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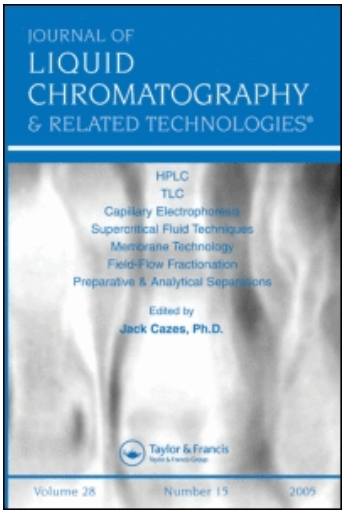

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IMMOBILIZATION OF PORCINE-PANCREATIC LIPASE ON SILICA SUPPORT TO STUDY LIPOLYSIS AND REVERSE HYDROLYSIS REACTION

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ABSTRACT

Porcine-pancreatic lipase has been immobilized on epoxypropylsilanized PartiSphere-5. The hydrolysis of olive oil on this column provided oleic acid as the major reaction product. The reversal of the hydrolysis reaction was tested by esterification of acetic acid and benzoic acid under nonaqueous conditions.

INTRODUCTION

Cambou and Klibanov (1,2) immobilized yeast lipase on the Sepharose support. The entrapment of enzyme in this porous cellulosic support exhibited poor reproducibility. In the present investigations, immobilization of lipase has been

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achieved on epoxypropylsilanized PartiSphere-5 by chemical reaction. the performance of this enzyme immobilized phase was demonstrated by hydrolysis of olive oil under aqueous conditions. The reversal of hydrolysis reaction was achieved by using nonaqueous solvent such as hexane.

EXPERIMENTAL

Materials

Porcine-pancreatic lipase and olive oil were purchased from Sigma Chemical Co., Inc. (St. Louis, MO). Potassium dihydrogen phosphate, calcium chloride, sodium chloride, gum acacia, sodium taurocholate, sodium hydroxide, acetic acid, benzoic acid chloroform and tetrahydrofuran were bought from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Packing Material

Epoxypropylsilanized PartiSphere-5 and PartiSphere-5 WCX were obtained from Whatman Manufacturing, Inc. (Clifton, NJ).

Sample Preparation

Solutions of olive oil was prepared by dissolved 2 g of the same in 2 mL of tetrahydrofuran. Solutions of acetic acid and benzoic acid were prepared by dissolving 50 mg of each in 5 mL water containing 0.5 mL of ethanol.

Assay of Lipase Activity

Lipase bound silica phase was assayed with emulsified olive oil as substrate according to the method as described by Marchis et al. (4). 0.05 M $CaCl_2$, 3 M NaCl and 14 mg/mL of sodium taurocholate solutions were prepared for this purpose.

Preparation of Packing Material

Lipase was immobilized by reacting 5 g of lipase with 5 g of epoxypropylsilanized PartiSphere-5 in 0.1 M KH_2PO_4 solution pH (4.5). The column was packed by slurrying enzyme immobilized bonded phase in methanol and applying a pressure of 6000 psi.

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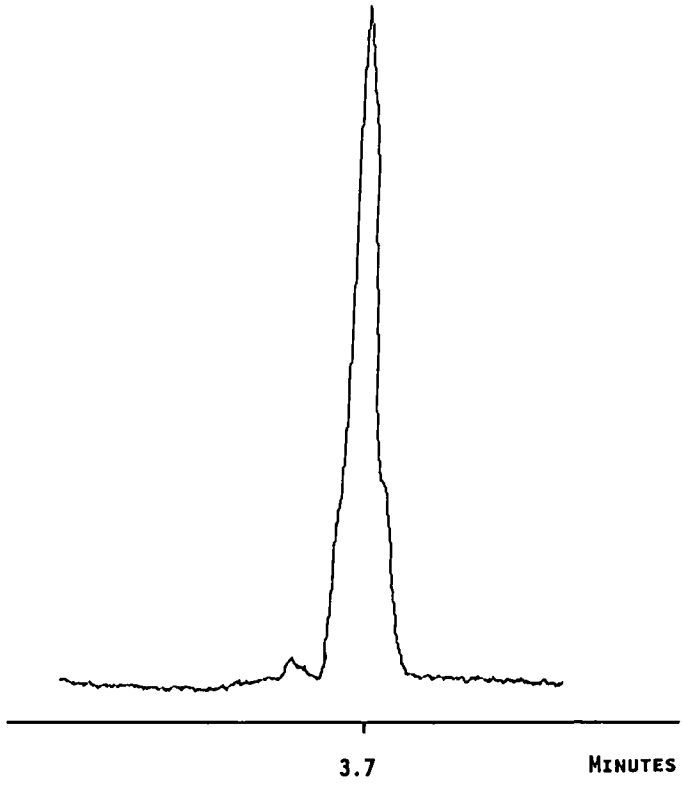

Fig. 1. The hydrolysis of olive oil on Porcine-pancreatic lipase immobilized epoxypropylsilanized PartiSphere-5 column (10 cm x 4.6 mm, I.D.). Mobile phase: water containing 0.3 g $CaCl_2$ and 1 g NaCl; flow rate: 0.7 mL/min; λ_{max} : 203 nm; sample: olive oil (2 g/2 mL tetrahydrofuran); sample size: 15 µL.

HPLC Analysis

HPLC was performed by using a variable wavelength UV detector, Spectroflow monitor SF-770 (Kratos Analytical, Ramsey, NJ), and Differential Refractometer, R 401 (Waters Associates, Milford, MA); a programmable solvent delivery system, Series 3B (Perkin-Elmer Corp., Norwalk, Conn.); a manual injection valve,

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with 50 μL loop (Valco Instruments Co., Houston, TX) and a chart recorder (Laboratory Data Control, Riviera Beach, FL).

500 mL water containing 0.3 g $CaCl_2$ and 1 g NaCl was used as a mobile phase on the lipase immobilized phase. The hydrolyzed olive oil from lipase phase was further characterized and analyzed on PartiSphere-5 WCX column by using water as a mobile phase. The lipase immobilized phase was first washed with ethanol and then run with hexane to perform reverse reaction. Authentic samples of ethyl acetate and ethyl benzoate were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI) and used to identify as the products of reverse reaction.

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RESULTS AND DISCUSSION

Immobilization of pancreatic lipase from porcine has been achieved on epoxypropylsilanized support. An assay run showed that this phase has 8000 units/g of activity. Figure 1 shows the result of injection of olive oil sample on lipase column. Incorporation of $CaCl_2$ in the mobile phase was found to be necessary for the enzymatic activity. The hydrolyzed olive oil was collected from the lipase immobilized column and analyzed on PartiSphere-5 column (Figure 2) by using water as a mobile phase.

Table 1. Esterification of acetic acid and benzoic acid with ethanol on lipase immobilized column at 40°C.

Acid	% of Esterification
Acetic acid	12
Benzoic acid	17

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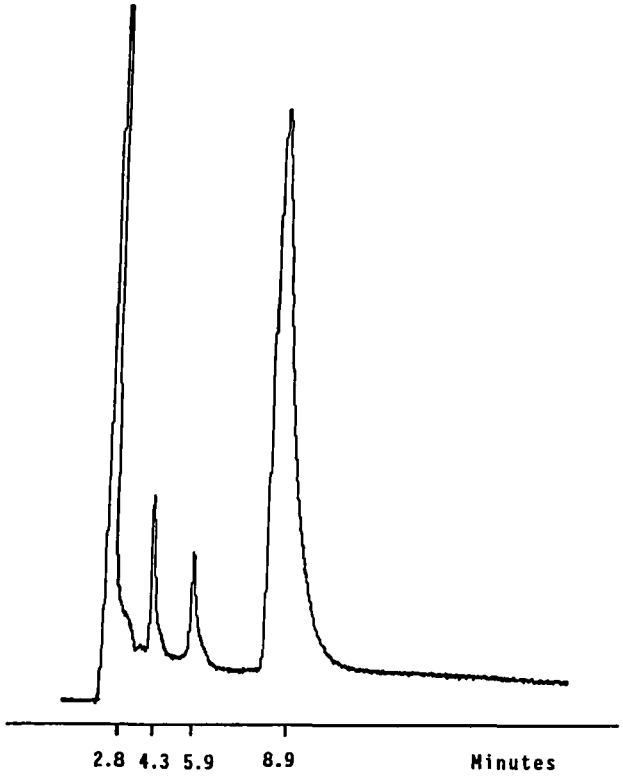

Fig. 2. Analysis of hydrolyzed products from lipase immobilized epoxypropylsilanized PartiSphere-5 column (Figure 1) on PartiSphere-5 WCX column (20cm x 4.6 mm, I.D.). Mobile phase: water; flow rate: 0.35 mL/min; λ_{max} : 203 nm; sample size: 170 µL + 15 µL caffeic acid as internal standard.
1. Tetrahydrofuran, 2. oleic acid (40%), 3. glycerides (60%), 4. caffeic acid.

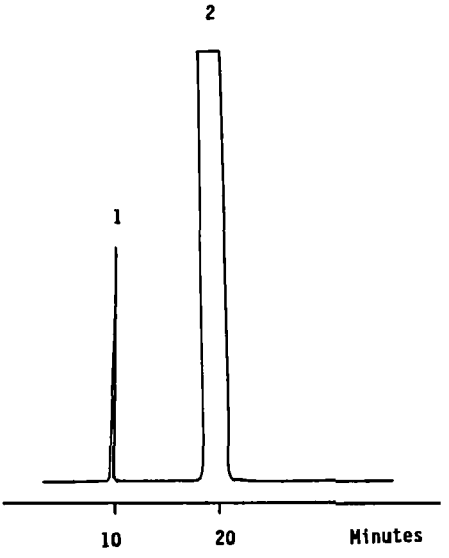

Fig. 3. Esterification of acetic acid with ethanol on lipase immobilized epoxypropylsilanized PartiSphere-5 column (20 cm x 4.6 mm, I.D.). Mobile phase: hexane; flow rate: 0.3 mL/min; sample: acetic acid + ethanol (2 mL/6 mL ethanol); sample size: 20 µL; detector: refractive index.

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Oleic acid was identified as one of the hydrolyzed products. The other products of hydrolysis such as mono- and diglyceride were found to coelute on this column. Olive oil triglyceride was absent in the mixture which shows that it underwent complete hydrolysis.

The reversal of hydrolysis reaction was accomplished by washing the lipase bound column with ethanol and then running it with nonaqueous solvents like hexane. Esterification of organic acids such as acetic and benzoic acid was attempted. Figure 3 and 4 represent the esterification of acetic acid and benzoic acid with ethanol respectively on the lipase column. The esters

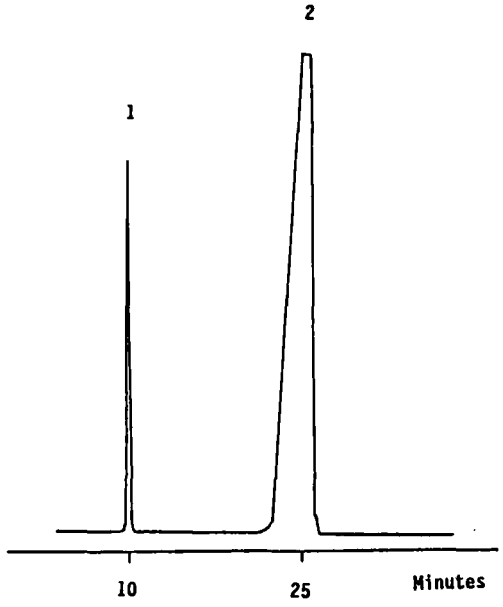

Fig. 4. Esterification of benzoic acid with ethanol on lipase immobilized epoxypropylsilanized PartiSphere-5 column (20 cm x 4.6 mm, I.D.). Mobile phase: hexane; flow rate: 0.3 mL/min; sample: benzoic acid + ethanol (2 mL/6 mL ethanol); sample size: 20 µL; detector: refractive index.
1. ethyl benzoate, 2. benzoic acid + ethanol.

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thus obtained were further analyzed on WCX column (Figure 5 and 6) by using a mixture of hexane, ethanol and water as a mobile. The result of their analysis is shown in the Table 1.

Figure 6 exhibits the resolution of ethyl benzoate from benzoic acid and *p-nitroaniline on WCX column.* Similar pattern of resolution was obtained when a mixture containing ethyl acetate, p-nitroaniline and acetic acid was attempted.

A partial reversal of hydrolysis reaction was feasible due to decreased concentration (activity) of water which shifted the

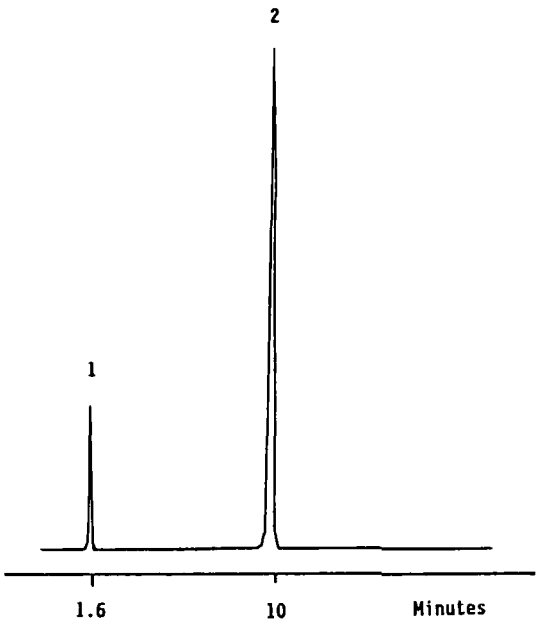

Fig. 5. Analysis of ethyl acetate as collected from lipase immobilized epoxypropylsilanized PartiSphere-5 column on PartiSphere-5 WCX column (10 cm x 4.6 mm, I.D.)(2 columns). Mobile phase: hexane: ethanol: water (300:30:0.75, v/v); flow rate: 0.2 mL/min; detector: refractive index; sample size: 15 µL of ethyl acetate as collected from lipase immobilized epoxypropylsilanized PartiSphere-5 column + 2 µL of p-nitroaniline as internal standard (10 mg/mL ethanol).

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thermodynamic equilibrium in (lipase catalyzed) hydrolysis in favor of esters (3). In nearly anhydrous organic media, the hydrolysis reaction can be completely reversed if an appropriate reaction time is given. It will be possible only if the reversal of hydrolysis reaction is catalyzed with the lipase immobilized phase as such in the open vessel instead of carrying out inside the packed column.

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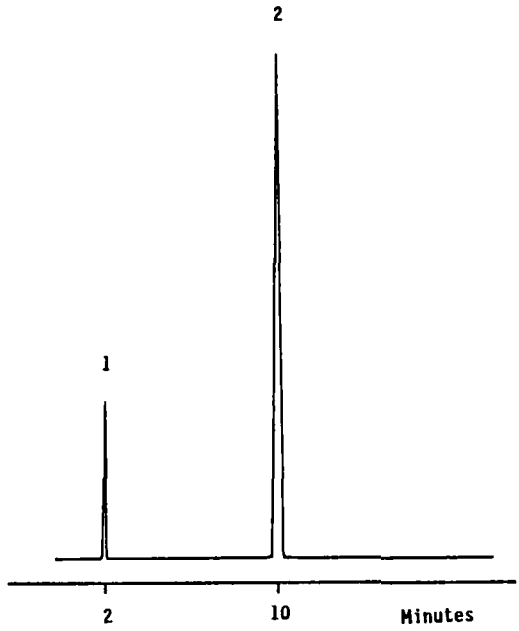

Fig. 6. Analysis of benzoate collected from lipase immobilized epoxypropylsilanized PartiSphere-5 column on PartiSphere-5 WCX column (10 cm x 4.6 mm, I.D.)(2 columns). Mobile phase: hexane: ethanol: water (300:30:0.75, v/v); flow rate: 0.2 mL/min; λ_{max}: 280 nm; sample size: 15 µL of ethyl benzoate as collected from lipase immobilized epoxypropylsilanized PartiSphere-5 column + 2 µL of p-nitroaniline as internal standard (10 mg/mL ethanol).
1. ethyl benzoate 2. p-nitroaniline.

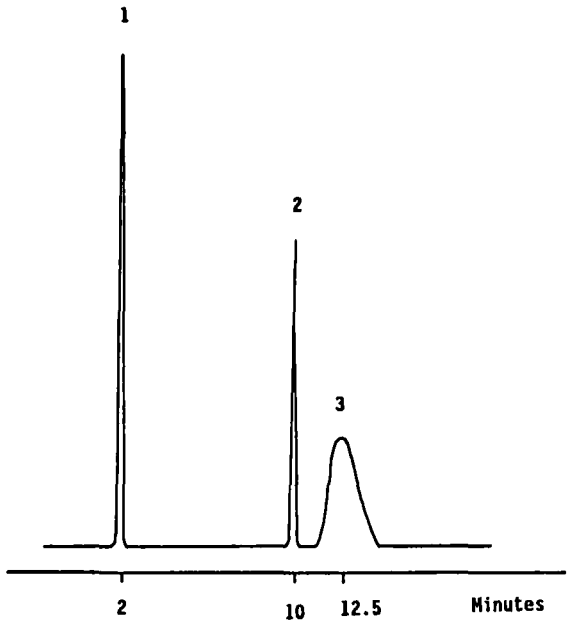

Fig. 7 Resolution of ethyl benzoate on PartiSphere-5 WCX column (10 cm x 4.6 mm, I.D.)(2 columns). Mobile phase: hexane: ethanol: water (300:30:0.75, v/v); flow rate: 0.2 mL/min; λ_{max}: 280 nm; sample size: 5 µL of ethyl benzoate (50 mg/3 mL ethanol) + 5 µL of benzoic acid (50 mg/3 mL ethanol) + 2.5 µL of p-nitroaniline (10 mg/3 mL ethanol).
1. ethyl benzoate, 2. p-nitroaniline, 3. benzoic acid.

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