한국농화학회지 제 39권 제 1호(1996)
Agricultural Chemistry and Biotechnology
Vol. 39, No. 1, pp. 95~98(1996)

NOTE

One-Step Separation of the Lipase Catalyzed Interesterification Mixture by HPLC

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(Received September 4, 1995; accepted December 28, 1995)

Introduction

The microbial lipases from *Rhizopus delemar* catalyze the hydrolysis of fatty acids specifically from the outer 1-and 3-positions of triglycerides.[1] On the other hand, if the 1,3-specific lipase reaction between triglycerides and free fatty acids carried out under conditions in which the amount of water in the reaction system is restricted, hydrolysis of the triglycerides can be minimized so that lipase catalyzed 1,3-specific interesterification becomes the dominant reaction.[1-4]

From the above results, the use of lipase in non-aqueous media has extended for fats and oils modification during the last decade.[2-7] Very often it is sufficient to follow the appearance or disappearance of free fatty acids in the reaction mixture. However, the amount of all lipid species in the reaction mixture have to be determined for the study of enzymatic reaction mechanisms.

For example, in the case of interesterification between stearic acid and triolein, eight compounds which can be detected in the reaction mixture, namely, oleic acid, stearic acid, 2-monooleoyl glycerol(2-MO), 1,2-dioleoyl glycerol(OO-OH), 1-stearoyl-2-oleoyl glycerol(SO-OH), triolein(OOO), 1,2-dioleoyl-3-stearoyl glycerol (SOO), 1,3-distearoyl-2-oleoyl glycerol(SOS) have to be measured.

Although it was reported that oleic acids and its glycerides formed by the enzymatic synthesis between oleic acids and glycerols were simply analyzed by high performance liquid chromatography(HPLC),[8,9] a suitable method for one-step separation of the more complex reaction mixture formed by the interesterification has not been found.

Erickson *et al.*[10] have analyzed the interesterification reaction products between trilaurin and palmitic acid catalyzed by 1,3-specific lipase using gas chromatography (GC) after silation with (N,O)-bis(trimethylsilyl) trifluoroacetamide. Yokozeki *et al.*[11] who carried out the reaction in n-hexane have separated the products by the thin-layer chromatography (TLC)/GC method. Many other reports are similar,[2-7] and these methods are time consuming and require additional techniques for quantitation of the fractions.

From this point of view, the work described here was carried out to develop a one-step system which would separate and quantify the 1,3-specific lipase catalyzed interesterification products between triolein and stearic acid by HPLC with refractive index(RI) detector.

Materials and Methods

Reagents

Oleic acids, Stearic acids, 2-MO, OOO, SOO and SOS (all 99% pure) were purchased from Sigma (St. Louis, Mo, USA), OO-OH(99%) from Fluka(Buchs, Switzerland) and SO-OH(99%) from Nakarai Chemical Co.(Japan). Acetonitrile, tetrahydrofuran and dichloromethane of HPLC grade were obtained from Supelco (Bellefonte, PA, U.S.A) and acetic acid from Wako Chemicals(Japan). The lipase purchased from Seikagaku Kogyo Co. Ltd(Japan) was of *Rhizopus delemar* origin and its activity was 600 unit/mg-solid. One unit corresponded to liberating 50 μmol equivalent of fatty acid from olive oil emulsion in the reaction period of 150 hrs at 30℃.

Chromatography

The separation and quantitation of the mixtures of the oleic acid, stearic acid, 2-MO, OO-OH, SO-OH, OOO, SOO and SOS dissolved in tetrahydrofuran was carried out using a HPLC (JASCO, Hachiyochi, Japan) equipped with a TRIROTAR pump with shodex SE-11 RI detector (Showa Denko Co., Ltd). The reverse phase column used was an Inertsil ODS, 5 μm(4.6 mm×250 mm, Gasukuro Kogyo Co., Ltd).

The solvent system employed was acetonitrile-tetrahy-

Key words: Lipase reaction, interesterification, HPLC, refractive index, separation
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drofuran-dichloromethane-acetic acid (70 : 20 : 20 : 0.8, v/v). Aliquots of 10 μl of the sample solution were introduced with a microsyringe at a solvent flow rate of 0.9 ml/min. The chromatographic analysis was carried out at a constant temperature of 20± 2℃.

Lipase Reaction

A glass vessel of 60 ml volume into which 0.2g immobilized lipase(150 mg lipase/g-Duolite weak anion-exchange resin), 0.2 g triolein, 0.2 g stearic acid and 40 ml of water-saturated n-hexane as a reaction medium were loaded, was used in the reaction experiments. To prevent volatililzation of the n-hexane, a cooler similar to a Liebig condenser was connected to the top of the reactor. The reactor was then put into a bath at a constant temperature and then magnetic stirring was started to initiate the reaction. The reactor was cooled with tap water to stop the reaction and 20 ml of n-hexane was immediately taken out of the reactor and subjected to analysis.

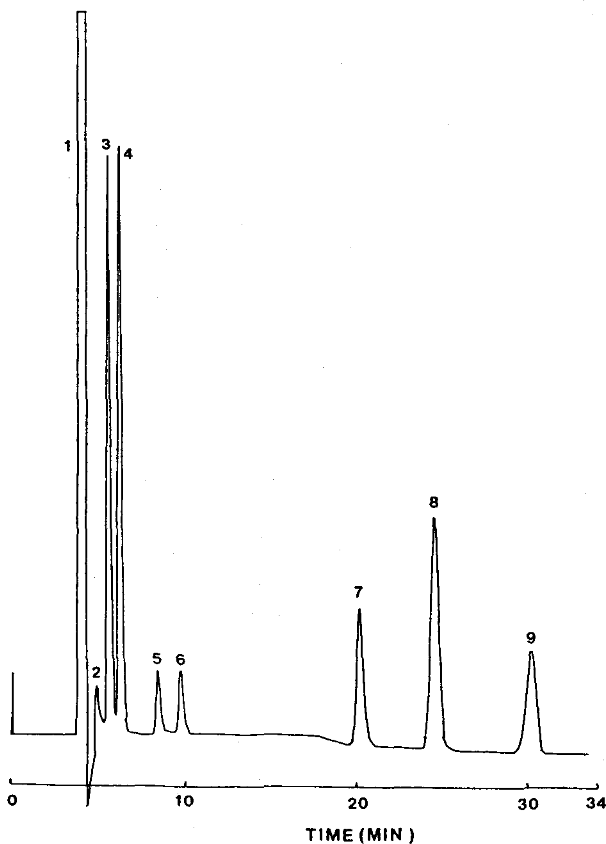

Fig. 1. HPLC separation of standard mixture. Chromatographic conditions are as given in Experimental. Peaks: 1=tetrahydrofuran, 2=2-MO, 3=oleic acid, 4=stearic acid, 5=OO-OH, 6=SO-OH, 7=OOO, 8=SOO, 9=SOS. The amounts of each lipid species injected into the column were: 2-MO, 30 μg; oleic acid, 180 μg; stearic acid, 200 μg; OO-OH, 30 μg; SO-OH, 30 μg; OOO, 50 μg; SOO, 100 μg; SOS, 50 μg.

Results and Discussion

The major advantage of UV detection over RI detection is that it permits solvent programming and is more sensitive to the lipid species when the double bond is

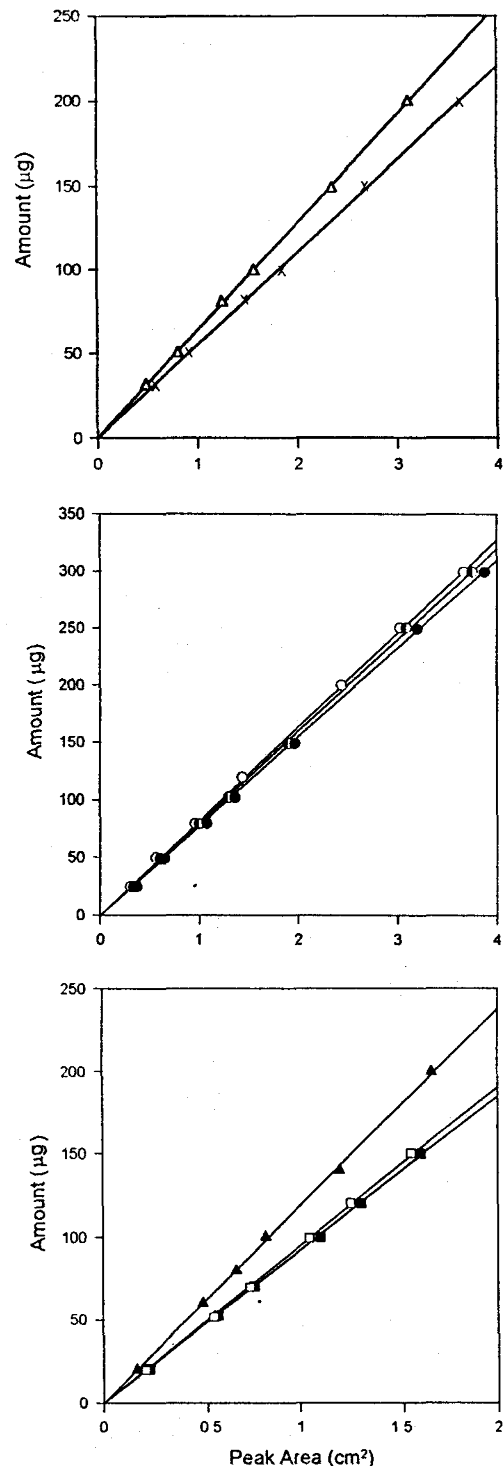

Fig. 2. Standard calibration curves of eight compounds which can be detected in the interesterification reaction between stearic acid and OOO. △−△, oleic acid; ×−×, stearic acid; ▲−▲, 2-MO; □−□, OO-OH; ■−■, SO-OH; ○−○, OOO; ◐−◐, SOO; ●−●, SOS.

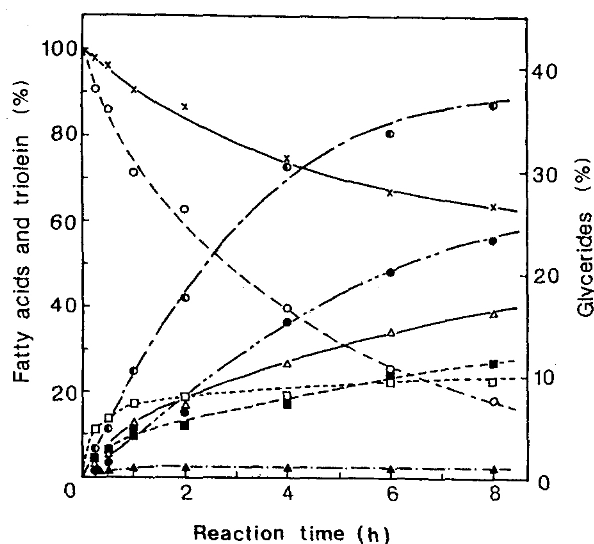

Fig. 3. Interesterification of triolein and stearic acid with a *Rhizopus delemar* lipase immobilized on Duolite at 50℃. The HPLC method described in Experimental was used to monitor the reaction. △—△, oleic acid; ×—×, stearic acid; ▲—▲, 2-MO; □—□, OO-OH; ■—■, SO-OH; ○—○, OOO; ◐—◐, SOO; ●—●, SOS.

present.[12] However, where solvent programs are employed, some problems can be encountered at short UV wavelengths (200~215 nm) with regard to baseline stability and spurious peaks from solvent contaminants.[13] In addition, short wavelength UV detection is unsuitable for saturated free fatty acids, as they exhibit almost no absorbance in this region.[14]

In order to separate free fatty acids successfully by HPLC, it is necessary to use solvent containing a small amount(ca. 1%) of an organic acid, e.g. formic or acetic acid and this approach is suitable for RI detection, but not UV or IR detection system as demonstrated by Ritchie and Jee.[13]

From these previous results, the system of RI detector and a solvent containing 0.8% acetic acid was chosen.

Fig. 1 shows the HPLC separation of standard mixtures of free fatty acids(oleic acid, stearic acid), 2-MO, diglycerides (OO-OH, SO-OH) and triglycerides (OOO, SOO, SOS). All components were satisfactorily separated within 32 min without overlapping and the elution times for each lipid species at 20±2℃ are; 2-MO, 4.60 min; oleic acid, 5.50 min; stearic acid, 6.18 min; OO-OH, 8.55 min; SO-OH 9.30 min; OOO, 19.40 min; SOO, 23.65 min; and SOS 29.20 min. But if the column temperature was varied, the elution times was also varied.

Fig. 2 shows the relationship between the peak area and the amount of each lipid species loaded onto a column for the standard calibration curve. The linearity and the reproducibility was good in the range of 20 μg~200 μg(2-MO), 30 μg~200 μg(oleic acid), 30 μg~200 μg(stearic acid), 20 μg~150 μg(OO-OH), 20 μg~150 μg (SO-OH), 25 μg~300 μg(OOO), 25 μg~300 μg(SOO) and 25 μg~300 μg(SOS). These calibration curves were used for monitoring the lipase reaction products.

Fig. 3 shows the time course of the interesterification reaction conducted in n-hexane with immobilized 1,3-specific lipase. Neither 1(3)-monoolein nor 1,3-diolein was accumulated in the reaction mixture by lipase from *Rhizopus delemar* and the positional specificity of *Rhizopus delemar* lipase towards the outer 1,3 positions of triglycerides was confirmed.

As described in this paper, it has been possible to successfully monitor the 1,3-specific lipase catalyzed interesterification reaction, not only for substrates and products of the reaction, but also for intermediate products of the reaction without any previous modification. This study thus will be helpful in elucidating the lipase reaction mechanisms and supports the view that HPLC can provide a relatively rapid and reproducible method for monitoring bench scale and pilot plant scale reaction involving hydrolysis and synthesis of fats using lipases.

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High Performance Liquid Chromatography와 Refractive Index 검출기를 이용한 Lipase 반응 혼합물의 분리

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