ノ - ト

Synthesis of Estolides During Hydrolysis of Castor Oil by *Geotrichum candidum* Lipase

Susumu OKUMURA, Mieko IWAI, and Yoshio TSUJISAKA

Osaka Municipal Technical Research Institute
(1-6-50, Morinomiya, Joto-ku, Osaka)

It was proved that the phenomenon of decrease in acid value which was once increased during incubation of castor oil with *Geotrichum candidum* lipase was caused by the polymerization of liberated ricinoleic acid catalyzed by the lipase. Products from ricinoleic acid by the action of *Geotrichum candidum* lipase were purified to homogeneity and their structures were investigated. Two of them were found to be dimer and trimer of ricinoleic acid.

1 Introduction

In the case of the hydrolysis of castor oil by lipase from *Geotrichum (Geo.) candidum* ATCC 34614¹⁾, acid value of the reaction mixture once increased and gradually decreased. The phenomenon was not recognized in the case of the hydrolysis of ordinary oils and fats by the same lipase, or in the case of the hydrolysis of castor oil by the lipase from *Rhizopus (Rh.) delemar* ATCC 34612 (C-lipase)²⁾ as shown in **Fig.-1**. The present study was undertaken to clarify the cause of this phenomenon.

Reaction mixture containing 1 g of castor oil, 5 ml of 0.05 M acetate buffer (pH 5.6), 0.5 ml of 0.1 M CaCl_2 and 0.5 ml of lipase (1000 U) was stirred at 200 rpm at 30°C. Reaction was stopped by addition of 20 ml of ethanol and free acid was titrated with 0.5 M KOH.

Fig.-1 Time courses of degradation of castor oil by two lipases.

2 Materials

Pure glycerol triricinoleate and pure ricinoleic acid were kindly supplied by Dr. S. Watanabe and Dr. H. Seino (Kitasato University). Lipases from *Geo. candidum*¹⁾ and *Rh. delemar*²⁾ were purified as previously reported. Lipase activity was assayed as described previously³⁾. One unit (U) was defined as the amount of enzyme which liberated 1 μmol equivalent of fatty acid per min from olive oil.

3 Results and discussion

Glycerol triricinoleate, the dominant constituent of castor oil, was incubated with lipase from *Geo. candidum* or *Rh. delemar*, and its reaction products were examined by TLC. In the case of *Rh. delemar* lipase, free ricinoleic acid, glycerol monoricinoleate and glycerol diricinoleate were detected (**Fig.-2**). In contrast, many products other than free acid and partial glycerides were formed by the action of *Geo. candidum* lipase. Further, the amount of free ricinoleic acid decreased remarkably after 5 h's reaction, compared with that obtained after 0.5 h's reaction.

In this regard, judging from the alcohol specificity of *Geo. candidum* lipase for ester synthesis⁴⁾, the authors presumed a possibility of polymerization of liberated ricinoleic acid.

Reaction mixture containing 1g of glycerol triricinoleate, 5 ml of 0.05 M acetate buffer (pH 5.6), 0.5 ml of 0.1 M CaCl_2 and 0.1 ml of lipase (200 U) was stirred at 500 rpm at 30°C . Products were extracted with ethyl ether and developed four times on silica gel plate in benzene/methanol/acetic acid (95 : 5 : 1 vol/vol) and visualized by spraying 50% H_2SO_4 and heating at 150°C for 20 min. Product-B and glycerol triricinoleate were not completely separated and overlapped each other. RA, ricinoleic acid; TR, glycerol triricinoleate.

Fig.-2 Products from glycerol triricinoleate by lipases from *Geo. candidum* and *Rh. delemar*.

To prove this presumption, reaction mixture consisting of 8g of ricinoleic acid, 4ml of 2% casein solution, 16ml of water and 1ml of *Geo. candidum* lipase (2000 U) was incubated with stirring at 200 rpm, at 30°C for 40 h. As a result, many reaction products were detected on thin layer chromatogram (**Fig.-3**). The products were separated by high performance liquid chromatography (μ -porasil, 4.6×600 mm, *n*-hexane/ethyl ether 6 : 4 vol/vol), and pure product-I and -II (**Fig.-3**) were obtained.

The IR spectra of the product-I and -II exhibited strong absorption bands at 1160 cm^{-1} and 1730 cm^{-1} which are characteristic of ester

Reaction conditions are noted in the text. Developing conditions of TLC are the same as described in **Fig.-2**.

Fig.-3 Synthesized products from ricinoleic acid by *Geo. candidum* lipase.

a) Ricinoleic acid; b) product-I; c) product-II.
Fig.-4 ^1H -NMR spectra of products and ricinoleic acid.

linkage. The two compounds also exhibited the absorption band at 1710 cm^{-1} due to C=O linkage of carboxylic acid and broad bands in the range of $3500\sim 3000\text{ cm}^{-1}$ which are assigned to the stretching vibrations of alcoholic and carboxylic OH.

In the NMR spectra (**Fig.-4**), intensity of the signal of hydroxy protons (H) due to carboxylic and hydroxyl groups at 6~7 ppm and that of methylene protons (M) of unbranched long chain at 1.4 ppm were compared. The H/M ratios of ricinoleic acid, product-I and product-II were 2/13, 2/26 and 2/41, respectively. Signals at 3~4 ppm and 4~5 ppm indicated $-\text{CH}=\text{CH}-$ and $\text{H}-\text{C}-\text{OH}$ functions, respectively.

The results of IR and NMR suggest that product-I has ester linkage, hydroxyl and carboxyl groups and long chain of twice as long as

that of ricinoleic acid. In a similar manner, product-II was assumed to have ester linkage, hydroxyl and carboxyl groups and alkyl chain of three times as long as that of ricinoleic acid.

Molecular weights of product-I and -II were determined by use of a vapor pressure osmometer (Mechrolab Inc.). Calibration of molecular weight was carried out by using 10~100 mM biphenyl-toluene solution. Molecular weights of product-I and -II were estimated to be approximately 600 and 1000, respectively.

Contents of carboxyl group of the products were determined by alkalimetry. One hundred milligrams of the sample was titrated with 0.05 M KOH. Titration values per unit weight of product-I and -II were half and one third of that of ricinoleic acid, respectively.

From the results described above, it was considered that these products were estolides between ricinoleic acid molecules, and product-I was dimer and product-II was trimer. Product-A and -B in Fig.-2 were proved to be the same compounds as product-I and -II in Fig.-3 by TLC and high performance liquid chromatography, respectively.

In this manner, it was clarified that decrease in acid value during incubation of castor oil with *Geo. candidum* lipase is mainly caused by the polymerization of liberated ricinoleic acid catalyzed by the lipase. Besides, the possibility of the formation of estolides between liberated ricinoleic acid and ricinoleic residue combined with glycerol still remains, although the authors have not identified these compounds yet.

The reason why ricinoleic acid was polymerized by *Geo. candidum* lipase while it was not polymerized by *Rh. delemar* lipase is

presumed as follows. Namely, *Geo. candidum* lipase has the ability to catalyze the synthesis of esters from secondary alcohols and fatty acids⁴⁾. Since *Rh. delemar* lipase has no activity on secondary alcohols⁴⁾, estolides were not synthesized as shown in Fig.-2. In addition, product-C, -D and -E were seemed to be tetramer, pentamer and hexamer of ricinoleic acid, respectively, judging from their behavior on the plate of thin layer chromatography.

Acknowledgement

The authors wish to thank Mr. T. Miyata for NMR analysis.

(Received Dec. 23, 1982)

References

- 1) Y. Tsujisaka, M. Iwai, and Y. Tominaga, *Agric. Biol. Chem.*, **37**, 1457 (1973).
- 2) M. Iwai and Y. Tsujisaka, *Agric. Biol. Chem.*, **38**, 1241 (1974).
- 3) J. Fukumoto, M. Iwai, and Y. Tsujisaka, *J. Gen. Appl. Microbiol.*, **9**, 353 (1963).
- 4) S. Okumura, M. Iwai, and Y. Tsujisaka, *Biochim. Biophys. Acta*, **575**, 156 (1979).

Geotrichum candidum リパーゼによる ひまし油分解に伴うエストロドの合成

奥村 晋・岩井美枝子・辻阪好夫

大阪市立工業研究所 (大阪市城東区森之宮 1-6-50)

ひまし油に *Geotrichum candidum* ATCC 34614 のリパーゼを作用させると、反応液の酸価はいったん上昇したあと低下した。この原因を明らかにするために、ひまし油の主成分であるグリセリン=トリリシノレート(精製品)に上記のリパーゼを作用させ、反応生成物を高速液体クロマトグラフィーにより分離し、その構造をIR や NMR により検討したところ、それらはリシノール酸の重合物であることがわかった。これらの結果から、*Geotrichum candidum* リパーゼによるひまし油分解における酸価の低下は、このリパーゼによりエストロドが生成することによることを明らかにした。