⁂Enzymatic Synthesis of Carbohydrate Esters of Fatty Acid (I) Esterification of Sucrose, Glucose, Fructose and Sorbitol

HAJIME SEINO* and **TSUYOSHI UCHIBORI**, School of Hygienic Sciences, Kitasato University, Kitasato 1-15-1, Sagamihara-shi, Kanagawa 228, Japan, and
TOSHIYUKI NISHITANI and **SACHIKO INAMASU**, Dai-Ichi Kogyo Seiyaku Co., Ltd., 55 Higashikubo-cho, Nishishichi jo Shimogyo-ku, Kyoto 600, Japan

ABSTRACT

The authors attempted to synthesize carbohydrate esters of fatty acids enzymatically in order to overcome the problems associated with the chemical processes for the synthesis of commercial sucrose esters. The enzymes used were lipases from microorganisms belonging to *Rhyzopus, Enterbacterium, Aspergillus, Pseudomonas, Chromobacterium, Candida, Mucor* and *Penicillium*. Fatty acids (stearic, oleic and linoleic) and carbohydrates (sucrose, glucose, fructose and sorbitol) used for the reaction were obtained from commercial sources. The enzyme reaction was performed by mixing the enzyme and the substrates in the buffer solution and incubating at 40 C; after freeze-drying the mixture, the products were extracted and subjected to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). It was observed by TLC and HPLC that carbohydrate esters of fatty acid were produced by the enzyme reaction, and their structures were confirmed by infra red (IR) and nuclear magnetic resonance (NMR) spectrometries. The lipase from *Candida cylindracea* was the most enzyme active on the synthesis of carbohydrate esters. The optimum conditions for its activity were as follows: molar ratio of carbohydrate to fatty acid: 0.05mol/l : 0.2mol/l; amount of lipase: 4g/l; pH of the reaction mixture: 5.4 in phosphate buffer; reaction period: 72 hr.

INTRODUCTION

Sucrose esters are potentially important as emulsifiers in foods, cosmetics and medicines.

The first paper describing a practical commercial process for the preparation of sucrose esters of fatty acid was reported by Osipow et al. (1). This process involved a transesterification reaction between sucrose and the methyl ester of a fatty acid in the presence of a basic catalyst and mutual solvent, such as dimethylformamide. Osipow developed another process (2) in which the sucrose is dissolved in propylene glycol and the methyl ester emulsified into this solution. The glycol was distilled off and, in the distillation process, transesterification takes place. A solvent-free interesterification process was reported by Feuge et al. (3). In this process, interesterification of molten sucrose and fatty acid esters at temperatures between 170-187 C is performed with the aid of lithium, sodium and potassium soap as catalyst and solubilizer.

The disadvantage of these processes is coloring of the products caused by heating or the toxity of the solvent used. These problems might be avoided if the sucrose esters could be synthesized by the action of enzymes. It is further expected that mono-, di- or triesters could be obtained selectively if the substrate specificity of the enzyme would be effected on the primary hydroxyl groups of the sucrose molecule.

Several papers have been reported on the enzymatic synthesis of triglycerides (4,5,6,7,8). But the enzymatic synthesis of carbohydrate esters has not yet been reported. It was supposed by the authors that some of the lipases which could hydrolyze or synthesize triglycerides would show the activity to synthesize carbohydrate esters. So the selection of enzymes was based on their hydrolytic activities.

In addition to sucrose esters, the authors tried to prepare esters of the monosaccharides glucose, fructose and sorbitol. These esters are expected to have not only surface activity but also antitumor and plant growth inhibiting activities as has been previously reported (9,10,11,12,13,14).

*To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Enzymes, carbohydrates and fatty acids which were used for the experiments were obtained from commercial sources.

The enzymes were the microbial lipases selected on the basis of their activities for hydrolysis. Their origins and hydrolytic activities were:

Rhyzopus	30,000 units/g
Enterbacterium	9,000 units/g
Aspergillus	40,000 units/g
Pseudomonas	30,000 units/g
Chromobacterium	4,000 units/g
Candida	30,000 units/g
Mucor	9,000 units/g
Penicillium	4,000 units/g

Carbohydrates used were sucrose, glucose, fructose and sorbitol. Purities of the stearic, oleic and linoleic acids were more than 99% as determined by gas liquid chromatography (GLC).

Procedure for the Reaction

Enzymes and the substrates were mixed in the buffer solution and incubated at 40 C with stirring by a magnetic stirrer. The reaction conditions were varied as follows.

Molar ratio of carbohydrate to fatty acid: from 0.02mol/1:0.2mol/l to 0.5mol/1:0.02mol/l.

Amount of lipase: 1g/l, 2g/l, 4g/l.

pH of the solution: 5.0, 5.4, 6.2, 7.3, 8.0.

Reaction period: 24-96 hr.

After the reaction the mixture was freeze dried and the products were extracted as shown in Figure 1.

The extracted products were subjected to TLC and HPLC. Analytical conditions of TLC and HPLC were as follows:

TLC

Plate: Merck DC-Fertigplatten Kieselgel 60F 254, 5X10cm, 0.25mm.

Developing system: chloroform:methanol:acetic acid: water=81:9:8:2 (v/v/v/v).

Detecting reagent: Anisaldehyde-sulfric acid.

HPLC

Instrument: Hitachi 635 Liquid Chromatograph.

Column: Finepack GEL-201, 2cm X50cmX2 (2 columns connected).

Mobil phase: tetrahydrofurun (THF).

Flow rate: 3.0ml/min.

Pressure: 40kg/cm^2.

Detector: RI (Shodex RI Model SE-11).

Sample size: 50μl (500mg/10mlTHF).

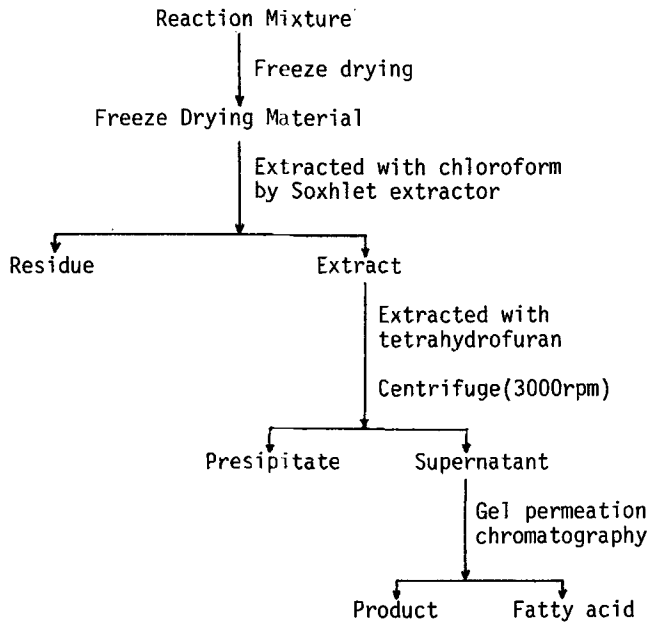


FIG. 1. Separation of the products from the reaction mixture.

Isolation and Confirmation of the Products

The components of the products which were obtained from the reaction of carbohydrates (sucrose, glucose, fructose, and sorbitol) with oleic acid under the following conditions were isolated through preparative HPLC. Their structures were confirmed by the determination of IR and C-13 NMR spectra. The instruments used were Hitachi 215 Infrared spectrometer and JNM-FX-90Q NMR Spectrometer.

Reaction conditions: Carbohydrate 0.05mol, oleic acid 0.2mol, and lipase from *Candida cylindracea* 4.0g in 1 liter of phosphate buffer solution, 40 C, 72 hr.

Conversion of Fatty Acid

The extent of conversion of fatty acid to carbohydrate esters was determined as follows: A 10-ml aliquot of the reaction mixture was taken periodically during the reaction, and the concentration of residual fatty acid was determined by titrating with 0.1N-KOH/ethanol. The extent of conversion was calculated.

RESULTS AND DISCUSSION

Activities of Enzymes

Results of TLC on the products extracted from reaction mixtures of sucrose, glucose, fructose and sorbitol with oleic acid in the presence of the lipase from *Candida cylindracea* are shown in Figure 2. The results of HPLC are shown in Figures 3, 4 and 5.

It was observed from the results of TLC and HPLC that several compounds were produced by the reaction of every carbohydrate with oleic acid. They were considered to be carbohydrate esters compared with TLC and HPLC of the authentic samples obtained by the chemical reaction of sucrose. Retention volumes of the peaks in the HPLC chromatogram of sorbitol-oleic acid reaction products were slightly different from those of sorbitan esters obtained by the chemical reaction. So it was supposed that uncyclized sorbitol esters would be synthesized by the enzyme reaction.

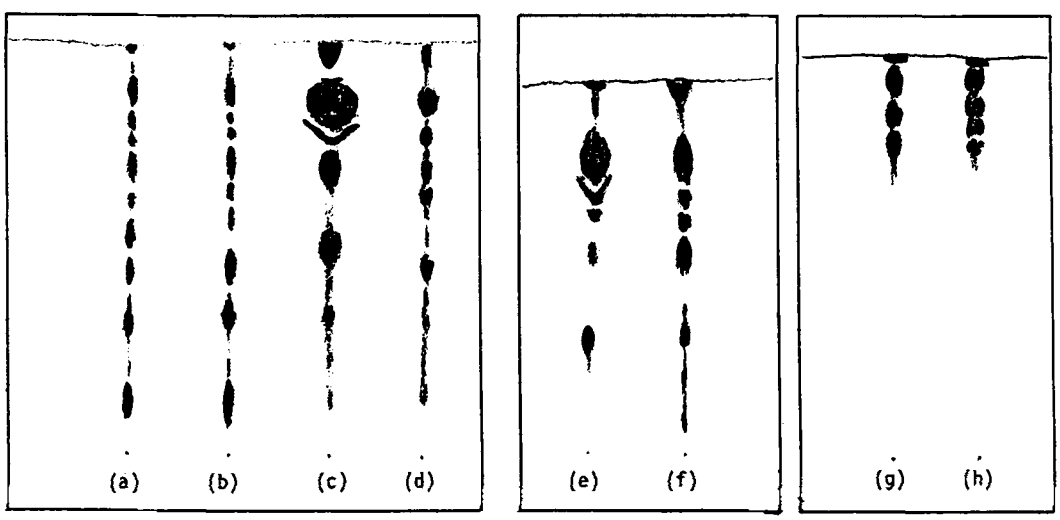
It also was observed that linoleic acid esters of these carbohydrates were produced by the enzyme action of the lipase from *Candida cylindracea.* But the esters of stearic acid were obtained in only small amounts. The low yield of stearic acid ester was considered to be due to the insufficient dispersion of the solid fatty acid in the reaction mixture.

It was found from the results of TLC and HPLC that the lipase from *Candida* was the most active enzyme and that the optimum pH of the reaction mixture for the activity was 5.0 to 7.0. The maximum activity was observed at the concentration of 4g/liter and pH 5.4.

Lipase from *Pseudomonas* and *Enterbacterium* also showed the activities to synthesize carbohydrate esters. But the activities were not so high as that of *Candida* lipase under the conditions examined.

Isolation and Confirmation of the Products

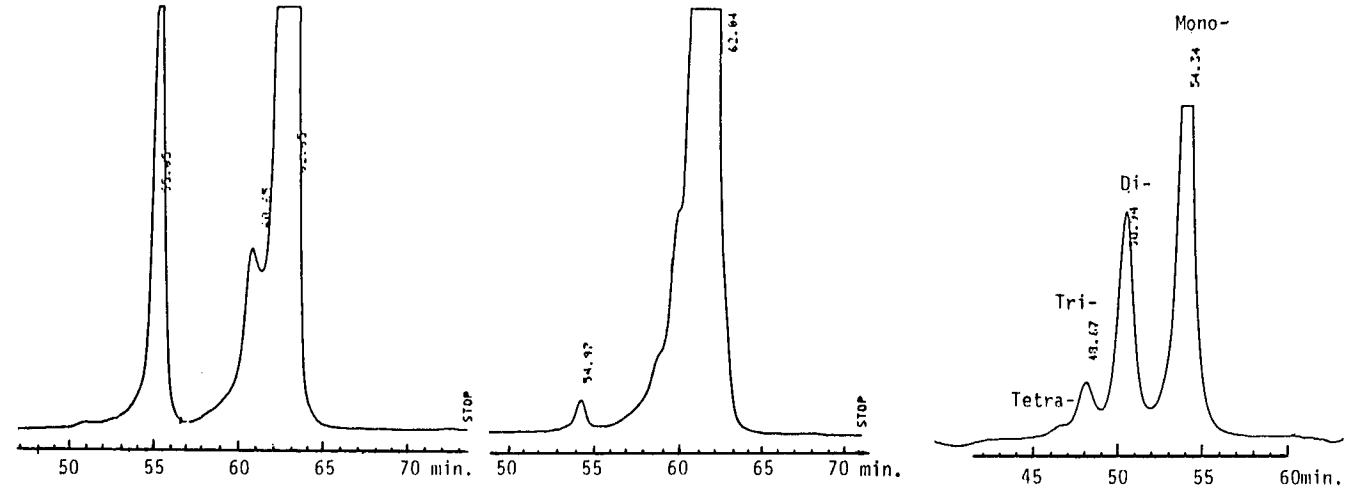
Figure 6 shows the result of TLC of the compounds



(a) Sucrose-stearic acid ester*
(b) Sucrose-oleic acid ester*
(c) Sucrose-oleic acid reaction product
(d) Sucrose-stearic acid reaction product
(e) Sorbitol-oleic acid reaction product
(f) Sorbitan-oleic acid ester*
(g) Glucose-oleic acid reaction product
(h) Fructose-oleic acid reaction product

*, Obtained by chemical reaction

FIG. 2. TLC of the reaction product of carbohydrate with fatty acid.



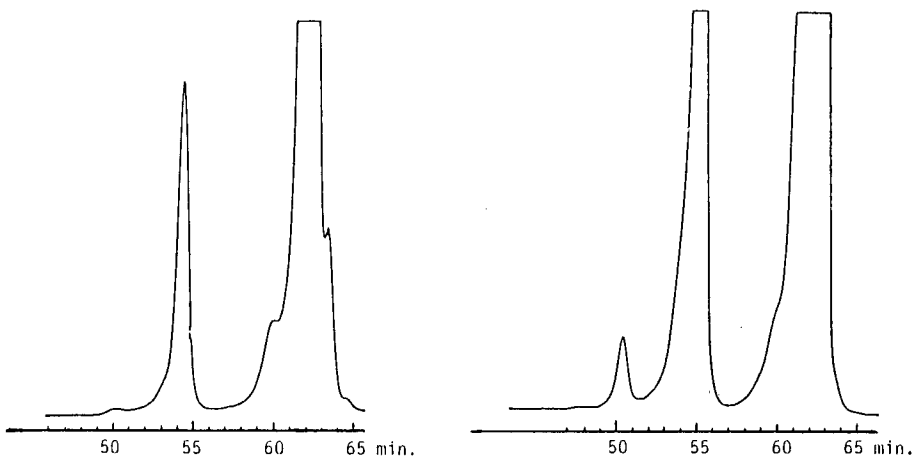
Sucrose-oleic acid reaction product

Sucrose-stearic acid reaction product

Sucrose-oleic acid esters*

*, obtained by chemical reaction.

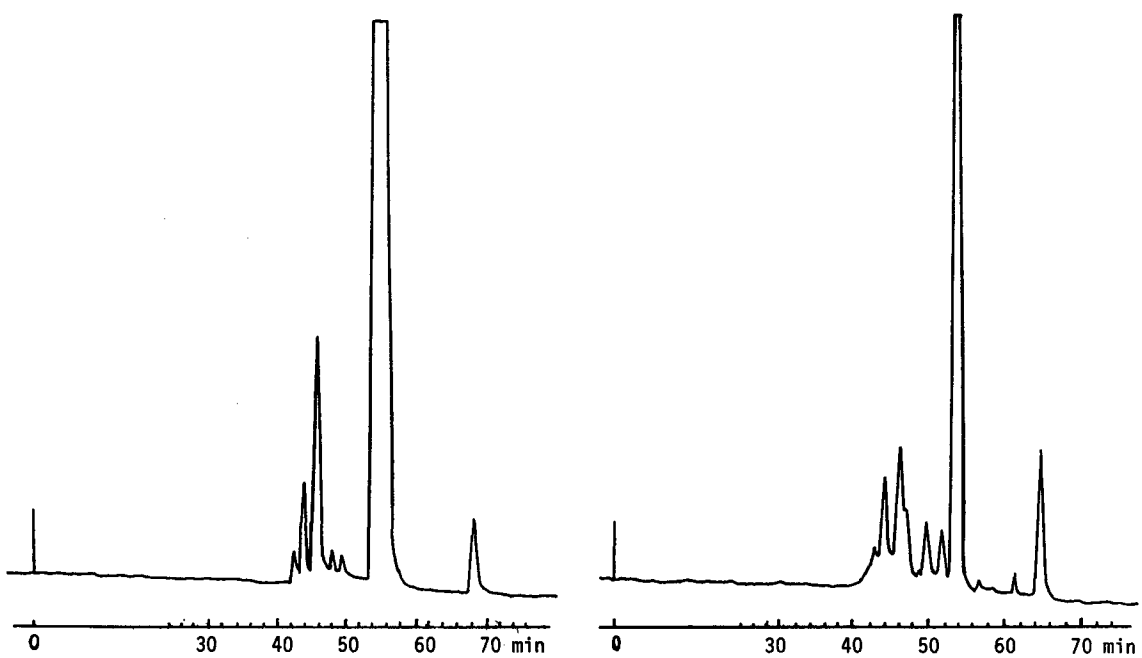
FIG. 3. HPLC of the reaction products of sucrose with fatty acid.



Glucose-oleic acid reaction product

Fructose-oleic acid reaction product

FIG. 4. HPLC of the reaction products of monosaccharide with oleic acid.



Sorbitol-oleic acid reaction product

Sorbitan-oleic acid ester*

*; Obtained by chemical reaction

FIG. 5. HPLC of the reaction products of sorbitol with oleic acid.

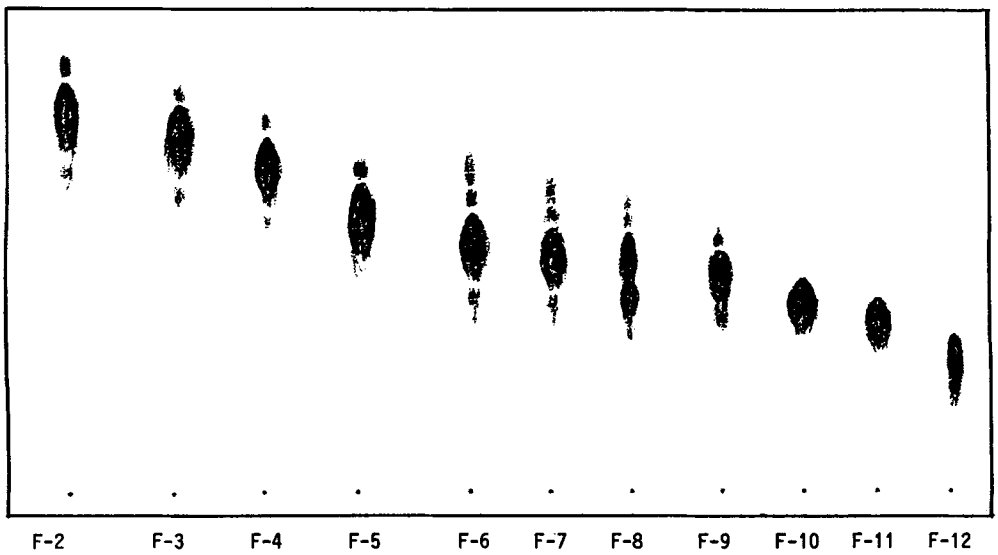


FIG. 6. TLC of the components of the reaction product of sucrose with oleic acid.

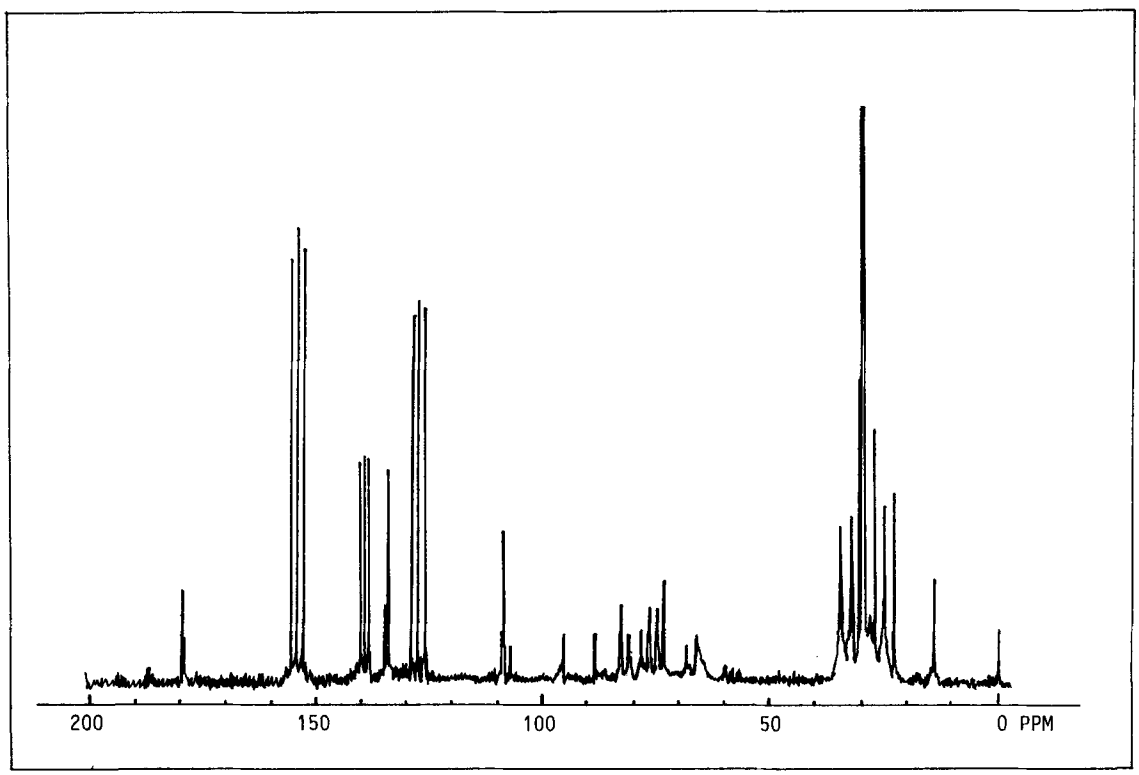


FIG. 7. ^{13}C-NMR spectrum of a sucrose ester (F-10, Fig. 7) in pyridine-d_5.

isolated from the reaction products of sucrose with oleic acid.

In some of the IR spectra of these compounds, absorption bands at 3420cm^{-1} (hydroxyl group), 1740cm^{-1} (ester carbonyl) and 920cm^{-1} (pyranose ring) were observed. These absorption bands also were observed in the IR spectra of the isolated compounds from the reaction products of glucose and fructose with oleic acid. In the IR spectra of the products of sorbitol and oleic acid, absorption bands caused by ester carbonyl and hydroxyl group also were observed.

One of the C-13 NMR spectra of the products of sucrose and oleic acid is shown in Figure 7.

In the spectrum signals of methyl and methylene carbons of oleoyl group (14-35ppm), carbons of sucrose skeleton (67-110ppm), double bonded carbons (129.2-130.0ppm) and carbonyl carbons (172.7-173.5ppm) were observed. These signals also were observed in the spectra of the products from other carbohydrates.

It was confirmed from these results of IR and NMR that sucrose esters were synthesized by the action of the lipase. It was confirmed in the same way that esters of glucose,

TABLE I

Conversion of Oleic Acid

Substrate		Phosphate Buffer	PH	Lipase	Reaction Time	Conversion
Sucrose 17.0g	Oleic acid 57.0g	1000ml	5.40	4.0g	72hr	60.1%
Glucose 3.6	Oleic acid 22.6	1000	5.39	2.0	72	27.7
Fructose 3.6	Oleic acid 22.6	1000	5.39	2.0	72	68.1
Sorbitol 14.6	Oleic acid 22.6	1000	5.40	4.0	72	62.1

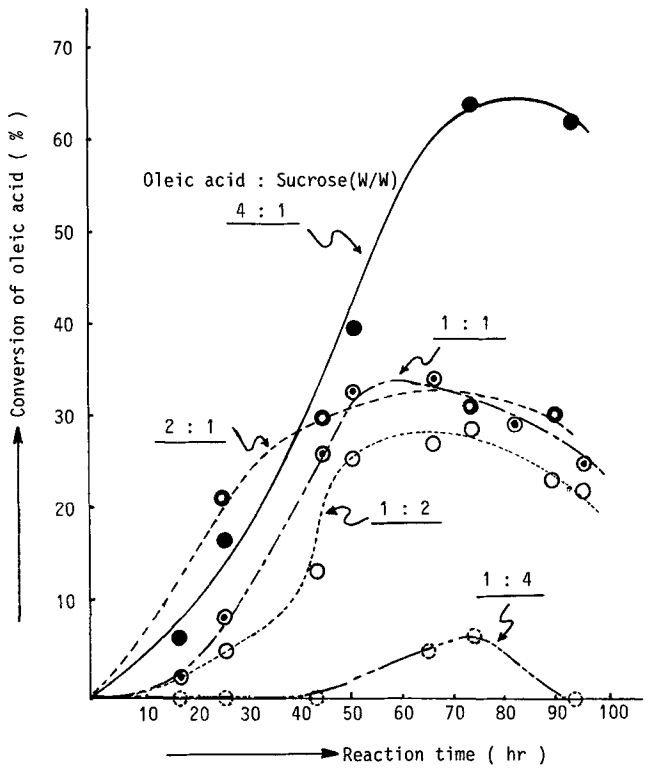


FIG. 8. Change of the concentration of oleic acid reacted in the course of reaction with oleic acid.

fructose and sorbitol also were formed under the same reaction conditions. The detailed discussion on the spectral data of the individual esters of these carbohydrates will be reported later.

Conversion of Fatty Acid

The change of the concentration of oleic acid reacted during the course of the reaction with sucrose in the presence of 4g/l lipase from *Candida* is shown in Figure 8.

The conversion of oleic acid was found to be the highest when the concentration of sucrose and oleic acid were 0.05mol/l and 0.2 mol/l, respectively. The conversion of oleic acid showed the maximum value at 72 hr. It was considered that hydrolytic action of the enzyme would be effected at that time. The highest enzyme activity of the lipase from *Candida cylindracea* also was observed with the other carbohydrates when the concentration of the lipase was 4g/l and the molar ratio of carbohydrate to oleic acid was 1 to 4. The conversion (%) of oleic acid is shown in Table I. Conversions of oleic acid were more than 60% for sucrose, fructose and sorbitol, although that for glucose was about 28%.

ACKNOWLEDGMENT

Meito Sangyo Co. Ltd. and Amano Pharmaceutical Co. Ltd. provided enzymes.

REFERENCES

1. Osipow, L.I.; F.D. Snell; D. Marra and W.C. York, Ind. Eng. Chem., 48:1459 (1956).
2. Osipow, L.I., and W. Rosenblat, JAOCS, 44:307 (1967).
3. Feuge, R.O.; H.J. Zeringue Jr.; T.J. Weiss and M. Brown, JAOCS, 47:56 (1970).
4. Iwai, M., and Y. Tsujisaka, J. Gen. Appl. Microbiol., 9:353 (1963).
5. Tsujisaka, Y.; S. Okumura, and M. Iwai, Biochem. Biophys. Acta, 489:415 (1977).
6. Okumura, S.; M. Iwai, and Y. Tsujisaka, Ibid., 575:156 (1979).
7. Linfield, W.M.; R.A. Barauskas; L. Sivieri; S. Serota and R.W. Stevenson Sr., JAOCS, 61:191 (1984).
8. Hoq, H.M.; T. Yamane, and S. Shimizu, Ibid., 61:776 (1984).
9. Nishikawa, Y., et al., Chem. Pharm. Bull. 23:387 (1975).
10. Nishikawa, Y., et al., Ibid., 24:756 (1976).
11. Nishikawa, Y., et al., Ibid., 25:1717 (1977).
12. Nishikawa, Y., et al., Ibid., 25:2378 (1977).
13. Nishikawa, Y., et al., Ibid., 27:2011 (1979).
14. Nishikawa, Y., et al., Ibid., 29:878 (1981).

[Received June 14, 1984]