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Kinetic Study of the Lipase-Catalyzed Interesterification of Triolein and Stearic Acid in Nonpolar Media

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Abstract : The kinetics of the interesterification of triolein and stearic acid catalyzed by immobilized *Rhizopus delemar lipase* were studied in a batch operation. In order to clarify the mechanisms of this reaction, three models are discussed under various conditions in terms of the ratio of triolein and stearic acid. The rate constants involved in the proposed model were determined by combining the numerical Gauss-elemination method, and the trial-and-error method so as to fit the calculated results with the experimental data. The accuracy of the obtained rate constants was confirmed after they were substituted for simultaneous differential equations and the equations simulated using an adaptive step-size Runge-Kutta method. Finally, the model which agrees with the calculated results and the experimental data was selected.
Key words : kinetics, 1,3-specific lipase, modeling, rate constants, simulation.

Fats and oils are almost exclusively composed of triacylgycerol esters of long chain fatty acids. Thus, the physical properties of fats and oils depend not only on satuaration or unsaturation of the attached acyl groups, but also on the position of binding of these groups. For example, the physical behavior of cocoa butter as a function of temperature results from a distribution of largely saturated fatty acids, e.g. palmitic or stearic acid in the 1- and 3-position and oleic acid in position 2 (Okumura *et al.*, 1976).

In order to modify the properties of triglyceride mixtures, the chemical interesterification method has been used in the oils and fats industry (Sonntag, 1979). But it is difficult to introduce the desired acyl group into the desired position of glycerol by chemical interesterification because the reaction shows no positional specificity (Macrae, 1983). Some microbial lipases catalyze the hydrolysis of fatty acids specifically from the outer 1- and 3-positions of triglycerides in the aqueous phase (Okumura *et al.*, 1976; Neidleman and Geigert, 1984). On the other hand, under conditions in which the amount of water in the reaction system is restricted, hydrolysis of triglycerides can be minimized so that lipase catalyzed 1,3-specific interesterification becomes the dominant reaction (Goderis *et al.*, 1986). For this reasion, a number of studies have been made on the production of cocoa butter-like fat from inexpensive olive oil and stearic acid using a 1,3-positional specific lipase in nonpolar media (Macrae, 1983; Neidleman and Geigert, 1984; Goderis *et al.*, 1986; Schuch and Mukherjee, 1987; Goto *et al.*, 1994).

Although this reaction is carried out in water-immiscible organic solvent, water is absolutely required for the catalytic capacity of enzymes. This is because water participates in all noncovalent interactions that maintain the active conformation of enzymes (Schulz and Schirmer, 1979; Zaks and Klibanov, 1985; Klibanov, 1986). Therefore, the addition of water around the enzyme molecule is responsible for the reaction in organic solvents. However, the problem is how much water is necessary. If more water than that required for the maintenance of enzyme conformation is present in the reaction mixture, the rest of it plays a role as substrate, and as a result the interesterification reaction is suppressed by thermodynamically unfavorable hydrolysis (Klibanov, 1986).

Even though the role of water in the enzymatic reaction in organic solvents was recognized well, two actual problems were encountered in regards to the difficulty of removal of excess water from the enzyme molecule during the reaction and the dependence of reaction rate on the concentration of water in the reaction mixture (Zaks and Klibanov, 1984). It is therefore necessary to decide what the purpose of the reaction is, that is, the thermodynamically favorable conditions to reaction equilibrium or reaction rate. If the decision was made to prefer the reaction equilibrium, the amount of

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water initially supplied to the reaction system has to be restricted extremely. A number of papers have shown that, even under microaqueous conditions, not only interesterification but also hydrolysis occured (Zaks and Klibanov, 1985; Kyotani *et al.*, 1988a, 1988b). For this reason, when the interesterification catalyzed by lipase is carried out in an organic solvent, it is difficult to avoid that the interesterification and the hydrolysis reaction take place simultaneously.

In the present study, the kinetics of enzymatic interesterification reaction between triolein and stearic acid in organic solvents is discussed. Recently, a few investigators have attempted to understand the functional properties of lipase in the process of modification of the properties of fats and oils (Malcata *et al.*, 1990; Malcata *et al.*, 1992a, 1992b). However, modeling of the lipase-catalyzed interesterification in nonpolar media has received relatively little attention by the scientific community.

From this point of view, a general model is proposed to elucidate the mechanism of lipase reaction in very low-water-content conditions and at the same time, the possibility of application of it for controlling bench scale production is considered.

Materials and Methods

Reagents

Triolein and stearic acid (both 99% pure) were purchased from Sigma (St. Louis, USA) and used as the substrates. The lipase obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) was of *Rhizopus delemar* origin and its activity was 600 U/mg-solid. One unit corresponded to liberating a 50 μmol equivalent of fatty acid from olive oil emulsion in the reaction period of 150 h at 30°C. The supports (Duolite A-568, weak anion-exchange resin) used for immobilization were purchased from Sasaki Chemical Co., Ltd. (Tokyo, Japan) and n-hexane used as the reaction medium was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other reagents were of analytical reagent grade.

Immobilization

Enzyme immobilization was carried out in n-hexane. That is, 3 g of a support, which contained ca. 24% (w/w) 0.3 M TES buffer (*i.e.*, N-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid, pH 6.5), was put into 100 ml of water-saturated n-hexane and then 450 mg of lipase powder was added little by little with mild magnetic stirring. After adsorptive immobilization, the n-hexane was decanted.

Control of water content

Before the use of an immobilized enzyme, its water content was decreased through dehydration for 48 h in a vacuum desiccator containing P_2O_5 powder. The water content of the desiccated immobilized enzyme was calculated by comparision of its weight before and after it was heated at 105°C for 3 h.

Time course of interesterification

A glass vessel of 60 ml volume into which 0.2 g immobilized lipase (150 mg lipase/g-Duolite), different ratios of triolein and stearic acid and 40 ml of water-saturated n-hexane as a reaction medium were loaded, was used in the reaction experiment. To prevent volatilization 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. The solvent containing reaction products was evaporated and then the residue was redissolved in 5 ml of tetrahydrofuran. This solution was analyzed by HPLC using the previously described method (Chi, 1996).

Modeling

To describe mathematically the interesterification in the batch reaction, it was assumed that *Rhizopus delemar* lipase acts only on the 1- and 3-position of the triolein (Okumura *et al.*, 1976). The three models discussed in this paper are shown in Fig. 1. Model I shows that the interesterification between triolein and stearic acid and the hydrolysis of triolein occur simultaneously since they generally compete with each other in the microaquous organic solvent. But the hydroxyl group of the produced diolein and monoolein from triolein can not accept new fatty acid. Model II shows that the interesterification has a consecutive mechanism of hydrolysis and esterification. In this model, triglyceride is first converted into diglyceride and native fatty acid by hydrolysis, followed by esterification between the diglyceride and new fatty acid. Model III is the mixed type of model I and II. This model means not only that the interesterification and hydrolysis have to be considered at the same time, but also that the hydroxyl group of the di- and monoglyceride can accept new fatty acid. In detail, the lipase forms a triglyceride-enzyme complex in the first step, followed by a diglyceride-enzyme complex and fatty acid in the second step, and finally the diglyceride-enzyme complex combines with the new fatty acid to turn into the other triglyceride-enzyme complex. Through the last stage of

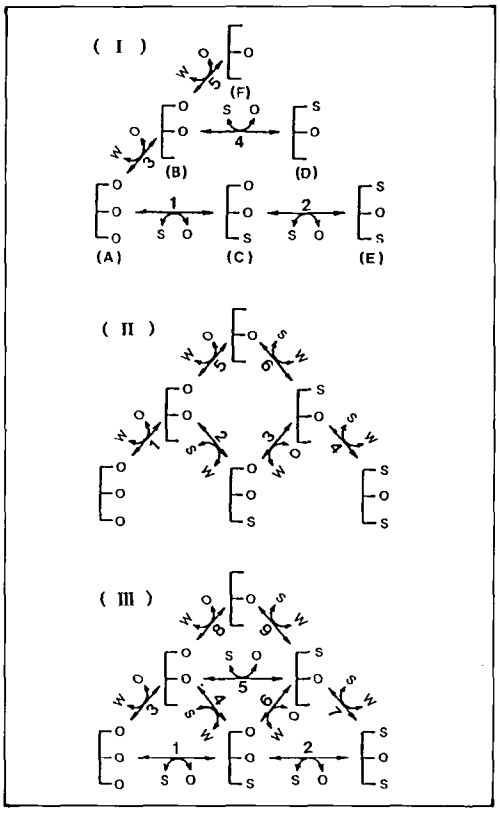

Fig. 1. Proposed reaction schemes of interesterification by 1,3-specific lipase between triolein and stearic acid in nonpolar media. (I): Interesterification and hydrolysis reaction compete with each other and the hydroxyl group of the mono- and diglyceride can not react with new fatty acids. (II): Interesterification proceeds with a consecutive mechanism of hydrolysis and esterification. (III): Mixed type of (I) and (II). Interesterification and hydrolysis compete with each other and the hydroxyl group of the mono- and diglyceride can accept new fatty acids. Each integer consists of two reaction rates. The one is forward (from left to right), the other is backward (from right to left). (A): Triolein (OOO), (B): 1,2-Dioleoyl glycerol (OO-OH), (C): 1,2-Dioleoyl-3-stearoyl glycerol (SOO), (D): 1-Stearoyl-2-oleoyl glycerol (SO-OH), (E): 1,3-Distearoyl-2-oleoyl glycerol (SOS), (F): 2-Monooleoyl glycerol (2-MO), (O): Oleic acid, (S): Stearic acid, (W): Water.

dissociation of the triglyceride-enzyme complex, interesterification is accomplished. Of course, lipase acts in the same manner on diglyceride and monoglyceride. Hydrolysis also occurs through the same manner with the exception of using water instead of fatty acid.

Kinetics

From the above models, the rate equations may be written as follows in a generalized form (Hayashi and Sakamoto, 1986).

$$\frac{dX_i}{dt} = \sum_{j=1}^{N} (-k_j A_{ij} + k_{-j} B_{ij}) \quad (1)$$

where i,j =1, 2, ···, 9, in the case of model III, X_i is the concentration of acylglycerol, fatty acids or water, k_j and k_{-1} are the rate constant (forward and backward) in the j-reaction step, A_{ij} and B_{ij} are respectively two species of reactant participating in the j-reaction step of production and consumption of i component, and N is the total number of reaction steps.

If we consider the lipase reaction based on model III, the rate equation is given by the following simultaneous differential equations:

$$\frac{d[A]}{dt} = -k_1[A][S] + k_{-1}[C][O] - k_3[A][W] + k_{-3}[B][O] \quad (2)$$

$$\frac{d[B]}{dt} = k_3[A][W] - k_{-3}[B][O] - k_4[B][S] + k_{-4}[C][W] - k_5[B][S] + k_{-5}[D][O] - k_8[B][W] + k_{-8}[F][O] \quad (3)$$

$$\frac{d[C]}{dt} = k_1[A][S] - k_{-1}[C][O] - k_2[C][S] + k_{-2}[E][O] + k_4[B][S] - k_{-4}[C][W] - k_6[C][W] + k_{-6}[D][O]) \quad (4)$$

$$\frac{d[D]}{dt} = k_5[B][S] - k_{-5}[D][O] + k_6[C][W] - k_{-6}[D][O] - k_7[D][S] + k_{-7}[E][W] + k_9[F][S] - k_{-9}[D][W] \quad (5)$$

$$\frac{d[E]}{dt} = k_2[C][S] - k_{-2}[E][O] + k_7[D][S] - k_{-7}[E][W] \quad (6)$$

$$\frac{d[F]}{dt} = k_8[B][W] - k_{-8}[F][O] - k_9[F][S] + k_{-9}[D][W] \quad (7)$$

$$\frac{d[O]}{dt} = k_1[A][S] - k_{-1}[C][O] + k_2[C][S] - k_{-2}[E][O] + k_3[A][W] - k_{-3}[B][O] + k_5[B][S] - k_{-5}[D][O] + k_6[C][W] - k_{-6}[D][O] + k_8[B][W] - k_{-8}[F][O] \quad (8)$$

$$\frac{d[S]}{dt} = -k_1[A][S] + k_{-1}[C][O] - k_{-2}[C][S] + k_{-2}[E][O] - k_4[B][S] + k_{-4}[C][W] - k_5[B][S] + k_{-5}[D][O] - k_7[D][S] + k_{-7}[E][W] - k_9[F][S] + k_{-9}[D][W] \quad (9)$$

$$\frac{d[W]}{dt} = -k_3[A][W] + k_{-3}[B][O] + k_4[B][S] - k_{-4}[C][W] - k_6[C][W] + k_{-6}[D][O] + k_7[D][S] - k_{-7}[E][W] - k_8[B][W] + k_{-8}[F][O] + k_9[F][S] - k_{-9}[D][W] \quad (10)$$

In order to calculate the rate constants, the Eq. (2)~(10) can be rewritten in matrix form (Hayashi and Sakamoto, 1986).

$$M \cdot K = T \quad (11)$$

Where M is a matrix with components of con-

centrations of chemical species. K is a column vector with elements of rate constants, and T is a column vector with elements of a derivative of concentrations of chemical species with respect to time. In the equation (11), the unknown is K, while the value of M and T can be obtained from the experimental data. Therefore, this matrix equation can be solved numerically by the Gauss-elimination method (Sugie *et al.*, 1988).

To confirm the calculated rate constants, they were substituted for simultaneous differential Eq. (2)~(10) and this set of differential equations was simulated using an adaptive step-size Runge-Kutta method (Sugie *et al.*, 1988). Finally, the calculated results of each model using the values of rate constants were compared with the experimental data.

Results and Discussion

Mass transfer considerarion

When a kinetic study is done using an immobilized enzyme, it must be considered how mass transfer limitations may affect the reaction. In this study, lipase was immobilized on Duolite (weak anion exchange resin) that has a specific surface area of about 200 m^2/g. Preliminary experimental data indicated that the reaction rate was sufficiently slow. Therefore, internal mass transfer limitations are negligible for these carriers. External mass transfer limitations between the bulk of the reaction mixture and the external surface of the carriers was also considered. The difference in substrate concentration between the solvent and the lipase surface was also negligible, because the reaction was carried out in a batch reactor under vigorous stirring. This conclusion is consistent with results of Miller *et al.* (1991) who observed no mass transfer effects in their study of the interesterification of triglycerides in cyclohexane.

Concerning the initial concentration of water

Water is both a reactant and product in the interesterification reaction as shown in the proposed model. An attempt to describe the kinetics of the interesterification reaction must be based on a knowledge of the initial concentrations of not only the acylglycerol and free fatty acids, but also that of water. When the enzyme is used in immobilized form in organic solvents, water is introduced in three forms in the reaction mixture. One is bound to the carrier , one participates in the noncovalent interaction that maintains the active conformation of enzymes, and one acts as a substrate. However, the problem is how much water is used as substrate. In this study, it was measured that 5.81 mM of water was present in the reaction mixture. For the sake of simplicity, 5 mM of water as an initial concentration was used as a substrate, when the calculation was done.

Table 1. Rate constants[a] estimated for model III of interesterification in n-hexane[b]

Forward reaction constants		Backward reaction constants	
k_1	0.364E-03	k_{-1}	0.480E-04
k_2	0.220E-03	k_{-2}	0.188E-03
k_3	0.120E-02	k_{-3}	0.104E-02
k_4	0.600E-04	k_{-4}	0.800E-04
k_5	0.240E-02	k_{-5}	0.416E-02
k_6	0.400E-05	k_{-6}	0.800E-04
k_7	0.400E-05	k_{-7}	0.400E-05
k_8	0.116E-03	k_{-8}	0.400E-05
k_9	0.400E-03	k_{-9}	0.400E-05

[a] The unit of rate constants is 1/mmol · min.
[b] Reaction was carried out at 50°C.

Kinetics

The rate constants involved in each model were first determined numerically by the Gauss-elimination method as mentioned earlier. Then they were substituted for Eq. (1), which was derived from each model. After the calculation of Eq. (1) using the Runge-Kutta method, the calculated curve and experimental data were compared. When the calculation results did not show agreement with the experimental data, rate constants were adjusted by trial-and-error method to enhance the fit between the calculated curve and experimental data. In spite of efforts to enhance the fit, the agreement between the experimental data and the simulated curves was not satisfactory when models I and II were used. However, in the case of model III, the agreement was satisfactory. The values of the rate constants are given in Table 1. The curves on Fig. 2~4 were calculated with the constants listed in Table 1 and reaction conditions were changed with respect to the ratio of triolein and stearic acid. As can be seen, it was assumed that the reaction steps of 1, 2, 3 and 5 are the main steps in the interesterification of triolein and stearic acid in nonpolar media. From the comparison of k_1 and k_3, one can see that hydrolysis is superior to interesterification thermodynamically in spite of carrying out the reaction in a nonpolar environment. Through an increase in the stearic acid concentration, interesterification was promoted to increase the amount of residual 1-stearoyl-2-oleoyl glycerol and 1,3-distearoyl-2-oleoyl glycerol, and correspondingly to decrease those of triolein and 1,2-dioleoyl glycerol (Fig. 2~

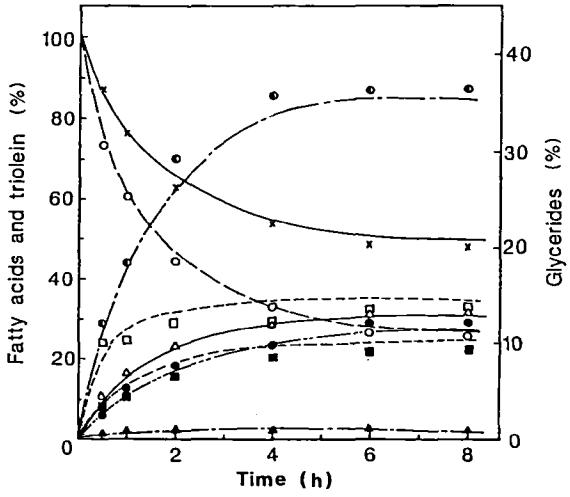

Fig. 2. Time course of the interesterification in n-hexane catalyzed by *Rhizopus delemar* lipase immobilized on Duolite at 50°C. The ordinate of the graph expresses the fraction of residual substrates or converted products. Substrate concentration ; Triolein 5.65 mM, Stearic acid 8.79 mM, Water 5.81 mM (2.09%, W/W-Duolite) for experiment and 5 mM for simulation. Symbols are of experimental value, and all lines are calculated curves based on model III. △ (——): Oleic acid, × (——): Stearic acid, ○ (— — —): OOO, ◐ (— · —): SOO, ● (— ·· —): SOS, □ (-------): OO-OH, ■ (---------): SO-OH, ▲ (— · —): 2-MO.

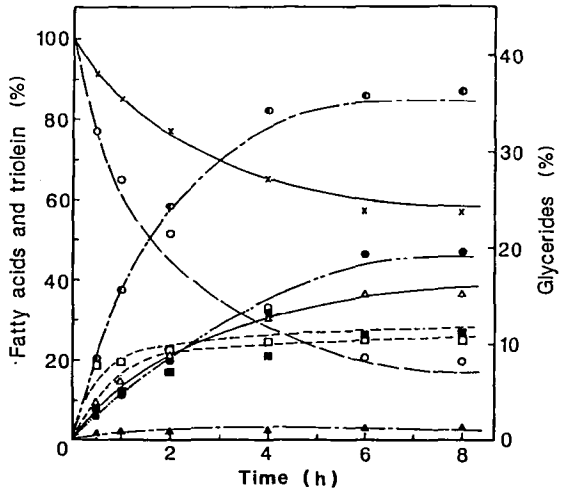

Fig. 3. Time course of the interesterification in n-hexane catalyzed by *Rhizopus delemar* lipase immobilized on Duolite at 50°C. Substrate concentration; Stearic acid 13.19 mM. The others are the same as in Fig. 2. For symbols, see Fig. 2.

4). However, the rate constants of each reaction steps were not changed, if the concentration of water was constant in reaction mixture, indicating that the mechanisms of *Rhizopus delemar* lipase can be explained using model III. The dependance of rate constants on temperature and water content in reaction

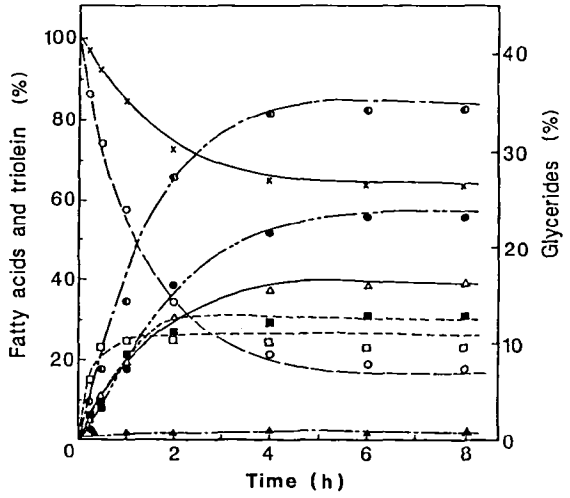

Fig. 4. Time course of the interesterification in n-hexane catalyzed by Rhizopus delemar lipase immobilized on Duolite at 50°C. Substrate concentration; Stearic acid 17.6 mM. The others are the same as in Fig. 2. For symbols, see Fig. 2.

mixture will be reported soon.

Conventionally, it has been assumed that the interesterification process proceeds through hydrolysis followed by esterification as in model II (Goderis *et al.*, 1986; Reys and Hill, 1993). However, from the results of this study, it was concluded that the interesterification and hydrolysis reaction compete with each other in the microaqueous organic solvent. This conclusion is consistent with the results of other groups (Kyotani *et al.*, 1988a; Kyotani *et al.*, 1988b). The disagreement between calculated results and experimental data in the case of model I, seems to be due to it having a deficient number of rate constants. Therefore, it is supposed that the hydroxyl group of diolein and monoolein produced from triolein reacts with the new fatty acids. As a conclusion, it was regarded that 1,3-specific lipase acts like model III.

Even though the results on 1,3-specific lipase action in organic solvents were obtained from the comparison of experimental data with that of calculation based on the proposed model, the real reaction mechanism of interesterification has not been elucidated. For this purpose, it is necessary to consider the existence of an enzyme complex with acylglycerol or with free fatty acid. However, it permits one to obtain some information on the general behavior of the system and the information obtained from the present study will be useful from an industrial point of view.

References

Chi, Y. M. (1996) *Agric. Chem. Biotech.* **39**(1), 95.

Goderis, H. L., Ampe, G., Feyten, M. P., Fouwe, B. L., Guffens, W. M., Van Cauwenbergh, S. M. and Tobbak, P. P. (1986) *Biotech. Bioeng.* **30**, 258.

Goto, M., Goto, M., Noriho, K. and Nakashio, F. (1994) *Biotech. Bioeng.* **45**, 27.

Hayashi, K. and Sakamoto, N. (1986) Dynamic Analysis of Enzyme Systems, Japan Scientific Societies Press, Tokyo.

Klibanov, A. M. (1986) *Chemtech* **6**, 354.

Kyotani, S., Fukuda, H., Morikawa, H. and Yamane, T. (1988a) *J. Ferment. Technol.* **66**, 71.

Kyotani, S., Fukuda, H., Morikawa, H. and Yamane, T. (1988b) *J. Ferment. Technol.* **66**, 567.

Macrae, A. R. (1983) *J. Am. Oil Chem. Soc.* **60**(2), 291.

Malcata, F. X., Hill, Jr. C. G. and Amundson, C. H. (1992a) *Biotech. Bioeng.* **39**, 984.

Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, Jr. C. G. and Amundson, C. H. (1990) *J. Am. Oil Chem. Soc.* **67**, 890.

Malcata, F. X., Reyes, H. R. Garcia, H. S., Hill, Jr. C. G. and Amundson, C. H. (1992b) *Enzyme Microb. Technol.* **14**, 426.

Miller, D. A., Prausnitz, J. M. and Blanch, H. W. (1991) *Enzyme Microb. Technol.* **13**, 98.

Neidleman, S. L. and Geigert, J. (1984) *J. Am. Oil Chem. Soc.* **61**, 290.

Okumura, S., Iwai, M. and Tsujisaka, Y. (1976) *Agric. Biol. Chem.* **40**(4), 655.

Reys, H. R. and Hill, Jr. C. G. (1993) *Biotech. Bioeng.* **43**, 171.

Schuch, R. and Mukherjee, K. D. (1987) *J. Agric. Food Chem.* **35**, 1005.

Schulz, G. E. and Schirmer, R. H. (1979) Principles of Protein Structure, Springer-Verlag, New York.

Sonntag, N. O. V. (1979) in Bailey's Industrial Oil and Fats Products, Vol. 1 (Swern, D., ed.) pp. 99-175, John Wiley & Sons, New York.

Sugie, H., Okazaki, A., Adachi, Y. and Okazaki, M. (1987) Numerical Calculation Method by FORTRAN 77, Baifukan, Tokyo.

Zaks, A. and Klibanov, A. M. (1984) *Science* **224**, 1249.

Zaks, A. and Klibanov, A. M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3192.