusted to a pH of 7.5 with Titrisol— containing 40 m*M* $CaCl_2$ and 1 m*M* EDTA). The mixture was stirred over night in a closed vial under nitrogen atmosphere by a magnetic stirrer at 37 ± 1°C. The solution was acidified with hydrochloric acid (5%, w/w) prior to the extraction of the free FAs with *n*-hexane (three times with 2 ml each) by shaking vigorously on a vortex shaker. Phase separation and settling of suspended matter were accelerated by centrifugation. The combined extracts were concentrated to a small volume (about 10 μl) under a flow of nitrogen gas at ambient temperature.

The samples were derivatized by freshly prepared or distilled ethereal solution of diazomethane at room temperature and 1 h reaction time. Diethyl ether and non-reacted diazomethane were evaporated under a flow of nitrogen gas. The lipids were then dissolved in *n*-hexane (5 ml) and ready for GC analysis.

2.3. Chromatographic conditions

The gas chromatograph used was an HRGC 5300 Mega series (Fisons Instruments, Milan, Italy) equipped with a flame ionization detector and optional split- or cold-on-column injection. Data acquisition was done by a PE Nelson Interface 900 series (Perkin-Elmer Nelson Systems, Cupertino, CA, USA), and injection of 1-μl sample with a 10-μl syringe.

Screening procedure

A Pyrex glass capillary column, 25 m × 0.32 mm I.D., was coated (0.15 μm film thickness) with OV-1701 OH (7% cyanopropyl-, 7% phenyl- and 86% methylpolysiloxane) [15]. The injection mode employed was cold-on-column and the inlet pressure of hydrogen, the carrier gas, 100 kPa. The oven temperature was programmed from 70 to 170°C at 25°C/min, then from 170 to 380°C at 10°C/min, and isothermal at 380°C for 15 min. The temperature of the detector was 380°C.

Quantification procedure

A fused-silica capillary column (J & W, Folsom, CA, USA), 20 m × 0.32 mm I.D., with a DB-1 phase (100% methylpolysiloxane, 0.1 μm film thickness) and cold-on-column injection was used. The inlet pressure of hydrogen was 50 kPa and the temperature programmed from 70 to 140°C at 25°C/min, from 140 to 200°C at 7°C/min, then from 200 to 260°C at 30°C/min, and isothermal at 260°C for 5 min. The temperature of the detector was held at 280°C.

3. Results and discussion

3.1. Optimization of reaction conditions

Lipase-catalyzed hydrolysis of acylglycerols in aqueous medium followed by esterification with diazomethane is advantageous in comparison to conventional sample preparation procedures, especially for the cleavage of triacylglycerols present in water-in-oil emulsions. The free FAs are not lost by this procedure. Reaction conditions are mild and the water content of the sample does not reduce the recoveries of FAs.

The buffer compositions proposed in the literature for lipase catalysis and tested for our application were: 25 m*M* borate (adjusted to pH 8.4) containing 33% methanol, 10 m*M* and 100 m*M* phosphate (adjusted to pH 7.0), and 10 m*M* Tris (adjusted to pH 8.0 and 7.45, respectively) [16]. These buffer systems have been used in combination with different lipase preparations for the hydrolysis of a mixture of triacylglycerols with triolein as a major constituent. Borate buffer containing methanol has been most promising because the reaction products, glycerol and FAs, are removed from the equilibrium. Borate complexes glycerol and methyl alcohol should react with FAs by lipase catalysis to the corresponding methyl esters. In a single high-temperature GC run we separated the acylglycerols from the FAs and determined the conversion rates. Our experiments showed that triacylglycerols were not hydrolyzed quantitatively when borate buffer containing methanol was used. Instead, partial acylglycerols and soaps have been formed in considerable amounts.

The best results we obtained with the Tris buffer (pH 7.45) and lipase from *Candida cyclindracea* (type VII and My). Hydrolysis of triacylglycerols was quantitative (Fig. 1). $CaCl_2$ and EDTA were added to the Tris buffer for the following reasons [16]: the calcium ions in the buffer protect the enzyme against product inhibition. According to the literature calcium may be considered as a co-reactant leading to calcium soaps. In order to prevent losses of free FAs by soap formation, the aqueous solution was acidified (pH 4) with hydrochloric acid prior to extraction with *n*-hexane. EDTA has been added to the buffer as a masking reagent that complexes heavy metal ions which catalyze autoxidation reactions. The cleavage of the triacylglycerols was performed additionally under nitrogen atmosphere.

The lipase from *Candida cyclindracea* shows no discrimination of the 1- and 2-position of triacylglycerols. Its specificity towards different types of FA residues (e.g. oleic acid is set free at higher rates than stearic acid [16]) results only in

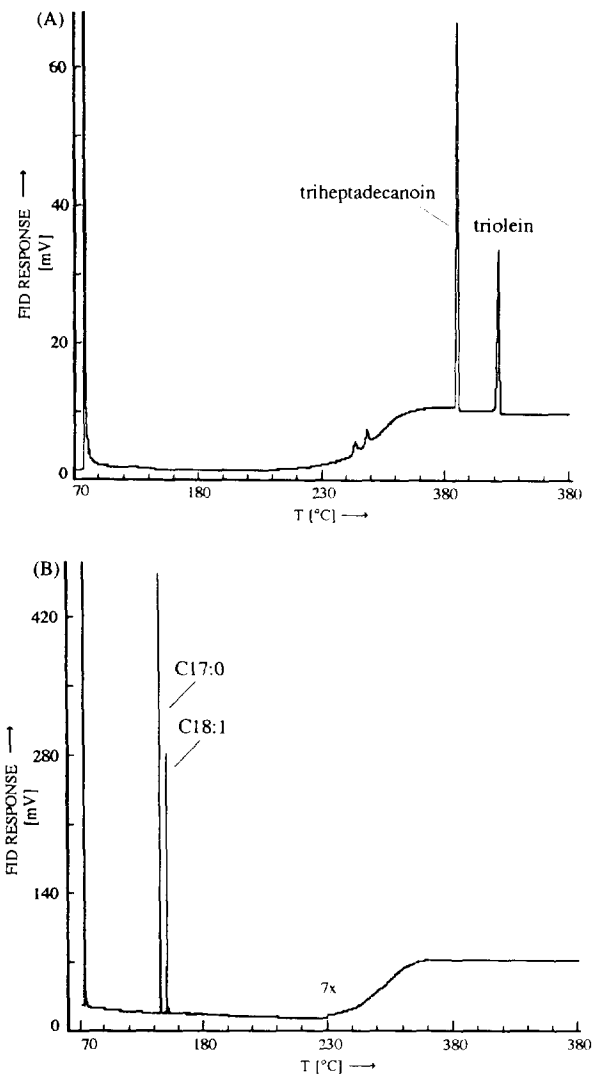

Fig. 1. High-temperature GC separation of (A) triolein and triheptadecanoin, and (B) their cleavage products oleic acid ($C_{18:1}$) and heptadecanoic acid ($C_{17:0}$) as their esters. For conditions, see Section 2.3, *Screening procedure*. FID = Flame ionization detection.

a kinetic discrimination and can be overcome by quantitative cleavage of the ester bonds. We observed no difference between the hydrolysis of triolein and triheptadecanoin (Fig. 1). Triheptadecanoin is, therefore, suitable as an internal standard for the quantitative analysis of unsaturated FAs released from acylglycerols.

The enzymatic reaction carried out by the lipase involves a water-soluble enzyme in the aqueous phase and a water-insoluble substrate in the organic hexane phase. This is an example of heterogeneous catalysis in which the catalytic events occur at a lipid-water surface. The addition of *n*-hexane improved the activity of the enzyme, but was also essential for the dissolution of triheptadecanoin and the ointment base (a water-in-oil emulsion with hydrocarbons as major constituents).

3.2. Derivatization of FAs

The free FAs produced by cleavage of the triacylglycerols were methylated for GC analysis by diazomethane at room temperature. Different procedures were tested for the diazomethane production. Consistently good results have been achieved with N-nitroso-N-methylurea [14], which is of advantage if large amounts of diazomethane are needed. The ethereal solution of diazomethane is stable in the freezer but has to be distilled before use. For freshly prepared diazomethane in small amounts Diazald [17] was employed. N-Nitrosoguanidine [18] gave unsatisfactory low yields of diazomethane. This may be a storage problem of the starting product [19].

3.3. GC analysis

Screening procedure

The screening by high-temperature GC used a fast temperature program. Only the total percentage of each lipid class (FAs as the products, mono- and diacylglycerols as intermediates, and triacylglycerols as unreacted starting material of the transformation) without separating them into individual components was of interest. The OV-1701 OH stationary phase is thermally very stable and has, therefore, a long lifetime. This makes the phase suitable for the development of methods for lipase catalysis which demands a large number of GC runs. Besides, this phase can be used to separate mixtures of *trans*- and *cis*-FAs containing, for example, elaidic and oleic acid by modified temperature programming. The cold-on-column injection is of advantage as the constituents of the sample have a broad range of boiling points.

Quantification procedure

Cold-on-column injection, but also split injection and a commercially available fused-silica capillary column coated with DB-1 were used in the quantification procedure so that this method can be employed in normal routine laboratories with conventional auto sampler equipment. More polar stationary phases than DB-1 are proposed for the separation of FAs but for our application the methyl polysiloxane phase was satisfactory.

Peak assignment for the quantitative analysis of γLn was done by spiking the sample solution with the methyl ester of γLn. Calibration was carried out by analysis of standard samples containing reference substance γLn and the internal standard heptadecanoic acid. The linearity of the detector response was checked. The recoveries of FAs comparing cold-on-column injection and split injection were the same.

Representative chromatograms for the quantitative analysis of γLn are presented in Fig. 2. The results showed that this method is well suited for the quantitative determination of polyunsaturated FAs which are released by cleavage of acylglycerols. This method should also be applicable for the analysis of triacylglycerols containing labile or reactive FAs.

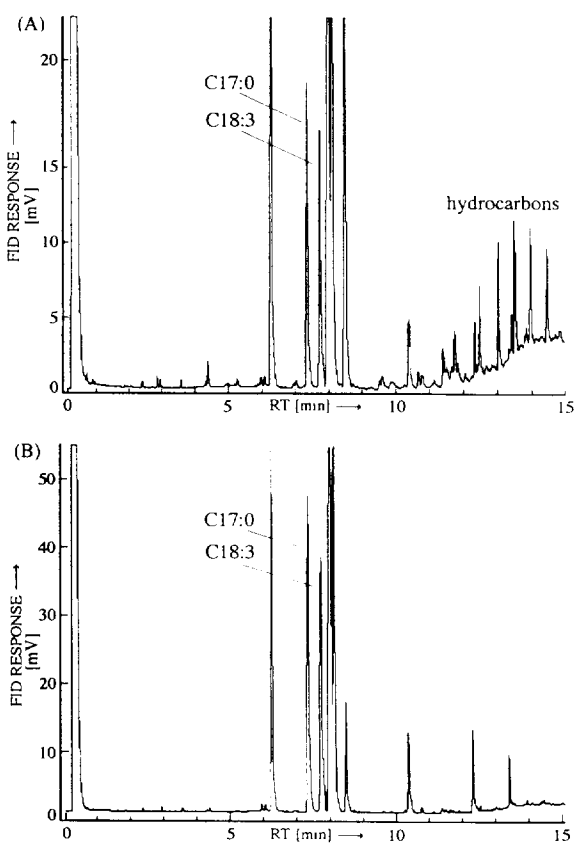

Fig. 2. Quantitative GC analysis of γLn ($C_{18:3}$) in (A) galenical preparation, and (B) borage oil by using an I.S. (heptadecanoic acid, $C_{17:0}$) as methyl ester. For conditions, see Section 2.3, *Quantification procedure*.

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