Chapter 2

Lipase Modification for Interesterification in *n*-Hexane

2-1 Selection of Surfactant-Modified Lipases for Interesterification of Triglycerides and Fatty Acids

Introduction

In this section, the interesterification activities of the modified lipases in organic solvent using various kinds of lipases and food-grade surfactants were investigated. The best combination of the surfactant modified lipase giving the highest activity and protein recovery was proposed. The effect of the lipase-surfactant ratio during the modification process was studied. Using the selected modified lipase, the interesterification activity in non-solvent system at high temperature was also investigated. Tripalmitin and stearic acid were used as model substrates, and interesterification reaction was carried out in *n*-hexane, in which palmitic and stearic acids are abundant fatty acids in vegetable oil. *n*-Hexane is commonly used in fat and oil industry, for instance, oil extraction from soybeans. For practical application, preliminary study in non-solvent system was also carried out to compare the reaction rates.

Materials and Methods

Materials

Lipases: Crude lipases, Newlase F (*Rhizopus niveus*), Lipase F Amano (*Rhizopus* sp.), Lipase AY 30 (*Candida rugosa*), Lipase A Amano 6 (*Aspergillus niger*) and Lipase M Amano 10 (*Mucor javanicus*) were supplied from Amano Pharmaceutical Co., Ltd. (Tokyo, Japan). Lipase A 5 (*Rhizopus japonicus*), Lipase Saiken 100, (*Rhizopus japonicus*), Lipase B 4 (*Rhizopus japonicus*) and Roosepase FD (*Rhizopus japonicus*) were supplied from Nagase Biochemicals Ltd. (Kyoto, Japan). Lipase OF (*Candida cylindracea*), Lipase MY (*Candida cylindracea*) and LIPASE PL (*Alcaligenes* sp.) were supplied from Meito Sangyo Co., Ltd. (Tokyo, Japan). Lipolase 100 T (*Aspergillus oryzae*) and Palatase M 1000 L (*Mucor miehei*) were supplied from Novo Nordisk A/S (Bagsvaerd, Denmark). Lipase Sankyo (*Aspergillus* sp.) was supplied from Sankyo Co., Ltd. (Tokyo, Japan). Talipase (*Rhizopus delemar*) was supplied from Tanabe Seiyaku Co., Ltd. (Tokyo, Japan). Lipase Asahi (*Chromobacterium viscosum*) was

supplied from Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Lipase Kurita (*Psudomonas* sp.) was supplied from Kurita Co., Ltd. (Tokyo, Japan) respectively.

Surfactants: Food-grade surfactants, sorbitan esters and sugar esters were used. Sorbitan esters: Emazol L-10 (F) (sorbitan monolaurate), Emazol P-10 (F) (sorbitan monopalmitate), Emazol S-10 (F) (sorbitan monostearate), Emazol S-30 (F) (sorbitan tristearate), Emazol O-10 (F) (sorbitan monooleate) and Emazol O-30 (F) (sorbitan trioleate) were supplied from Kao Co., Ltd. (Tokyo, Japan). Sorgen 30 (sorbitan sesquioleate) was supplied from Daiichi Kogyo Seiyaku Co., Ltd. (Tokyo, Japan). Nonion CP-08R (sorbitan monocaplirate) and Nonion OP-80R (sorbitan monooleate) were supplied from Nippon Oil & Fats Co., Ltd. (Tokyo, Japan) respectively.

Sugar esters: DK-Ester F-10, F-20, F-50, F-70, F-90, F-110, F-140 and F-160 (sucrose palmitate and stearate mixture) were supplied from Daiichi Kogyo Seiyaku Co., Ltd. (Tokyo, Japan). Ryoto Sugar Ester ER-190 and ER-290 (sucrose erurate) and O-170 (sucrose oleate) were supplied from Mitsubishi Kasei Corp. (Tokyo, Japan).

Phospholipids: Lecithin DX and Beisisu LG-10E were supplied from Nisshin Oil Co., Ltd. (Tokyo, Japan).

Chemicals: Tri-, di-, monopalmitin, tri-, di-, monostearin, 1-palmitoyl-3-stearoyl glycerol with more than 99% purity were purchased from Sigma Chemical Company (St. Louis, MO, USA). 1,2-dipalmitoyl-3-stearoyl glycerol (PPS) and 1,3-distearol-2-palmitoyl glycerol (SPS) with more than 98% purity were supplied from Unilever Research Colworth Laboratory, UK. Palmitic acid, stearic acid, *n*-hexane, ethanol, pyridine, molecular sieves (4A 1/16) and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) all of analytical grade were purchased from Wako Pure Chemical Ind. (Tokyo, Japan). Trimethyl chlorosilane (TMS) was purchased from GL Science Co., Ltd. (Tokyo, Japan).

Lipase modification procedure

Lipase, 3 g, was dissolved in 1 L ion-exchanged water. Surfactant, 0.75 g, dispersed in 20 mL ethanol (40°C) were added to the lipase solution. The mixture was sonicated at 600 W for 15 min and stirred at 600 rpm and 5°C for 2 h. The precipitate was obtained by centrifugation at 8000 rpm (7000 x g) and 5°C for 10 min, frozen and freeze dried. The modified lipase was obtained as a powder.

Interesterification experiment in n-hexane system

The substrates of tripalmitin (0.5 g) and stearic acid (0.5 g) were dissolved in 50 mL *n*-hexane where the water concentration was decreased by molecular sieves. The modified lipase (30 mg) was added to the reaction mixture and the interesterification reaction was carried out at 500 rpm stirring and 40°C for 3 h. Samples were taken from the reaction system at prescribed time intervals and were filtered (LCR13-LH, pore size 0.5 μm, Millipore Co., Milford, MA, USA) and then analyzed by Gas chromatography (GC).

Interesterification experiment in non-solvent system

Substrates of tripalmitin (5 g) and stearic acid (5 g) were melted at 75°C in a flask with lid. The modified lipase (300 mg) was added to the reaction mixture and interesterification was carried out at 500 rpm and 75°C for 3 h. Samples were taken from the reaction system at prescribed time intervals, dissolved in *n*-hexane and filtered prior to GC injection.

Interesterification activity

Interesterification activity of the modified lipase was described in terms of the specific reaction rate constant, k* [m^6/(mol•g•s)], which was based on two-substrates, two-products reversible reaction system, assuming second order reaction kinetics (detail

explanation is in Chapter 3-1). The value, k*, reflects the conversion rate of the tripalmitin to PPS and SPS per g protein of the modified lipase.

First lipase screening for the modified lipase

Nineteen different lipases were screened to obtain the modified lipase having high interesterification activity in the *n*-hexane system and high protein recovery. Emazol S-10 (F), sorbitan monostearate, was used for surfactant in each modification process.

Second lipase screening for the modified lipase

The modified lipases, which were obtained from the lipases and the surfactant in different buffer solutions of McIlvaine buffer[19] ranging pH 4 to 8, were analyzed for their interesterification activities and protein recoveries. Lipases selected by the first lipase screening were used for the investigation. The ratio of lipase protein weight and surfactant weight during the modification was the same in each modified lipase preparation.

Surfactant screening for the modified lipase

Twenty two kinds of surfactants were screened to use for modified lipase formation. Lipase used for the screening was the lipase selected by the second lipase screening.

Investigation of the lipase-surfactant ratio

To obtain the modified lipase efficiently, the weight ratio of lipase and surfactant used in the modification process was investigated. Lipase Saiken 100 as lipase and Emazol S-10 (F) as surfactant were used. The lipase-surfactant ratio was described as the R value which was the weight ratio of lipase protein amount and surfactant amount during the modification (lipase protein / surfactant). The modified lipases were obtained from the combination of the lipase (0.5 - 5.0 g) and the surfactant (0.4 - 5 g).

The interesterification activity and protein yield were investigated under the different R values.

Analysis method

For the analysis of the triglycerides and fatty acids, which existed in the reaction system, the sample was injected to the GC with internal standard (*n*-hexadecane). For partial glycerides, the sample was treated by the silylation method as follows[10]. The reaction solvent, *n*-hexane, was evaporated at 60°C with nitrogen flow from 0.5 mL of the sample. The dried sample was mixed with 1 mL of internal standard solution (*n*-hexadecane in pyridine), 0.2 mL BSTFA and 0.1 mL TMS. The mixture was kept at 70°C for 30 min to allow silylation of the carboxyl and hydroxyl groups. After cooling down to room temperature, the mixture was injected to the GC.

GC analysis was performed on a GC-14AH Gas Chromatography (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (Ulbon HR-TGC1, 0.25 mm internal diameter, 25 m column length, 0.1 μm film thickness, Shinwa Chemical Industries, Ltd., Tokyo, Japan) and a flame-ionization detector (FID). The operating temperature was from 80 to 360°C, programmed at the rate of 10°C/min, with a final hold of 5 min. The injector and detector temperatures were set up at 370 and 400°C respectively. Helium was used to as the carrier gas, 5 mL/min flow rate and 1:10 split ratio. Air, 0.5 kg/cm^2 and hydrogen, 0.6 kg/cm^2 were also used.

Water concentration of the reaction system was analyzed by Karl Fischer water determination, 684 KF Coulometer (Metrohm Ltd., Herisau, Switzerland). The sample of water analysis from the reaction system was injected to the coulometer without the filtration. The water concentration of *n*-hexane in which water content was decreased by molecular sieve was below 10 mg/L.

The protein contents of crude and modified lipases were analyzed by Automatic Nitrogen Analyzer, FP-428 (Leco Co., St Joseph, MI, USA).

Results and Discussion

Figure 2-1-1 shows the typical time course of the interesterification of tripalmitin and stearic acid using the modified lipase obtained from Lipase Saiken 100 and Emazol S-10 (F). The concentrations of tripalmitin and stearic acid decreased, and palmitic acid, 1,2-dipalmitoyl-3-stearoyl glycerol (PPS) and 1,3-distearoyl-2-palmitoyl glycerol (SPS) were produced with time. The reaction system seemed to reach steady state after 2 h reaction. Tristearin (SSS) was not produced at all, which implied the modified lipase had 1,3 specificity. Monoglycerides were not produced at all. The amount of diglycerides produced were less than 6% of the triglycerides. In this system, the initial water concentration was below 30 mg/L. The modified lipase had interesterification activity even at such low water concentrations.

To obtain modified lipase having high interesterification activity, the modified lipases which were obtained from various lipases and surfactants were analyzed for activity and protein yield.

First lipase screening

Table 2-1-1 shows the interesterification activity and protein yield of the modified lipases obtained from various kinds of lipases, which were classified by their 1,3 specificity. Sorbitan monostearate was used as surfactant, and all of the crude lipases could produce precipitates. The protein concentrations of the modified lipases varied from 1.15 to 18.56%. The k* values of the modified lipases obtained from the crude lipases having 1, 3 specificity were 3 - 32 [$10^{-9}m^6/(mol \cdot g \cdot s)$]. On the other hand, the k* values of the modified lipases obtained from the crude lipases having no 1,3 specificity were almost zero. Total activity of the modified lipase was evaluated by the relative activity yield, on the basis of unit of crude lipase protein, which was obtained from k* multiplied by the protein recovery. On the total activity evaluation, Lipase Kurita was determined to have the highest activity yield. From this first screening, the lipases having high total activity, Lipase Kurita (*Psudomonas* sp.),

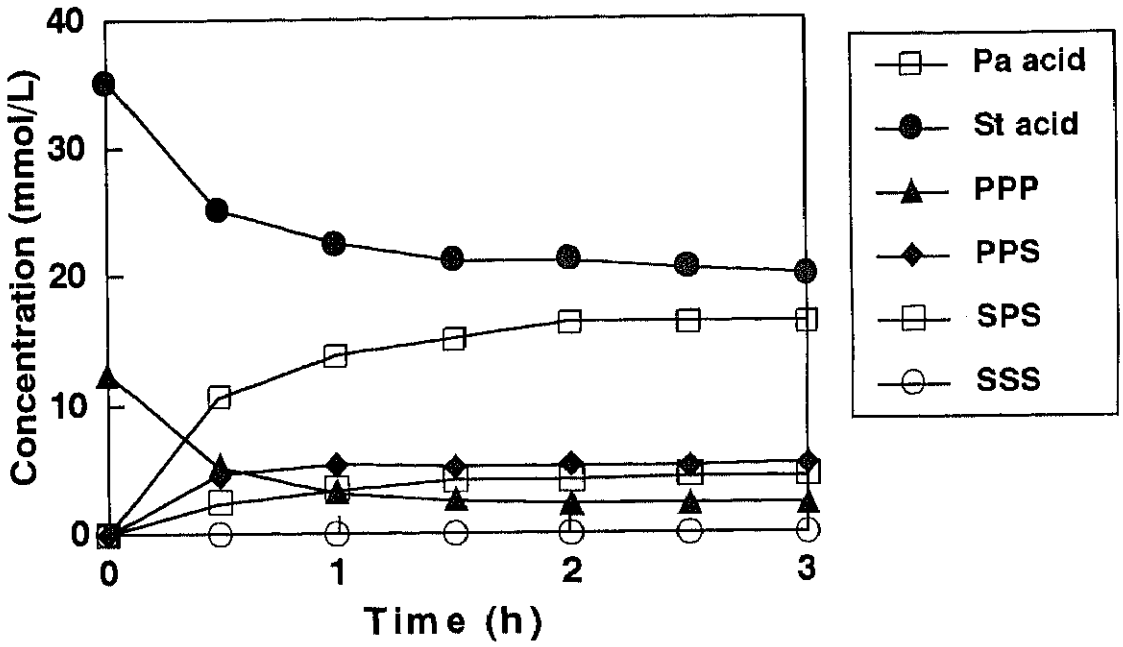

Fig. 2-1-1 Interesterification reaction time course using the modified lipase obtained from Lipase Saiken 100 (*Rhizopus japonicus*) and Emazol S-10(F) (sorbitan monostearate). Substrates were 0.5 g of tripalmitin and 0.5 g of stearic acid. The amount of the modified lipase was 30 mg. The reaction was carried out at 500 rpm stirring and 40°C in the *n*-hexane system.
Pa acid; palmitic acid, St acid; stearic acid, PPP; tripalmitin, PPS; 1,2-dipalmitoyl-3-stearoyl glycerol, SPS; 1,3-distearoyl-2-palmitoyl glycerol, SSS; tristearin.

Table 2-1-1 Modified lipase activities obtained from various kinds of lipases during first lipase screening

Lipase	Source	1,3 specificity* of crude lipase	Modified lipase amount (g/L)	Modified lipase protein (%)	Protein recovery(%)	Activity k* x 10^9 [m^6/(mol•g•s)]	Relative activity yield
Newlase F	*Rhizopus niveus*	Y	0.303	2.61	3.2	11	17
Lipase F Amano	*Rhizopus* sp.	Y	0.366	6.64	5.1	19	46
Lipolase 100 T	*Aspergillus oryzae*	Y	0.477	3.26	33.6	4	64
Palatase M 1000 L	*Mucor miehei*	Y	1.864	1.15	2.9	3	4
Lipase A Amano 6	*Aspergillus niger*	Y	0.329	4.05	6.2	3	9
Lipase M Amano 10	*Mucor javanicus*	Y	0.502	12.68	22.2	4	42
Talipase	*Rhizopus delemar*	Y	1.868	2.08	5.1	32	77
Lipase Saiken 100	*Rhizopus japonicus*	-	0.364	7.15	3.5	44	73
Lipase A 5	*Rhizopus japonicus*	-	0.392	2.84	7.0	17	56
Lipase B 4	*Rhizopus japonicus*	-	0.593	15.04	35.5	0	0
Roosepase FD	*Rhizopus japonicus*	-	0.869	15.01	45.6	0	0
Lipase PL	*Alcaligenes* sp.	-	0.449	14.57	19.3	3	27
Lipase Asahi	*Chromobacterium viscosum*	-	0.419	4.49	2.5	60	71
Lipase Kurita	*Pseudomonas* sp.	-	0.333	13.10	5.7	37	100
PPL	*Porcine pancreas*	-	0.538	18.56	12.4	0	0
Lipase AY 30	*Candida rugosa*	N	0.348	3.11	20.1	0	0
Lipase OF	*Candida cylindracea*	N	0.170	11.60	7.9	1	4
Lipase MY	*Candida cylindracea*	N	0.362	4.39	12.6	0	0
Lipase Sankyo	*Aspergillus* sp.	N	0.320	2.64	5.9	0	0

* Y : yes, N : no, - : unknown

Lipase Saiken 100 (*R. japonicus*), Talipase (*R. delemar*) and Lipase Asahi (*C. viscosum*) were selected for further investigation. Because it has been reported that the lipase from *M. miehei* had 1,3 specific interesterification activity with the immobilized form in organic solvent, *M. miehei* was also selected as a standard[47,26,77].

Second lipase screening

The modified lipases which were obtained from Lipase Kurita, Talipase, Lipase Saiken 100, Lipase Asahi, and Palatase M 1000 L, and surfactant, Emazol S-10 (F), in the buffer solution, McIlvaine buffer, pH4 to 8, were investigated for the effect of pH during the modification process. Table 2-1-2 shows the effect of pH on the protein recovery and interesterification activity. Total activity of the modified lipase can be evaluated by the relative activity yield. Except Platase M 1000 L, pH condition during the modification affected the modified lipase amount, modified lipase protein concentration, protein recovery and interesterification activity. The amount of the modified Lipase Kurita obtained at pH5 (2.153 g) was almost three fold that obtained at pH6 to 8. The protein recovery (14.9%) and the modified Lipase Kurita activity [80 x $10^{-9}m^6/(mol \cdot g \cdot s)$] at pH5 were high, giving the highest relative activity yield of all the modified lipases. In the case of Lipase Saiken 100, the amounts of the modified lipases obtained at pH5 and 6, were higher than those at pH7 and 8. The modified Lipase Saiken 100 obtained at pH5 had high protein recovery (13.7%) and activity [87 x $10^{-9}m^6/(mol \cdot g \cdot s)$], and the relative activity yield was the highest of all the modified lipases (same value as the modified lipase obtained from Lipase Kurita at pH5). In the case of Lipase Asahi, the amounts of the modified lipases obtained at pH5 and 6 were almost twice of those at pH7 and 8. The modified Lipase Asahi at pH6 showed the highest activity [100 x $10^{-9}m^6/(mol \cdot g \cdot s)$] among the all kinds modified lipases, and the relative activity yield reached 99%. In the case of Talipase, higher amount of the modified lipases were collected than the other kinds of modified lipases, however, the protein recovery and activity were low, then, the relative activity yields were 0.9 to

Table 2-1-2 Effect of pH during modification on the interesterification activities of selected modified lipases during the second lipase screening

Lipase	Source	pH	Modified lipase amount (g/L)	Modified lipase protein (%)	Protein recovery(%)	Activity $k^* \times 10^9$ [$m^6/(mol \cdot g \cdot s)$]	Relative activity yield (%)
Lipase Kurita	*Pseudomonas* sp.	5.0	2.153	5.30	14.9	80	100
		6.0	0.713	11.10	10.4	42	37
		7.0	0.333	13.10	5.7	37	18
		8.0	0.527	10.40	7.2	44	27
Talipase	*Rhizopus delemar*	4.0	2.335	0.83	2.6	4	0.9
		5.0	2.367	1.34	4.2	20	7
		6.0	1.042	1.22	1.7	30	4
		7.0	1.868	2.08	5.1	32	14
Lipase Saiken 100	*Rhizopus japonicus*	5.0	0.866	11.90	13.7	87	100
		6.0	0.491	10.30	6.7	59	33
		7.0	0.364	7.15	3.5	44	13
		8.0	0.159	7.95	1.7	49	7
Lipase Asahi	*Chromobacterium viscosum*	5.0	1.092	8.07	11.8	93	92
		6.0	1.248	7.09	11.8	100	99
		7.0	0.419	4.49	2.5	60	13
		8.0	0.694	3.11	2.9	99	24
Palatase M 1000 L	*Mucor miehei*	5.0	1.784	0.97	2.3	5	1
		6.0	1.547	1.28	2.6	3	0.7
		7.0	1.864	1.15	2.9	3	0.7
		8.0	1.574	1.36	2.9	0	0

14%. In the case of Palatase M 1000 L, relatively high amount of the modified lipases were collected, however, activities were very low.

Table 2-1-3 shows the diglycerides (DG) and monoglycerides (MG) production of modified lipases after 3 h reaction. The modified Lipase Kurita at pH5 produced 17.5wt% DG and 2.76 wt% MG after 3 h reaction. However, the modified Lipase Saiken 100 at pH5 produced 3.58 wt% DG and no MG at all. The DG produced by the modified Lipase Asahi at pH5 and 6 were 5.58wt% and 5.33wt% respectively, and no MG were produced by both the modified lipases. All modified lipases were freeze dried and all free water was assumed to be removed during the process. The initial water concentrations in all reaction mixtures ranged from 20 to 80 mg/L. The produced amount of DG and MG were dependent on lipases and modification conditions.

On the basis of the total activity yield, modified Lipase Kurita at pH5, modified Lipase Saiken 100 at pH5 and modified Lipase Asahi at pH6 were estimated to be appropriate. However, because of the considerable amount of DG and MG production, Lipase Kurita was not selected. Lipase Asahi was not selected because of the expensive cost for diagnostic use in medical area.

Concerning the interesterification activity, DG and MG production and the cost performance, Lipase Saiken 100 (*R. japonicus*) was selected as the most suitable lipase for the modified lipase formation.

Surfactant screening

Using Lipase Saiken 100 (*R. japonicus*) surfactant screening was carried out. Table 2-1-4 shows the surfactants used for the modified lipase formation and the obtained modified lipase activities. Three solid types and 6 liquid types of sorbitan ester were used. Eight solid types and 3 liquid types of sugar ester and 2 types phospholipids were also used. Sorbitan esters and sugar esters were classified by HLB as proposed by Griffin[31]. HLB describes the ratio of the hydrophilic radicals in a

Table 2-1-3 Monoglycerides (MG) and diglycerides (DG) produced by modified lipases after three hours

Lipase	Source	pH	MG (wt%)	1,3 DG (wt%)	1,2 DG (wt%)	DG total (wt%)
Lipase Kurita	*Pseudomonas* sp.	5.0	2.76	13.00	4.50	17.50
		6.0	0.00	5.24	2.02	7.26
		7.0	0.00	5.08	1.55	6.63
		8.0	0.00	5.11	1.62	6.73
Talipase	*Rhizopus delemar*	4.0	1.22	1.63	3.03	4.66
		5.0	0.94	1.14	4.14	5.28
		6.0	0.68	1.05	2.67	3.72
		7.0	0.75	2.45	3.02	5.47
Lipase Saiken 100	*Rhizopus japonicus*	5.0	0.00	0.00	3.58	3.58
		6.0	0.00	0.00	4.28	4.28
		7.0	0.00	0.00	5.99	5.99
		8.0	0.00	0.00	7.48	7.48
Lipase Asahi	*Chromobacterium viscosum*	5.0	0.00	4.38	1.20	5.58
		6.0	0.00	4.29	1.04	5.33
		7.0	0.76	4.82	1.97	6.79
		8.0	0.75	5.22	1.27	6.49
Palatase M 1000 L	*Mucor miehei*	5.0	0.80	1.06	4.05	5.11
		6.0	0.86	1.19	3.50	4.69
		7.0	0.00	1.78	4.59	6.37
		8.0	0.63	1.35	4.90	6.25

Table 2-1-4 Modified lipase activity obtained from various kinds of surfactants

Surfactant		HLB	Modified lipase amount (g/L)	Modified lipase protein (%)	Protein recovery (%)	Activity $k^* \times 10^9$ $[m^6/(mol \cdot g \cdot s)]$	Relative activity yield (%)
Sorbitan ester							
Emazol O-30(F)	Sorbitan trioleate*	1.8	NP**	-	-	-	-
S-30(F)	Sorbitan tristearate	2.1	0.238	3.80	1.9	0	0
O-10(F)	Sorbitan monooleate*	4.3	NP	-	-	-	-
S-10(F)	Sorbitan monostearate	4.7	0.364	7.15	3.5	44	100
P-10(F)	Sorbitan monopalmitate	6.7	0.236	6.08	3.0	32	62
L-10(F)	Sorbitan monolaurate*	8.6	0.133	7.51	2.1	8	11
Sorgen 30	Sorbitan sesquioleate*	3.7	NP	-	-	-	-
Nonion OP-80R	Sorbitan monooleate*	4.3	1.190	9.75	31.5	0	0
Nonion CP-08R	Sorbitan monocaplirate*	9.5	0.303	17.94	14.7	4	38
Sugar ester							
DK-Ester F-10	Sucrose palmitate / stearate	1.0	0.250	7.78	1.6	0	0
F-20	Sucrose palmitate / stearate	2.0	0.216	6.86	1.2	0	0
F-50	Sucrose palmitate / stearate	6.0	0.210	8.81	1.5	13	13
F-70	Sucrose palmitate / stearate	8.0	0.206	7.44	1.3	6	5
F-90	Sucrose palmitate / stearate	9.5	0.410	7.16	2.5	8	13
F-110	Sucrose palmitate / stearate	11.0	0.323	6.98	1.8	5	6
F-140	Sucrose palmitate / stearate	13.0	0.693	4.89	2.8	9	16
F-160	Sucrose palmitate / stearate	15.0	1.508	4.76	5.9	7	27
Ryoto Sugar Ester							
ER-190	Sucrose erurate*	1.0	NP	-	-	-	-
ER-290	Sucrose erurate*	2.0	NP	-	-	-	-
O-170	Sucrose oleate*	1.0	NP	-	-	-	-
Phospholipid							
Lecithin DX*		-	NP	-	-	-	-
Beisisu LG-10E*		-	NP	-	-	-	-

* : liquid, the others are solid

** : NP : no precipitate was observed

surfactant, and it varies from 0 to 20 (0 corresponds most hydrophobic, 20 corresponds most hydrophilic)[84].

In the case of sorbitan esters of HLB lower than 4.3, almost no precipitates were observed, or the obtained precipitates (modified lipases) showed no activity. With sorbitan monostearate of HLB 4.7, the highest k* and activity yield were obtained. With the other sorbitan esters of HLB 6.7 - 9.5, the activity yields were determined to be 12 - 68% of the sorbitan monostearate. In the case of sugar esters of HLB lower than 2, almost no precipitates were observed, or the obtained precipitates (modified lipases) showed no activity. With sugar esters of HLB in the range 6.0 - 13.0, the k* value, protein recoveries and relative activity yield did not depend on HLB. At HLB 15, the highest protein recovery of 5.9% and the highest relative activity yield of 27% were observed among the modified lipases with sugar esters. In the case of phospholipids, no precipitates were obtained and cloudy solutions were suspended after centrifugation. Among the 9 liquid surfactants (6 sorbitan esters and 3 sugar esters), 6 did not give any precipitates due to stable cloudy solution. The other 3 gave precipitates with considerable protein recoveries, however, the observed k* values were not so high, which is probably due to differences in fatty acid groups. From these results, EMAOL S-10(F), sorbitan monostearate, HLB 4.7, was selected as the most suitable surfactant for the modified lipase formation. Goto *et al.*[28] reported that the esterification activity of the surfactant modified lipase in organic solvent was influenced by the hydrophobic groups of the surfactant. They suggested that the surfactant, having a large number of hydrophobic groups, is better for lipase modification, because of its solubility in the organic solvent. From our results, both HLB and fatty acid group in surfactants significantly affected the activities and yields.

Investigation of lipase-surfactant ratio

Figure 2-1-2 shows the relationship of relative interesterification activity and protein yield against the R value (lipase protein/surfactant). As the R value increased

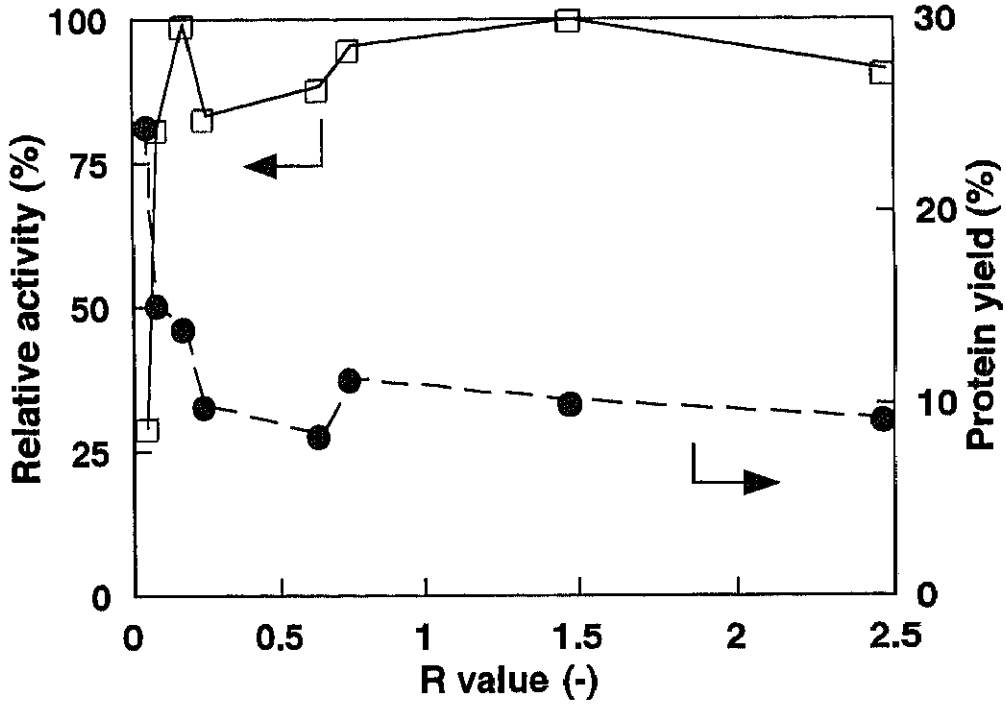

Fig. 2-1-2 Relative interesterification activity and protein yield of modified lipase obtained with different R values (lipase protein/surfactant).

between 0 and 0.5, protein yield became lower, however, interesterification activity became higher. At the R values higher than 0.5, interesterification activity and protein yield were not changed. Approximately one molecule of modified lipase was found to have 400 - 4000 molecules of surfactants at R values of 2.5 to 0.25 respectively. In my previous study, the modified lipase obtained at the R value of 1 could keep the original activity for more than 70 h in continuous operation (Chapter 4). An R value of 1 was selected as the most suitable for the modification process.

Summarizing the condition of the modification, Lipase Saiken 100 (*R. japonicas*) as lipase, Emazol S-10 (F), Sorbitan monostearate with HLB 4.7 as surfactant and the R value of 1 were chosen.

Interesterification in non-solvent system

The interesterification experiment of tripalmitin and stearic acid in non-solvent system was also carried out at 75°C using the modified lipase, which was obtained from the selected modification condition. Figure 2-1-3 shows the comparison of interesterification activity using *n*-hexane and a solvent free system in the reaction systems. The interesterification activity was described by the conversion of stearic acid incorporated into tripalmitin. The reaction temperatures in the non-solvent and the *n*-hexane systems were 75°C and 40°C respectively. Substrates were tripalmitin and stearic acid. The amount of the modified lipase was 3% of the amount of substrates in both systems. Changes in conversion from tripalmitin to PPS and SPS were almost the same in both systems. Both systems almost reached steady state after 2 to 3 hours. The activity of modified lipase at 75°C in non-solvent system was found to be almost same as that at 40°C in *n*-hexane system.

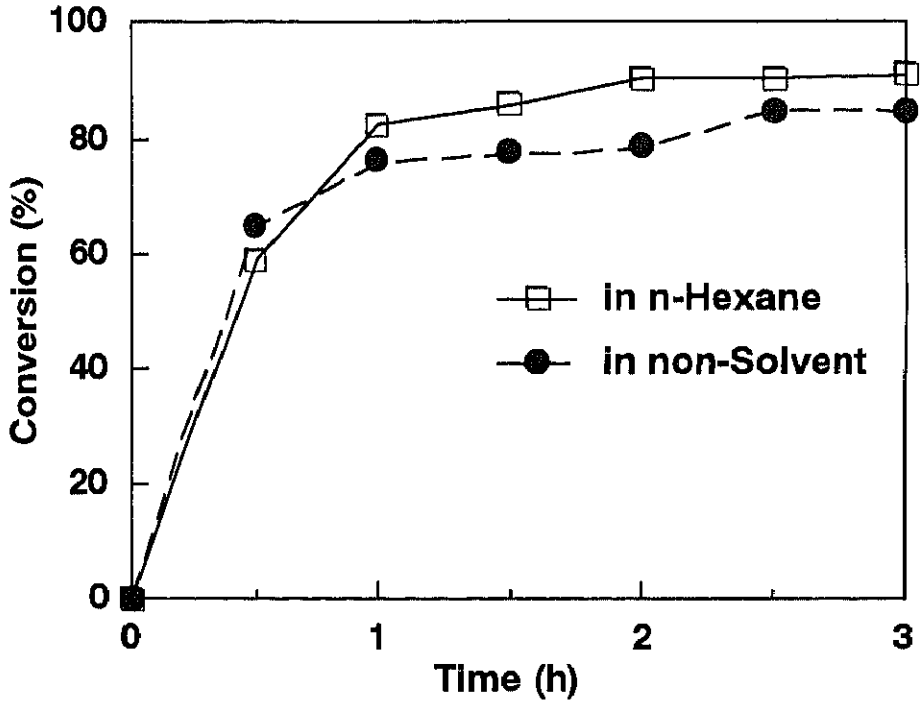

Fig. 2-1-3 Comparison of the interesterification activities in *n*-hexane and non-solvent in the reaction system. The modified lipase obtained from Lipase Saiken 100 (*Rhizopus japonicus*) and Emazol S-10(F) (sorbitan monostearate) was used. In *n*-hexane system, the reaction was carried out in 50 mL *n*-hexane containing 0.5 g of tripalmitin, 0.5 g of stearic acid and 30 mg of modified lipase at 40°C. In the non-solvent system, the reaction was carried out with 5 g tripalmitin, 5 g stearic acid, and 300 mg of modified lipase at 75°C.

2-2 Optimal Conditions for the Preparation of Modified Lipase Having High Interesterification Activity

Introduction

Generally, water activity is of vital importance in determining the activity of enzymes and the chemical equilibrium position of reactions in organic solvent systems. High water activity in the reaction system shifts the reaction towards hydrolysis, and reduced water activity shifts the reaction towards synthesis. Therefore, the activity of water is lowered so that the occurrence of undesirable hydrolysis products, such as di-, monoglycerides and glycerol, is minimized, but the water available is sufficient for the enzyme to retain its activity. Water content (as a measure for water activity in the reaction system) requirements for different enzymes vary considerably, but typically for interesterification reactions of triglycerides and fatty acids in organic solvents 1-4 wt% supplemented water is required[76,88,99,108].

In Chapter 2-1, the modified lipase obtained from Lipase Saiken 100 (*R. japonicus*) with sorbitan monostearate as surfactant had the highest interesterification activity in the *n*-hexane was described. However, the optimum reaction conditions in *n*-hexane system were not revealed yet. In this section, to make clear the characteristics of modified lipase activity, the specificity and activity of the modified lipase and the effect of water content in the reaction system on the activity were investigated. During the lipase modification process it was observed that the percentage of recovered protein in the modified lipase was only 10%, and the recovered hydrolysis activity was less than 50%. To obtain the modified lipase efficiently, higher recoveries of protein and activity are required. In this section, the repetitive modification to recover higher protein and activity was also investigated.

Materials and Methods

Materials

All chemicals and Lipase Saiken 100 used in this section were mentioned in Chapter 2-1. Lipase D Amano 20 (*Rhizopus delemar*; protein content, 27.7%) was supplied by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Lipozyme 10000 L

(*Rhizomucor miehei*; protein content, 5.0%) was supplied by Novo Nordisk A/S (Bagsvaerd, Denmark).

Analytical methods

The analysis of tri-, di-, and monoglycerides and fatty acids were done by GC and high-performance liquid chromatography (HPLC). GC analysis followed the method described in Chapter 2-1. For HPLC analysis, the n-hexane-free samples were mixed with 1 mL of internal standard solution (10 mg tripentadecanoin dissolved in 10 mL pyridine) and then analyzed by HPLC (model 600E; Waters, Milford, MA, USA; equipped with a Mass Detector, model 750/14; ACS, Macclesfield, UK). The column used to separate triglycerides was LiChrospher 100, RP-18 (5 μm) (Cica-Merck, Tokyo, Japan). The mobile phase was a gradient mixture of acetone and acetonitrile. The gradient set point and solvent compositions are given in Table 2-2-1.

Water content in the reaction system was measured with a Karl Fisher Titrator 684 KF. The modified lipase after freeze-drying contained 0.041 mg H_2O/mg biocatalyst. The water content in *n*-hexane was reduced to 10 mg/L by drying over molecular sieve. The modified lipase was desiccated below 5°C and all substrates were kept below 0°C.

Lipase modification

The modification procedure of the lipase with the surfactant performed for this study was as follows: Crude Lipase Saiken 100 (3 g, protein content 11 wt%) was dissolved in 1 L Tris (hydroxymethyl) aminomethane buffer adjusted to pH 5 and stirred magnetically at 4°C. The buffer solution was prepared by dissolving 0.61 g Tris (hydroxymethyl) aminomethane in 1 L de-ionized water and the pH was adjusted to a desired pH value by adding appropriately 5 M NaOH or 3 M HCl solutions. Sorbitan monostearate (0.75 g) dissolved in 20 mL ethanol was added dropwise into the stirred lipase solution. The mixture was sonicated for 15 min and then stirred for 2 h at 5°C.

Table 2-2-1 Solvent gradient for HPLC analysis[*1]

	Solvent composition[*2]		
Time (min)	A (%)	B (%)	Comment
0	55	45	Inject
3	55	45	
11	95	5	
12	55	45	Regenerating column
17	55	45	Next injection

[*1]Flow rate was set at 2 mL/min and the column temperature kept at 30°C

[*2]A, acetone and B, acetonitrile

The precipitate produced in the mixture was collected by centrifugation, frozen at -20°C, and then freeze-dried to remove water. The solid collected after freeze drying had a yellowish color.

The protein content of the modified lipase was measured by a nitrogen analyzer (Chapter 2-1), and was determined to be 11 wt%. The protein content of the complex was found to be dependent on the pH of the buffer solution and on the weight ratio of surfactant to protein used during the modification process. The solubility of the complex in most organic solvents dried over molecular sieve was found to be very poor. However, it was found that the solubility of the complex is very dependent on the content of water (estimated to be less than 0.5 g/L in *n*-hexane containing 25 mg/L of water). Throughout this section the measured protein content either of the crude Lipase Saiken 100 or of the modified lipase complex was considered to be the lipase content.

Interesterification experiment

In all experiments, *n*-hexane before use was dried over molecular sieve (250 g molecular sieves 5 Å/5 L *n*-hexane), and also the substrates, tripalmitin (PPP) and stearic acid were freeze dried before use to remove their water content. The interesterification reaction of PPP and stearic acid catalyzed by the modified lipase was performed in a 60 mL glass flask with Teflon-faced septa. Reactions were initiated by adding 30 mg of modified lipase into 55 mL *n*-hexane containing 0.55 g PPP (12.4 mmol/L) and 0.55 g stearic acid (35.1 mmol/L). The content of water in the reaction system was about 25 mg/L. The temperature of the reaction system was controlled at 40°C by immersion in a water bath, and the reaction system was magnetically stirred at 800 rpm. Samples (0.5 mL) were periodically withdrawn from the reaction system and filtered using 0.5 μm disposable Millipore filters. All experiments, unless stated otherwise, were carried out under the above conditions. Initial reaction rates were determined from the concentration of PPP in the first 30 min of the interesterification

reaction. And interesterification activity of the modified lipase was also described by specific reaction rate constant k* (Chapter 2-1).

To study the solubilization of water by the modified lipase complex and the effect of the solubilized water on the modified lipase complex, the following experiments were carried out: Four separate sets of 10 mL *n*-hexane containing 100 mg PPP, 100 mg stearic acid, and 10 mg water (1000 mg H_2O/L), were prepared. The prepared sets of solutions, each set composed of seven solutions, were treated differently as follows:

1. The surfactant concentration was varied in the first set; no lipase was present in the solution.
2. In the second set, the same surfactant concentration series was used as in the first set and in addition 11 wt% purified lipase, relative to the total amount of the surfactant and lipase, was added.
3. In the third set, the amount of the modified lipase (protein content 11 wt%) was changed in each solution, however, the concentrations of the surfactant and the lipase were similar to those in the second set of experiments.
4. As a control experiment, only purified Lipase Saiken 100 (purified by using ultrafiltration system, molecular weight cut of 6000, model PS-24001; Asahi Kasei, Tokyo, Japan) was added into the solutions, as in the second set of experiments; no surfactant was present in the solution.

All solutions were stirred magnetically at 800 rpm and thermostated at 40°C. Two samples of 1 mL each were taken after 4 h from each solution, and their water contents were analyzed by a Karl Fisher titrator. The first sample was taken during stirring of the solutions and the second sample was taken after 2 min from stopping stirring.

Hydrolysis experiment

Hydrolysis activities of the original, modified and supernatant lipases were analyzed by tributyrin assay, using pH-stat titrator, VIT 90 Video Titrator (Radio Meter, Analytical A/S, Copenhagen, Denmark)[80]. Tributyrin and emulsification reagent

(mixture of sodium chloride, potassium dihydrogene phosphate, glycerol and gum arabic) were mixed vigorously, then used as the substrate. Lipases were dissolved in water by stirring for an hour, then added to the substrate. Hydrolysis activity was described by LU (lipase unit). One LU is the amount of lipase which can hydrolyze 1 μmol butyric acid per min from emulsified tributyrin.

Repetitive lipase modification process

The batch modification process was repeated 3 times as shown in Fig. 2-2-1. First, each original lipase containing 300 mg protein was dissolved in 1L of the McIlvaine buffer solution[19]. Table 2-2-2 shows the lipase amount and the buffer pH used for the modification process. The pH used in the experiment was adjusted to optimize the modified lipase activity for each lipase (Chapter 2-1). Surfactant (0.75 g) dispersed in 20 mL ethanol at 40°C was added to the lipase solution. The mixture was stirred at 600 rpm and 5°C for 24 h. A precipitate was obtained by centrifugation at 9000 rpm (9000 x g) and 5°C for 20 min, frozen and freeze dried. The modified lipase (ML1) was obtained as a powder.

Using the supernatant (SP1), the second lipase modification process was carried out. Surfactant (0.75 g) dispersed in 20 mL ethanol at 40°C was added to SP1. The mixture was stirred at 600 rpm and 5°C for 24 h. The precipitate was obtained by centrifugation at 9000 rpm (9000 x g) and 5°C for 20 min, frozen and freeze dried. The modified lipase (ML2) was obtained as a powder. The supernatant (SP2) was used for further lipase recovery process. The modified lipase, ML3, was obtained from the supernatant, SP2, by the same procedure as for ML2. Protein concentrations of the supernatant and the modified lipase were determined by the Hartree method[37].

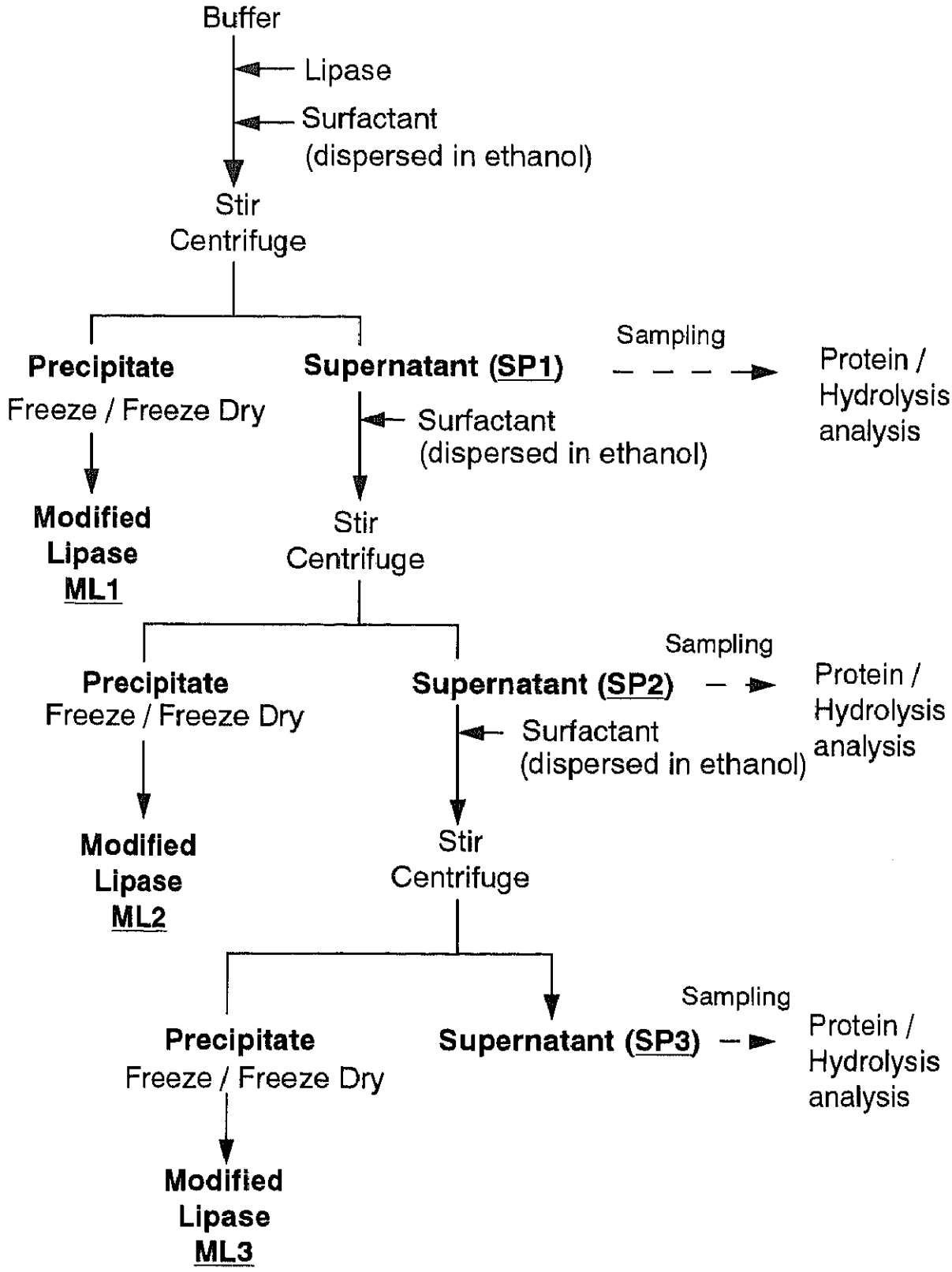

Fig. 2-2-1 Lipase modification process and protein recovery process from the supernatant. Lipases from *R. japonicus*, *R. delemar* and *R. miehei* were used. Surfactant used was Emazol S-10(F) (sorbitan monostearate).

Table 2-2-2 The amount of lipase and buffer used for lipase modification process

Lipase	Amount of lipase (g/L)	Protein amount (mg/L)	Buffer pH
Lipase Saiken 100	3.00	300	5.0
Lipase D Amano 20	1.10	305	6.0
Lipozyme 10000 L	6.00	300	6.0

Results and Discussions

Specificity of modified lipase towards type of fatty acids and triglycerides in comparison with those of crude lipase

Figure 2-2-2a shows that the modified lipase predominantly catalyzed the interesterification of stearic acid and PPP in the presence of a low content of water (25 mg/L). 1-Stearoyl-2,3-palmitoyl glycerol (SPP) and 1,3-stearoyl-2-palmitoyl glycerol (SPS) were produced. Steady state of the interesterification reaction was achieved after around 5 h. A fresh batch of 30 mg of modified lipase was added to the reaction mixture after 10 h reaction time in order to check whether the steady state had been reached after around 5 h because of equilibrium or the reaction was stopped due to lipase inactivation. Analysis results of the reaction mixture after a further 13 h stirring revealed that there were no changes in the composition, which demonstrates achievement of the equilibrium state in the reaction mixture.

The analysis results revealed that the modified lipase could also catalyze the hydrolysis of triglycerides to form diglycerides as byproducts of the interesterification reaction (Fig. 2-2-2b). 1,2-Diglycerides are chemically unstable, and therefore, undergo either spontaneous or enzymatically catalyzed acyl migration to form 1,3-diglycerides[41,61]. The isomerization of 1,2-dipalmitin (PPG) to 1,3-dipalmitin (PGP) explains the formation of 1-palmitoyl-3-stearoyl glycerol (PGS) in the reaction system. Subsequently, PGP and PGS undergo a further lipase-catalyzed interesterification reaction with stearic acid to form 1,3-distearin (SGS). The analysis results, however, showed that the percentage of total diglycerides produced in the reaction system did not exceed 6 wt% of the initial concentration of PPP. This percentage of the byproducts, diglycerides, is relatively low compared to more than 10 wt% of diglycerides obtained in other studies where the interesterification reaction was performed in microaqueous organic systems (20-10,000 mg H_2O/L)[63,72,73]. Equilibria were attained in less than 1h for both the hydrolysis of PPP and the interesterification reaction between PPG, PGP and stearic acid. These results indicate that water is more likely to be a faster

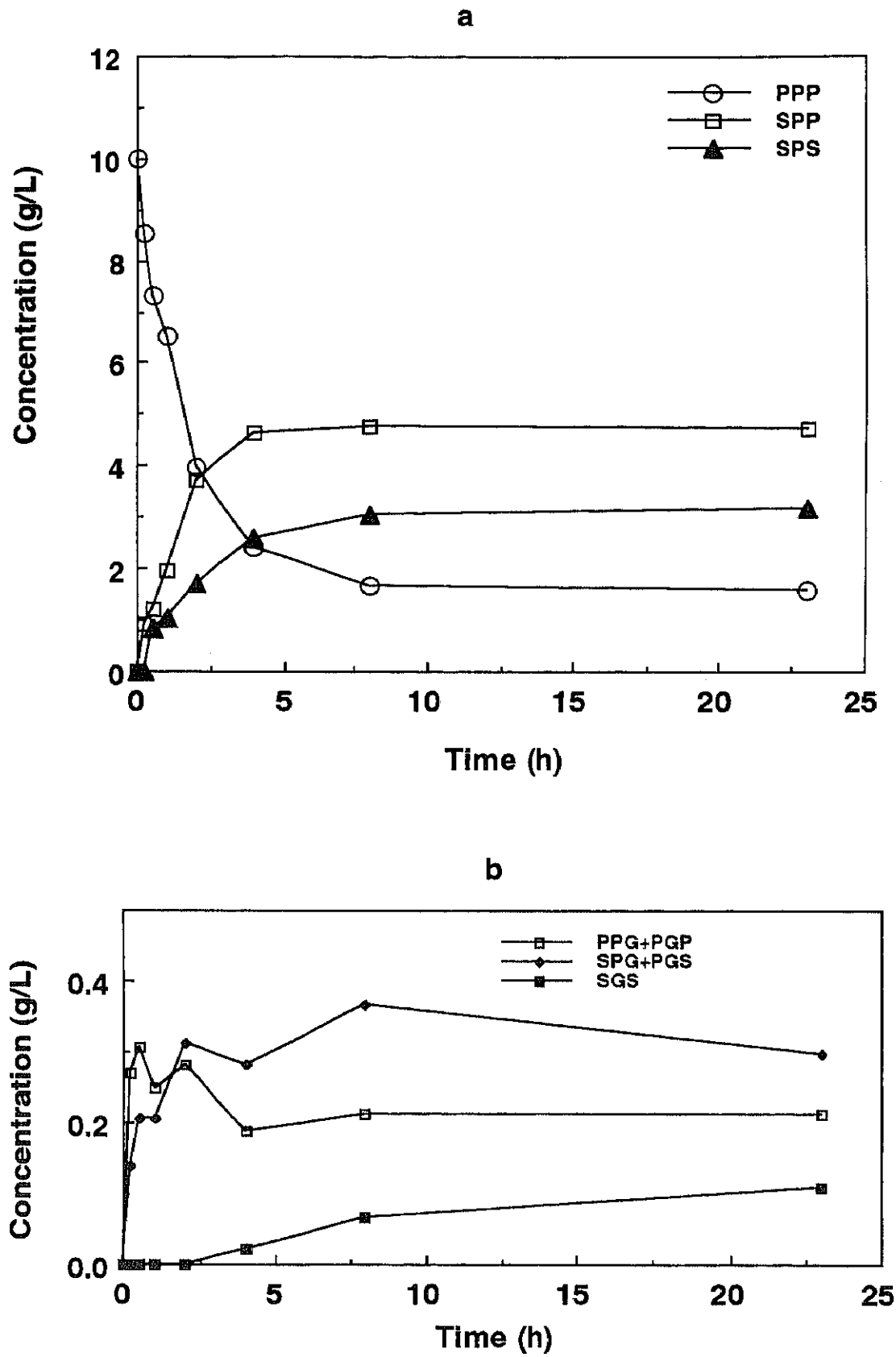

Fig. 2-2-2 Triglycerides (a) and diglycerides (b) concentration profiles resulting from the modified-lipase-catalyzed interesterification of tripalmitin and stearic acid. The reaction was carried out in 55 mL *n*-hexane containing 0.55 g tripalmitin (12.4 mmol/L), 0.55 g stearic acid (35.1 mmol/L) and 30 mg modified lipase. The reaction mixture containing 25 mg/L water was magnetically stirred at 800 rpm and thermostated at 40°C.

nucleophile than free fatty acids. Furthermore, tristearin (SSS) was not formed in the reaction system; therefore, this result provides the evidence of 1,3-positional specificity of the modified lipase.

To study the specificity of the modified lipase with respect to the type of fatty acid incorporated into the glycerol backbone, as well as with respect to the type of triglyceride, the interesterification reaction of SSS and palmitic acid was carried out under the same reaction conditions of PPP and stearic acid. The results of both interesterification reactions indicated that the modified lipase does not distinguish either between stearic and palmitic acid or between PPP and SSS used as substrates. Similar results were obtained in other studies using different lipase preparations[39].

The interesterification activity of the crude Lipase Saiken 100 was tested under the same conditions as the previous reactions. It was found that the crude lipase has no interesterification activity. To promote the interesterification reaction by crude lipase or purified Lipase Saiken 100, water was added in the range 100 - 1000 mg/L into the reaction system. The analysis results showed the occurrence of a very slow hydrolysis of triglycerides to produce diglycerides, which did not exceed 2 wt% conversion of triglycerides during 8 h reaction. Also, the addition of various concentrations of sorbitan monostearate and water to the reaction system containing PPP, stearic acid, and either crude Lipase Saiken 100 or purified lipase did not lead to the appearance of the interesterification products in the produced reverse micellar reaction system.

These results suggest that the modification process adopted in this study caused Lipase Saiken 100 to be 'locked' into a unique catalytically active conformation which is suitable for the catalysis of the interesterification reactions of triglycerides and fatty acids. The complexes or aggregates produced in a mixture of lipase and surfactant in *n*-hexane containing water had no interesterification activity. The results demonstrate that the modified lipase complex must be prepared in an aqueous solution and then used

in the *n*-hexane system. The proposed structure of the modified lipase complex is shown in Fig. 2-2-3.

Water solubilization in the reaction system and its effect on the interesterification reaction

The experimental procedure followed to investigate the capability of the modified lipase complex to solubilize water in a hydrophobic organic system is described in the Materials and Methods section. Figure 2-2-4 depicts the percentage of water solubilized by the surfactant in the organic system, where 100% water solubilization corresponds the water content of 10000 mg/L. Water measurements were determined from the samples taken during stirring the solutions in all sets of the experiments. The results showed that the content of water in all solutions of the control experiments measured by a Karl Fisher titrator was found to be constant at 125 mg/L, and it is independent on the protein concentration in the solutions. This value was considered to be the solubility of water in the system (compared to 103 mg/L of water-saturated pure *n*-hexane under the same conditions). As expected, increasing the concentration of the surfactant in the first set of the experiments caused an increase in the solubilized water confined in reverse micelles formed in the continuous organic solvent medium. A concentration of 12 g surfactant/L solvent was enough to completely solubilize the whole water content added to the system (10 mg).

In the second set of the experiments, the maximum amount of water solubilized in the *n*-hexane reached around 80% of the total initial water content when the concentration of the surfactant was 12 g/L. The rest of the water in the system has stuck on the bottom of the glass. The third set of experiments explicitly shows that the modified lipase is capable of solubilizing water in the continuous organic phase. This can apparently be accomplished because the presence of water molecules in the system causes the detachment of the surfactant molecules from the surface of the modified lipase complex to the bulk solvent. Then, the free molecules of the surfactant

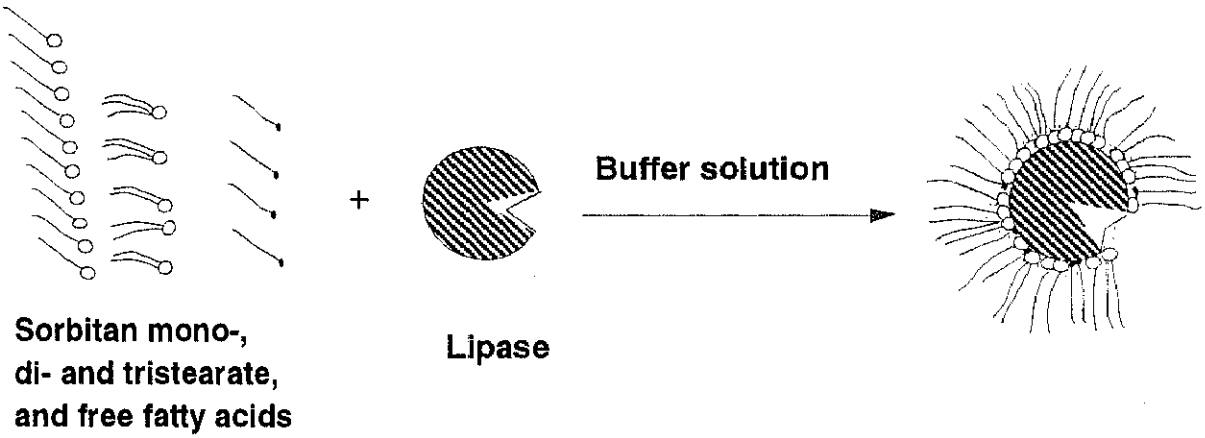

Fig. 2-2-3 Schematic illustration of the proposed structure of the surfactant-modified lipase.

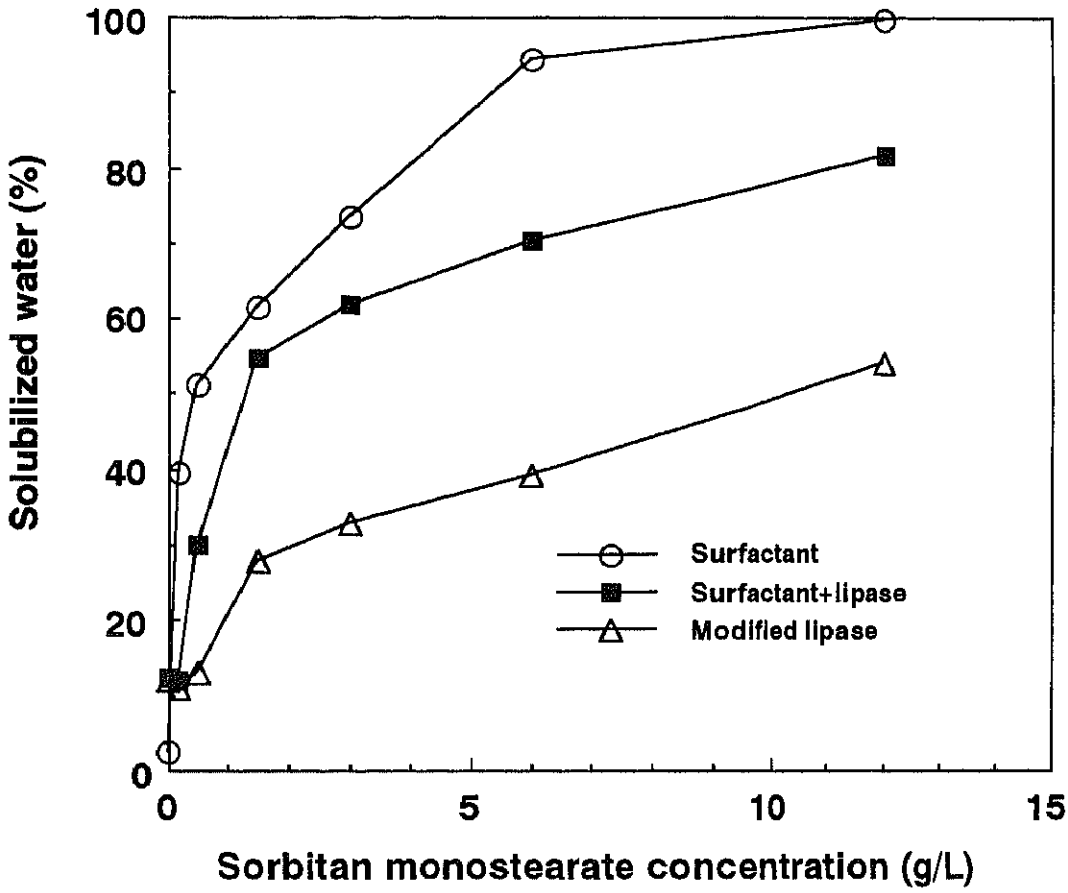

Fig. 2-2-4 Effect of sorbitan monostearate concentration on solubilization of water added into three sets of experiments. Seven identical solutions of 10 mL *n*-hexane were in each set. Each solution contained 100 mg tripalmitin, 100 mg stearic acid and 10 mg water. Each set of solutions was treated differently; (○) The concentration of sorbitan monostearate in the seven solutions was changed from 0 to 12 g/L. (■) The amount of sorbitan monostearate was kept the same as in the first set of experiments and purified lipase (11 wt% of the surfactant) was added to each solution. (△) The amount of modified lipase complex was changed in each solution whereas the concentration of the surfactant and that of the enzyme were the same as in the second set of experiments.

reorganize themselves to form aggregates containing solubilized water and lipase partially coated with surfactant[62]. Analyzing the content of water in samples which were taken 2 min after stopping the magnetic stirring gave identical results with samples taken from the same stirred solutions of the first set of experiments. In the second and the third sets of experiments, the water content in the samples taken 2 min after stopping stirring were 10-20% lower than those taken during stirring. This refers to the good stability of the reverse micellar system produced in the first set and fairly good stability of the reverse micellar system produced in the second and third sets of experiments against coalescence.

Figure 2-2-5a shows the effect of water content on the initial reaction rates r_i of the interesterification and hydrolysis reactions. When no water was supplemented to the reaction system, the solutions were cloudy after the addition of the modified lipase and became transparent when more than 20 mg/L of water was added to the reaction solutions. The interesterification reaction conducted at a water content of 5 mg/L was very slow and no hydrolysis occurred. This is probably because 40 mg of molecular sieves were added to the reaction system, which caused the decomposition of the modified lipase complex due to excessive desorption of water and surfactant molecules from the complex (no molecular sieves were added to the other reaction systems). In Fig. 2-2-5a, as the water content increases, the rate of PPP disappearance due to both interesterification and hydrolysis reactions increased significantly. The initial interesterification rates, which are shown as SPP and SPS production rates, increased with water content up to 200mg/L and increased again between 400 and 9000 mg/L. The initial hydrolysis rates increased when the water content increased from 5 to 18 mg/L and were kept almost constant at water contents lower than 400 mg/L. The increase in the rate of PPP disappearance at water contents lower than 200 mg/L was mainly due to the increase in the interesterification rate between PPP and stearic acid. However, at water contents higher than 400 mg/L, both interesterification and hydrolysis rates affected the PPP disappearance rate. There also appeared a decrease

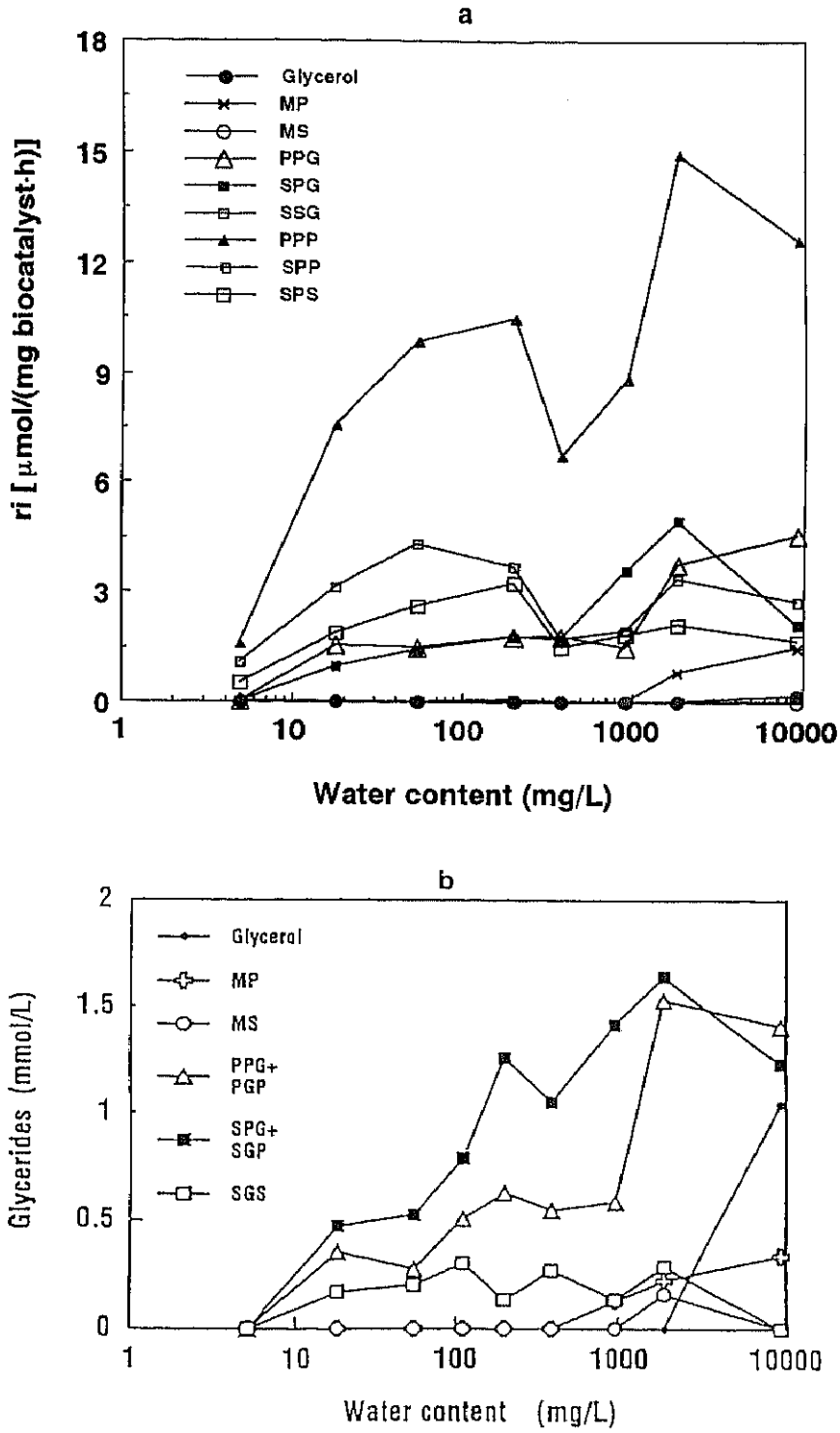

Fig. 2-2-5 (a) Effect of water content on the initial interesterification and hydrolysis reaction rates. (b) Effect of water content on the concentrations of diglycerides, monoglycerides, and glycerol after 24 h reaction. Reaction conditions: 55 mL *n*-hexane containing 0.55 g tripalmitin, 0.55 g stearic acid, and 30 mg modified lipase magnetically stirred at 800 rpm and thermostated at 40°C. The water content in the reaction system was as follows: 5, 18, 54, 109, 200, 383, 927, 1820 and 9090 mg/L.

in the rates of PPP disappearance and SPP and SPS production at water content of 400 mg/L, which might be due to some structural change such as detachment of the surfactant molecules from the surface of the modified lipase.

The modified lipase, similar to other lipases, requires some amount of water in order to function. Therefore, initially its activity increased with the increase of water content. At higher water content, the increase in water content may cause the desorption of a sufficient number of surfactant molecules so that they can reorganize themselves to form new structures of water pool containing modified lipase, where the initial hydrolysis rates became higher, and increased concentration of diglycerides may cause higher initial interesterification rates.

Figure 2-2-5b shows the effect of water content on the concentrations of diglycerides, monoglycerides and glycerol after 24 h reaction, where the steady states were almost attained except in the case of the lowest water content. The concentrations of diglycerides increased significantly with the increase of water content, and the concentrations of stearoyl palmitoyl glycerol (SPG, SGP) and dipalmitin (PPG, PGP) were higher than that of distearin (SGS). Monoglycerides were appeared only when the water content was higher than 2000 mg/L. Glycerol was also produced after more than 20 h reaction when the water content was higher than 9000 mg/L. The concentrations of diglycerides and monoglycerides were influenced significantly by the water content. Summarizing these results, the water content at low levels affected the initial interesterification rate more than the initial hydrolysis rates, and at higher water contents, hydrolysis occurred significantly, and the interesterification rate may be accelerated by the increased concentration of diglycerides.

Effect of pH on the modification process and on the modified lipase activity

The influence of pH on the modification process with respect to the amount of the precipitated modified lipase complex, the protein recovery from the buffer solution, the protein content of the complex after freeze drying, and its interesterification activity

was investigated. Figure 2-2-6 shows the effect of pH on the amount of the precipitated complex collected after the modification process. In parallel, control experiments using protein-free buffer solutions at different pH values were performed. It can be seen from the control experiments, where no lipase was present, that at pH values lower than 6 more than 90 wt% of the sorbitan monostearate dissolved in ethanol and then added to the prepared solutions was precipitated under the described experimental conditions. At pH values higher than 7, the solutions produced after the addition of sorbitan monostearate were emulsion-like and cloudy. The precipitated sorbitan monostearate amount was decreased sharply and the yield of the collected precipitate reached less than 4 wt% at pH values above 7.

The same tendency was seen with the solutions containing crude lipase. At pH values below 5 the yield of the precipitated complex collected was more than 90 wt% with respect to the added amount of sorbitan monostearate. At higher pH values the yield of the precipitate exponentially decreased and reached approximately 13 wt% at pH above 7. These results indicate that the sorbitan esters and protein produce insoluble aggregates preferably at low pH values which could be precipitated. Hydrolysis of the sorbitan esters at high pH values may occur. Therefore, free anions of fatty acids either from the surfactant preparation or produced from hydrolysis of the sorbitan esters can cause the formation of emulsion-like solutions[1].

Figure 2-2-7 shows that the protein recovery from the buffer solution of the crude lipase and the protein content in the modified lipase complexes after freeze drying, both increased when the pH of the solution increased from 3 to 5 and reached a maximum at pH 5. Increasing the pH to above 5 caused a sharp decrease in both protein recovery and protein content in the lipase complexes. These results indicate that interactions between the surfactant and the protein preferentially occur at pH 5. Above that pH value, changes in the ionization state of the lipase and in combination with the changes in the ionic state of the surfactant components prevented the formation of modified lipase precipitate.

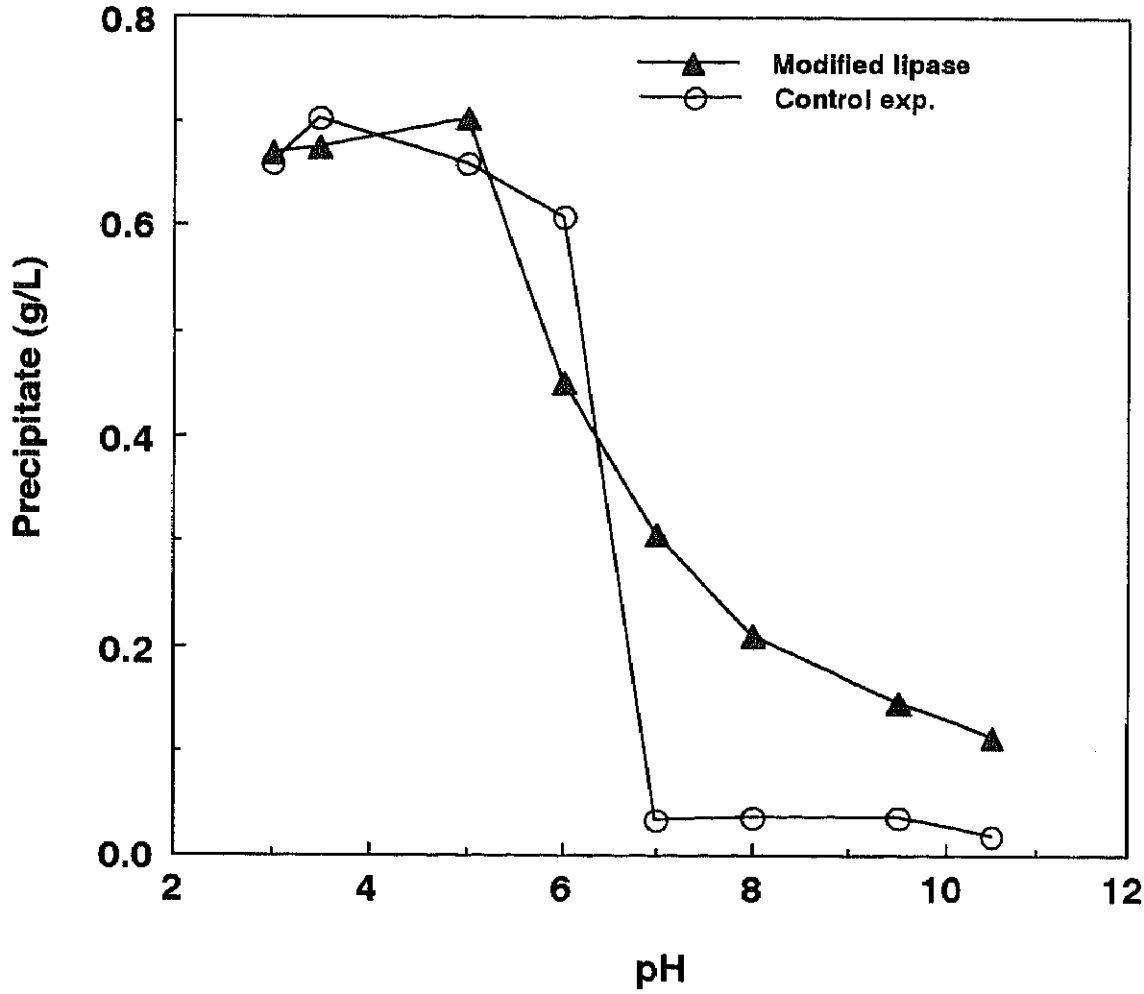

Fig. 2-2-6 Effect of pH on the amount of the precipitated lipase complex. Experimental conditions: Tris buffer solutions containing 3 g crude lipase were adjusted to various pH values and then treated with 750 mg sorbitan monostearate (total protein content in all solutions was 330 mg). The control experiments did not contain crude lipase.

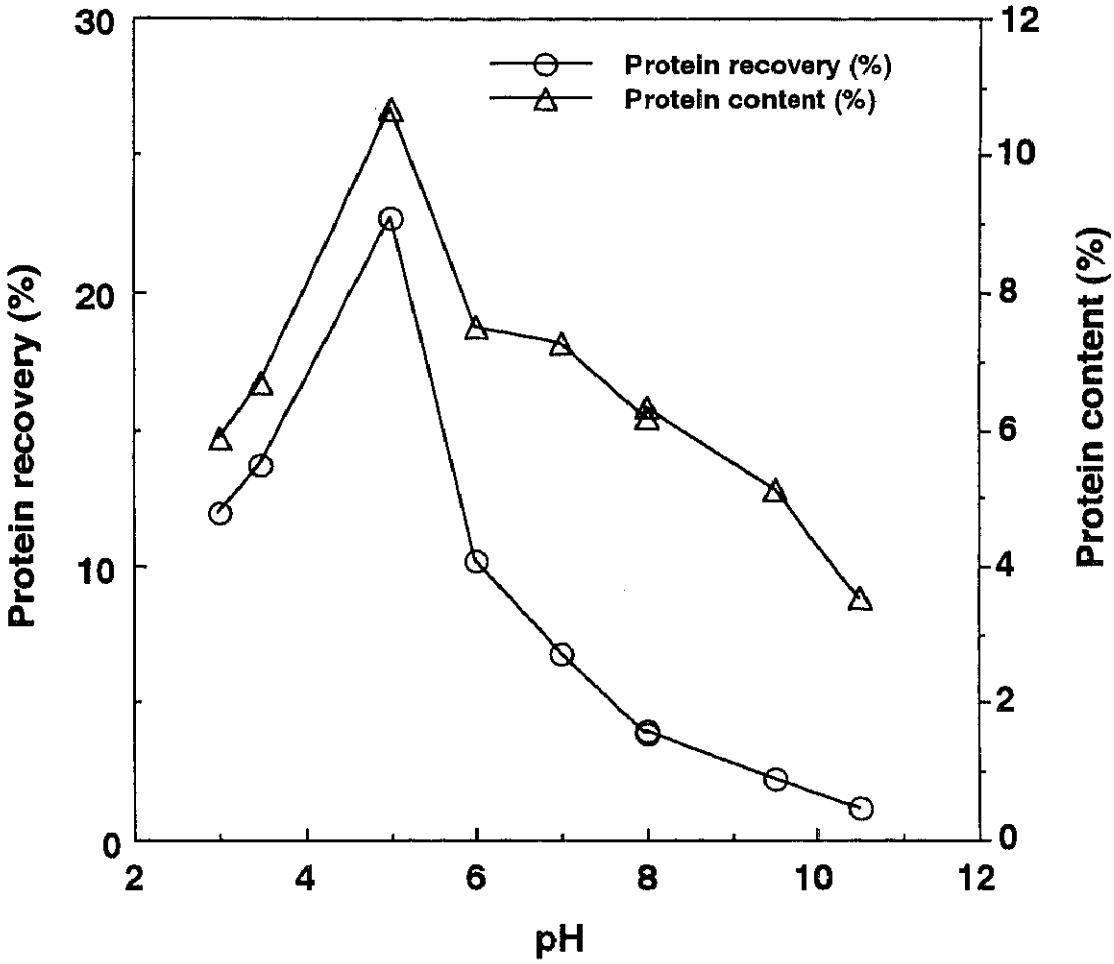

Fig. 2-2-7 Effect of pH on the protein content of the modified lipase complex and on the protein recovery from crude lipase solutions prepared at different pH values.

Modified lipases prepared at pH 3 and 3.5 were found to be catalytically inactive. All of the other modified lipases prepared at different pH values were found to catalyze the interesterification of triglycerides and fatty acids. Diglycerides produced in the reactions did not exceed 6 wt% of the initial concentration of PPP. Steady states in the reaction systems were attained after around 5, 15, 12, 9, 8 and 30 h using the biocatalysts prepared at pH 5, 6, 7, 8, 9.5 and 10.5, respectively. Initial reaction rates r_i expressed per mg modified lipase and per mg protein in the modified lipase as a function of the pH during the modification process are shown in Fig. 2-2-8. It can be seen that the interesterification activity has two regions of optimum pH values, where the initial reaction rate was sharply increased from zero with the increase in pH from 3.5 to 5 and decreased to almost a constant rate at pH 6-7. The pH values above 7 and up to 9.5 again caused a substantial increase in the initial reaction rates. These changes might be due to the ionization state of the lipase combined with the changes in the ionic state of the components of the surfactant, different concentration of surfactant in modified lipases and/or protein denaturation. Taking into account the interesterification activity of the complex, the recovery of the protein during the modification process, and the protein content of the precipitates, Figures 2-2-6 to 2-2-8 show that pH 5 is the optimum value for carrying out all of the lipase modification experiments in this study.

Effect of surfactant concentration on modification process and on activity of modified lipase complex

The influence of sorbitan monostearate concentration during the modification process on the modified lipase complex with respect to the collected precipitate weight, the recovered protein from the buffer solution, the protein content of the freeze-dried complex, and its interesterification activity was also checked and is shown in Fig. 2-2-9 and Fig. 2-2-10, where the concentration of the crude lipase was kept constant, whereas the concentration of sorbitan monostearate dissolved in ethanol was varied. The

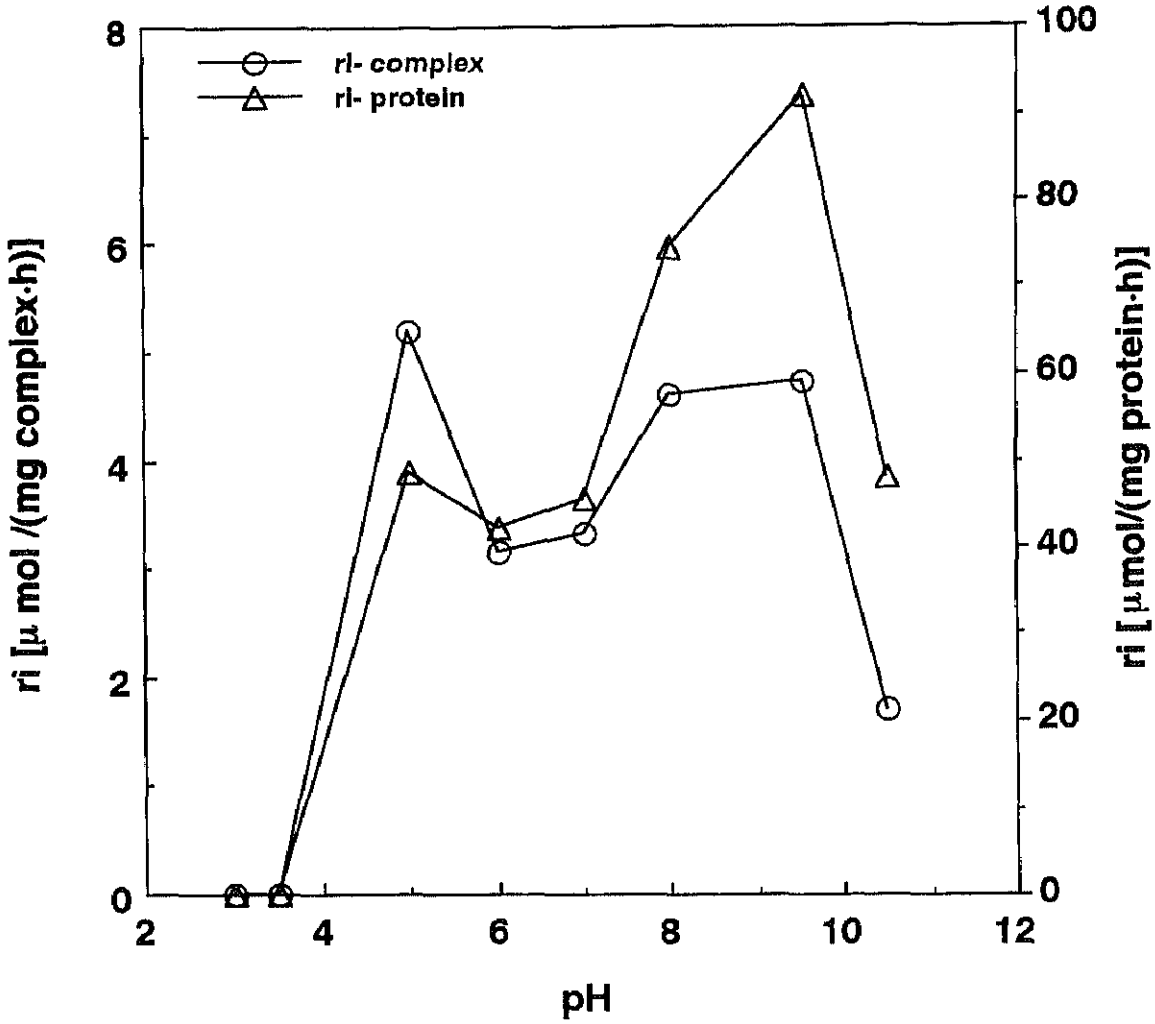

Fig. 2-2-8 Initial interesterification reaction rates using modified lipase prepared at different pH values. Rates were calculated per mg modified lipase complex and per mg of protein. The reactions were carried out in 30 mL *n*-hexane containing 250 mg tripalmitin, 250 mg stearic acid, and 30 mg modified lipase. Water content was 25 mg/L.

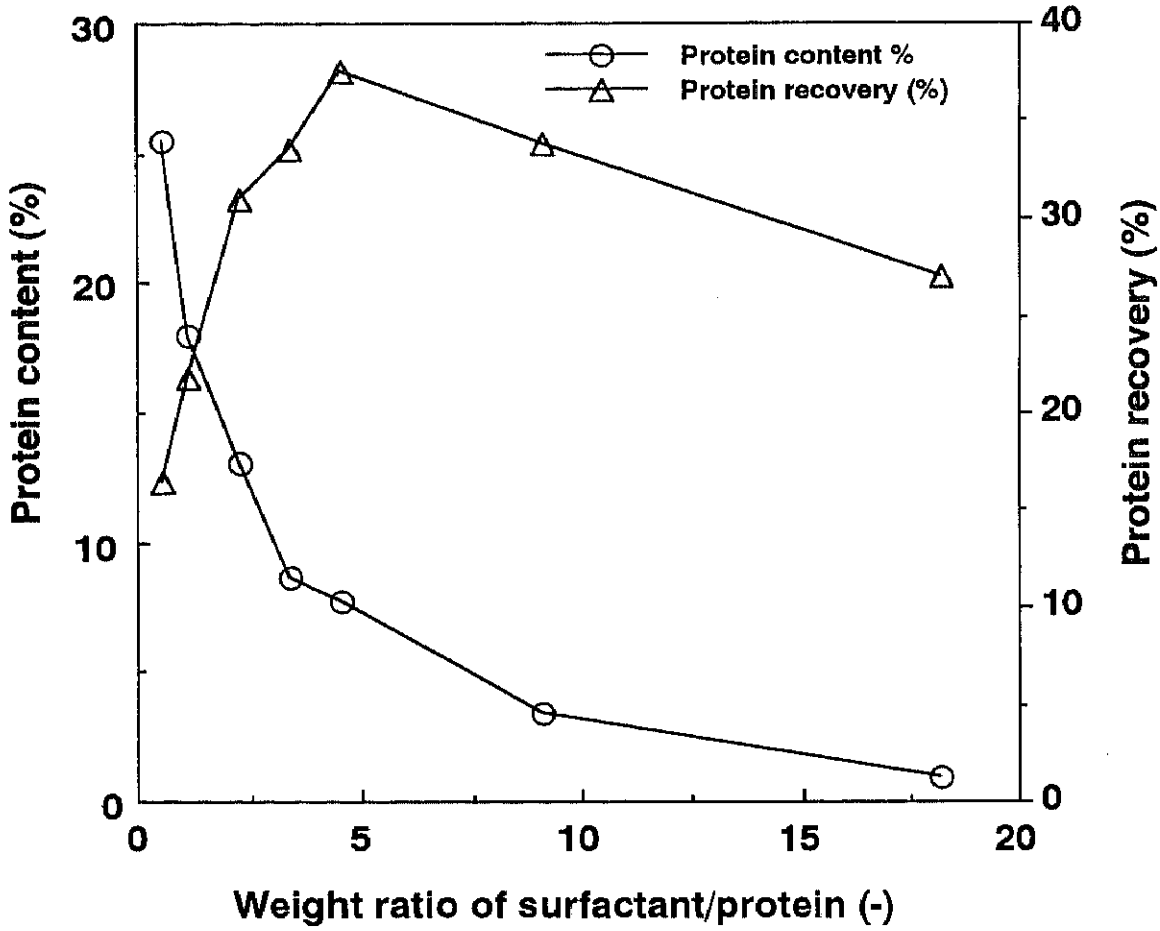

Fig. 2-2-9 Effect of weight ratio of sorbitan monostearate/protein on the protein recovery and on the protein content of the modified lipase complex.

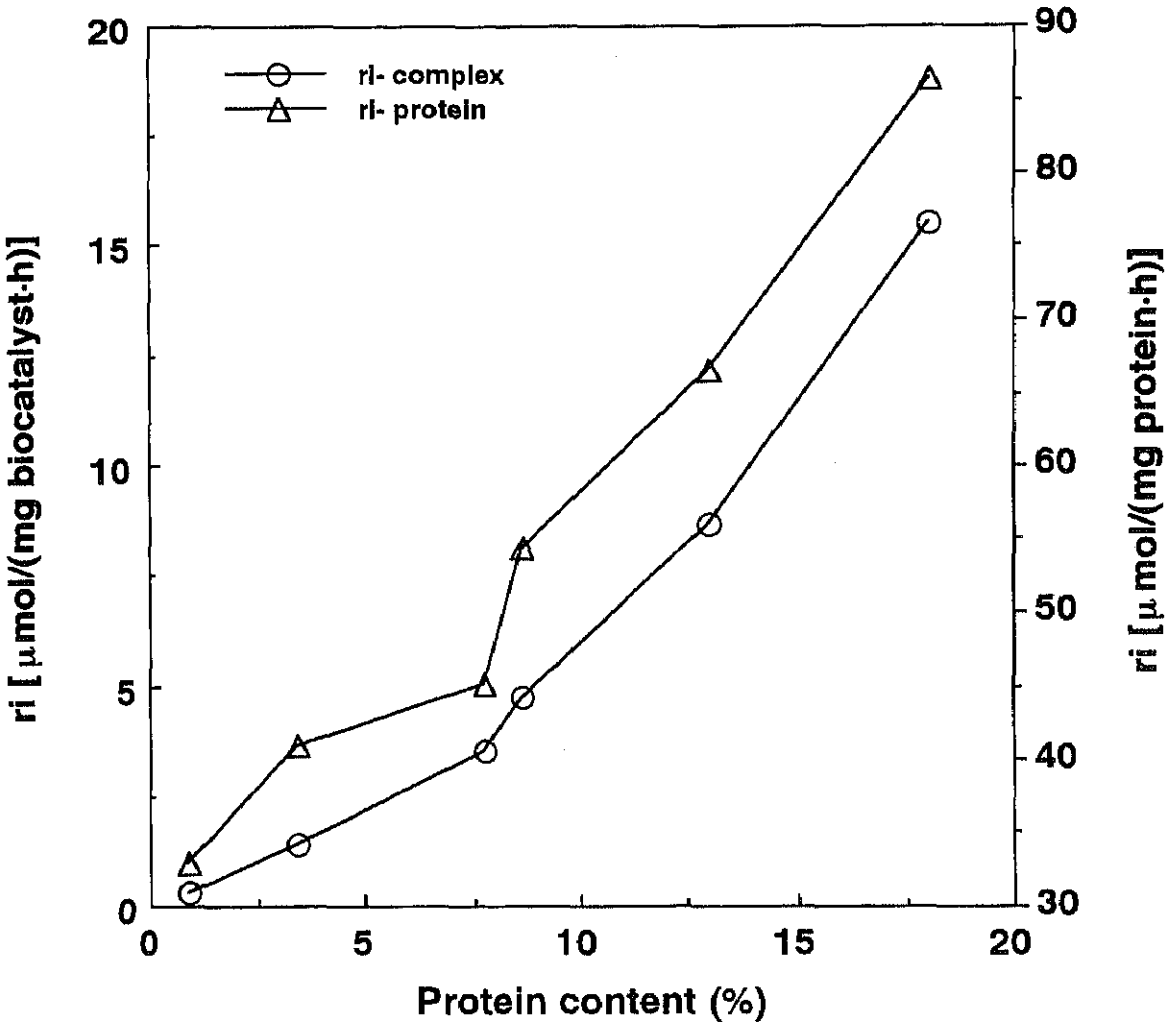

Fig. 2-2-10 Initial interesterification reaction rates (from disappearance of tripalmitin) using modified lipases prepared at different weight ratios of surfactant/protein. Rates are expressed per mg lipase complex and per mg protein. Reaction conditions as in Figure 2-2-8.

amounts of the modified lipase collected after drying were about 90-100% of that of the surfactant because of low solubility in water solution. It can be seen from Fig. 2-2-9 that increasing the weight ratio of surfactant to protein led to an increase in the protein recovery from the solution. A maximum protein recovery of 37 wt% was reached at weight ratio of surfactant to protein at about 5. A further increase in the concentration of surfactant caused a slight decrease of the protein recovery. This trend can be explained by the limited solubility of the surfactant which caused the precipitation of the surfactant prior to its exposure to protein molecules.

Figure 2-2-9 also shows the protein content of the modified lipase as a function of the weight ratio of surfactant to protein in the solution. It is clearly shown that a small number of surfactant molecules attached to the protein surface is sufficient to precipitate the modified lipase complex. Furthermore, the results showed that changing the weight ratio of sorbitan monostearate to protein in the complex did not affect the specificity of the modified lipase. All of the modified lipases contained different ratios of sorbitan monostearate to protein, catalyzed mainly the interesterification of triglycerides and fatty acids in microaqueous *n*-hexane system, and yielded around 6 wt% diglycerides as byproducts.

Modified lipases containing 18 wt% and 13 wt% protein catalyzed the interesterification reaction relatively fast, and the steady states were reached within less than 3 h. Steady state was not reached after even more than 10 h in interesterification reactions where modified lipases contained less than 8 wt% protein were used, which suggests that the number of molecules of the surfactant loaded on one protein molecule has an important role on the activity of the complex. Because the commercial surfactant used in this study is a mixture of sorbitan mono-, di- and tristearate, free sorbitan, and free stearic and palmitic acids, the number of molecules of surfactant attached to one molecule of lipase could not be calculated. The fact that modified lipases overloaded with surfactant molecules were active indicates that the surfactant molecules associate with only one specific part of the lipase, forcing the lipase structure

to a unique conformation so that the active site of the lipase is easily accessible by substrates in a microaqueous organic media.

Figure 2-2-10 shows the initial interesterification reaction rates r_i calculated per mg modified lipase and per mg protein as a function of the protein content in the modified lipase. It can be seen that initial reaction rates calculated per mg modified lipase increased exponentially with the increase in protein content of the complex. Initial rates calculated per mg protein increased slowly with the increase of protein content of the complex from 0.9 - 7.7 wt%. Using modified lipases containing more than 7.7 wt% protein caused a significant and linear increase in the initial reaction rates calculated per mg protein. This trend is most likely to be attributed to steric effects imposed by the lipophilic tails of the attached surfactant molecules on the protein surface so that the substrate access to the active site of the lipase is restricted.

Protein recovery and interesterification activity

The protein recovery and interesterification activity in modified lipase by the lipase modification process is shown in Table 2-2-3. In the case of the lipase from *R. japonicus*, protein recovery in the ML1 was 11.8% from the first lipase modification process. By means of the second and third lipase modification processes, protein recoveries of the ML2 and ML3 were 3.34% and 2.64% respectively, which were much lower than that of the ML1. As a whole, 48.5 mg protein was recovered in 3.43 g modified lipase, the protein concentration was 1.38%, and the protein recovery reached 17.1%. In the control test without addition of lipase no precipitate was observed. The interaction between lipase and surfactant gave significant precipitate, and most of the surfactant was transferred to the precipitate.

The original lipase of *R. japonicus* had no interesterification activity at all. No activity was also observed with addition of the surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed high interesterification activity. The interesterification activity of the ML1 was 80.0 x 10^{-9} $m^6/(mol \cdot gPr \cdot s)$.

Table 2-2-3 Protein recovery and interesterification activity in modified lipase

Modification process	ML amount*1 (g)	Protein content (%)	Protein recovery in each process (%)	Interesterification activity k* x 10^9 [m^6/(mol·gPr.·s)]
R. japonicus				
Lipase*2	-	10.0	-	0
1st	1.12	2.80	11.8	80
2nd	1.10	0.77	3.34	24
3rd	1.21	0.53	2.64	0
R. delemar				
Lipase*2	-	27.7	-	0
1st	1.28	2.38	10.5	13
2nd	1.17	3.78	16.8	2.9
3rd	1.39	1.99	12.9	0.8
R. miehei				
Lipase*2	-	5.0	-	0
1st	1.49	1.49	7.88	6.4
2nd	1.38	1.38	8.10	0.6
3rd	1.32	1.32	14.1	0.1

*1Total dry weight of recovered modified lipase

*2 Lipase before modification

The interesterification activity of ML2 decreased to 24.0 x 10^{-9} m^6/(mol•gPr•s). The concentration of produced diglycerides was about 4wt% of the triglycerides after 24 h reaction. Monoglycerides were not produced at all. In the ML3, the interesterification reaction did not occur during 24 h. The interesterification activities of the supernatant, the SP1, SP2 and SP3 of *R. japonicus* were also investigated. The SP1, SP2 and SP3 were frozen, freeze dried and used for interesterification reaction with sorbitan monostearate in *n*-hexane and, no reaction products were observed at all during 24h reaction (data not shown). The surfactant which is combined with lipase was not one of the substrates for the interesterification and hydrolysis. As a blank test for the interesterification reaction, only the modified lipase was stirred in *n*-hexane at 500 rpm and 40°C for 24 h. After GC analysis of the final product, no related surfactant or no chemicals composed the sorbitan monostearate were detected. In the blank test for the hydrolysis reaction, no surfactant or chemicals composed of the sorbitan monostearate were detected.

In the case of the lipase from *R. delemar*, protein recovery of the ML1 was 10.5%. By the second and third processes, protein recovery of the ML2 and ML3 were 16.8% and 12.9% respectively, a similar amount of protein was recovered in each modification process. As a whole, 102 mg protein was recovered in 3.84 g modified lipase, the protein concentration was 2.66%, and the protein recovery reached 35.4%.

The original lipase from *R. delemar* had no interesterification activity at all. No activity was also observed with the addition of the surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed an interesterification activity of 13.0 x 10^{-9} m^6/(mol•gPr•s). The concentration of diglycerides was about 4wt% of the triglycerides, and monoglycerides were not produced after 24 h reaction. The interesterification activities of the ML2 and ML3 were 2.90 x 10^{-9} m^6/(mol•gPr•s) and 0.80 x 10^{-9} m^6/(mol•gPr•s) respectively.

When the lipase from *R. miehei* was used for the lipase modification process, protein recovery of the ML1 was 7.88%. Following by the second and third processes,

protein recovery of the ML2 and ML3 were 8.10% and 14.1% respectively. As a whole, 77.5 mg protein was recovered in 4.19 g modified lipase, its protein concentration was 1.85%, and the protein recovery reached 27.3%.

In the case of *R. miehei,* the original lipase had no interesterification activity at all. No activity was also observed with addition of the surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed an interesterification activity of 6.40 x 10^{-9} m^6/(mol•gPr•s). The concentration of diglycerides was about 12wt% of the triglycerides, and monoglycerides were not produced after 24 h reaction. The interesterification activities of the ML2 and ML3 were 0.60 x 10^{-9} m^6/(mol•gPr•s) and 0.10 x 10^{-9} m^6/(mol•gPr•s) respectively. The concentration of diglycerides was 3 times higher than those in the modified lipases obtained from *R. japonicus* and *R. delemar.*

The protein recovery of *R. japonicus* was not significantly increased by the repeated modification processes. However, in the case of *R. delemar* and *R. miehei,* the lipase recoveries increased significantly with repeated lipase modification processes. *R. japonicus* seems to have different characteristics of association with the surfactant from the other two lipases. In Chapter 2-1, the optimum weight ratio of *R. japonicus* and sorbitan monostearate during the modification process to obtain the high actively modified lipase was investigated. At a ratio of lipase protein to surfactant between 0.2 and 2.5, protein recoveries were kept constant at 10-12%. From these results, it is speculated that a limited part of the protein in *R. japonicus* was associated with the surfactant. The modified lipase in this study was obtained by the interaction of lipase and surfactant in aqueous media, which seems important for the modified lipase to give the interesterification activity. It is speculated that significant lipase modification by the surfactant did not occur in *n*-hexane so that there was no interesterification activity. SP1, SP2 and SP3 did not have the interesterification activities, which is probably due to the low concentration of the surfactant-lipase complex.

Hydrolysis activity recovery

The hydrolysis activity recoveries in modified lipases are shown in Table 2-2-4. Initial activities show the total hydrolysis activities of original lipases in the pH adjusted buffer solution. In the case of *R. japonicus*, the activity of 3570 LU was recovered in the ML1 from the initial activity of 11200 LU by the first process, i.e., the activity recovery was 31.9%. In the second and third processes, the activities of 549 LU and 159 LU were recovered in ML2 and ML3 respectively, and total recovered activity reached 38.2%. The specific hydrolysis activity of original *R. japonicus* was 38.1 LU/mg Protein. The specific activity of the ML1 increased to 106 LU/mg, which was about 3 times that of the initial specific activity. The specific activities of ML2 and ML3 increased 10-15 times compared to those of SP1 and SP2. From these results it can be concluded that the proteins which have higher hydrolysis activity (mainly lipase) were recovered selectively in the modified lipases from the original lipase, and the other proteins which have less activity remained in the supernatants. These results suggest that the surfactant modification process may be a useful lipase purification process for *R. japonicus*.

In the case of *R. delemar*, the activity of 2340 LU was recovered in the ML1 from the initial activity of 9100 LU by the first modification process, i.e., the activity recovery was 25.7%. In the second and third modification processes, 641 LU and 465 LU were recovered in the ML2 and ML3 respectively, and total activity recovered reached 37.8%. By the first modification, the specific lipase activity of the ML1 was increased to 76.8 LU/mg, which was 2.5 times the original activity. However, in the second and third processes the specific activities of the ML2 and ML3 were similar to the specific activities of the SP1 and SP2.

For *R. miehei* lipase, the recovered hydrolysis activity in the ML1 was 11200 LU from the initial activity of 52700 LU by the first modification process, i.e., the activity recovery was 21.2%. By the second and third modification processes, 2080 LU and 1060 LU were recovered in the ML2 and ML3 respectively, and total activity recovered

Table 2-2-4 Hydrolysis activity recovery in modified lipase by the lipase modification process

Modification process	Activity in SPs (LU)	Specific activity in SPs (LU/mgPr.)	Residual activity in SPs (%)	Recovered activity in MLs (LU)	Specific activity in MLs (LU/mgPr.)	Recovered activity (%)
R. japonicus						
Initial	11200	38.1	-	-	-	-
1st	1530	5.87	13.7	3570	106	31.9
2nd	431	1.75	3.90	549	64.4	37.8
3rd	0.00	0.00	0.00	159	25.0	38.2
R. delemar						
Initial	9100	30.5	-	-	-	-
1st	5090	19.0	55.9	2340	76.8	25.7
2nd	3950	18.1	43.4	641	14.5	32.7
3rd	3250	17.4	35.7	465	16.8	37.8
R. miehei						
Initial	52700	179	-	-	-	-
1st	18400	67.6	34.8	11200	500	21.2
2nd	11600	47.6	22.1	2080	96.6	25.1
3rd	10200	49.4	19.3	1060	31.4	27.1

ML: Modified lipase

SP: Supernatant

was 27.1%. By the first modification process, the specific activity of the ML1 increased 2.8 times of the original activity.

For *R. japonicus*, *R. delemar* and *R. miehei* lipases, the specific hydrolysis activities of the ML1s increased 2.5-2.8 times those of the original lipases. More than 60% of the total recovered activity was found to be obtained in the ML1s by the first lipase modification process in each lipase. In the second and third processes of *R. delemar* and *R. miehei*, specific activities of the modified lipases were almost same as the respective specific activities of supernatant lipases, which implies that there is little selectivity for proteins. However, in the case of the second and third processes of *R. japonicus*, the specific activities of modified lipases were 10 to 14 times higher than respective specific activities of supernatant lipases, which suggests that the surfactant interaction with the lipase has a higher selectivity.

Checking the activity balance of *R. japonicus*, the total recovery of hydrolysis activity was 38.2% after the third modification process, however, the residual activity in the SP3 showed no activity at all. 61.8% of the initial activity was reduced during modification. The total activities in all the modified lipases and residual activity in the SP3 were also lower than the initial total activity in *R. delemar* and *R. miehei*, which might be described by two reasons. The modified lipase is thought to be a complex of lipase and surfactant, and is not easily soluble in water. In the hydrolysis experiments, the activity of the non-soluble modified lipase might be estimated to be lower than that of soluble lipase. The other reason is that some deactivation or activity inhibition may occur due to the surfactant modification effect.

As explained above, both hydrolysis and interesterification activities of ML1 were much higher than in the ML2 and ML3 for all 3 lipases. All the specific hydrolysis activities in the ML1s were about 3 times higher than the original specific activities. It suggests that the modification process is effective for not only interesterification but also for lipase purification. Further investigation for lipase purification by surfactant modification is required.

2-3 Evaluation of Lipid-Modified Lipase for Interesterification and Hydrolysis Reactions in *n*-Hexane

Introduction

In Chapters 2-1 and 2-2, it was described that the lipase modification with surfactant was effective to catalyze the interesterification of triglyceride and fatty acid in organic solvent. The best combination of lipase and surfactant, and the optimal lipase modification condition and reaction condition were reported. From these studies, stearic acid associated surfactants were found to be effective as the lipase modification. In this section, further evaluation on the suitability of various stearic acid associated substances for modifying Lipase Saiken 100 was carried out to asses the effect of lipid/lipase interactions on activity. Stearic acid modification was investigated in more detail, such as preparation conditions affecting activity and characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Other lipases were also investigated with crude and modified forms compared.

Materials and Methods

Materials

All chemicals were supplied from Wako Pure Chemicals Ltd. (Osaka, Japan). Lipases used in this section were mentioned in Chapter 2-1 except for the Lipolase (Novo Nordisk A/S, Bagsvaerd, Denmark). Before use *n*-hexane was dried over molecular sieves (200 g molecular sieves 4 A / 3 L *n*-hexane) to give a water concentration of 10 mg/L.

Analytical methods

Interesterification and hydrolysis samples (0.4 mL) were removed from the reaction media, filtered (0.5 μm, Millipore) and analyzed by GC (Chapters 2-1 and 2-2) and thin layer chromatography with a FID[49].

Protein content of lipase and the modified lipase were measured by Hartree method[37].

Modified lipase preparation

Lipase Saiken 100 (90 mg) was added to 30 mL of 5 mmol/L tris (hydroxymethyl) aminomethane solution (pH 5) and stirred (600 rpm) at room temperature. 50 mg of stearic acid (or other lipid) dissolved in 0.6 mL ethanol (40°C) was added dropwise to the stirred solution. The mixture was sonicated for 15 min and then stirred for 12 h at 5°C. The precipitate was recovered by centrifugation (7000 x g for 10 min), dried under vacuum for 8 h and stored overnight over silica gel at room temperature. The modified lipase was kept at 5°C until used and had a water content of around 5 wt %.

Stearic acid-modified lipase had a protein content of 7.3 wt %, and Lipase Saiken 100 was previously shown to have 1, 3- positional specificity (Chapter 2-1).

Protein electrophoresis

Modified lipase protein was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Phast System of Pharmacia Fine Chemicals [97].

Reaction conditions

The hydrolysis reaction was between 121 mg tripalmitin (6 mmol/L) and 10 mL additional water (400 mg/L), while interesterification was carried out between 250 mg tripalmitin (12.4 mmol/L) and 250 mg stearic acid (35 mmol/L) with an additional water concentration of 0.5 mL (20 mg/L). The reactions were initiated by addition of 20 mg crude or modified lipase to each flask containing 25 mL dry *n*-hexane, substrates and additional water. The flasks were held in a water bath at 40°C and magnetically stirred at 600 rpm. Interesterification activity is expressed as the specific interesterification reaction rate constant k* (Chapter 2-1). In Table 2-3-3, the interesterification activity is expressed as mmol PPS (1,2-palmitoyl-3-Stearoyl glycerol) / (g initial protein•h) in order to compare different types of lipase practically.

Hydrolysis is expressed as mmol fatty acid/(g•h). The g protein refers to g modified lipase protein. The hydrolysis data given in Tables 2-3-1 and 2-3-2 were determined using the Novo Nordisk tributyrin assay[80].

Results and Discussion

Effect of various lipids on lipase modification and activity

During lipase modification, electrostatic interactions with cationic and anionic surfactants resulted in poor activity, with the latter type denaturing the protein structure and unable to form complexes due to electrostatic repulsion[29]. As a result, saturated and unsaturated fatty acids, mono-, di-, and tri-stearin, stearyl alcohol and methyl stearate were evaluated for Lipase Saiken 100 modification (Table 2-3-1).

Of all the saturated fatty acids tested (capric, palmitic, stearic and behenic), the largest precipitate of 1.24 g/L and highest protein recovery of 15.2 % was achieved using stearic acid. The corresponding interesterification and hydrolysis activities of 24.5 L^2/(mol•g•h) and 2.1 mmol/(g•h) were also the highest (Table 2-3-1). Interesterification and hydrolysis activities were enhanced with saturated fatty acids up to carbon length C18; however, behenic acid (C22) showed very poor activity (Fig. 2-3-1). With unsaturated fatty acids, activities were reduced compared to stearic acid (C18) and with degree of unsaturation there was a concomitant decrease in activity (Fig. 2-3-2). It is not clear why there is such a variation in these results. Short chain length and unsaturation enhance the fluidity of fatty acids and their derivatives. Horiuti and Imamura[45] found that long chain fatty acids (up to C16) and unsaturated fatty acids were the most effective for increasing lipase (*Chromobacterium*) activity.

With mono-, di- and tri-stearin lipase modification, only mono-stearin resulted in good interesterification and hydrolysis activity (Table 2-3-1). Tri-stearin addition resulted in a stable emulsion, hence an ultrafiltration membrane was used to concentrate the modified protein from the aqueous phase. As a result, a high percentage (57 %) of protein was recovered (Table 2-3-1). Despite the vast difference between protein

Table 2-3-1 Effect of lipid on ML precipitate (Ppt) yield, Ppt protein content, protein recovery, interesterification and hydrolysis activities

Modifying agent	Ppt yield (g/L)	Ppt protein content (%)	Protein recovery (%)	Interesterification [L^2/(mol•g•h)]	Hydrolysis [mmol/(g•h)]
Capric acid	0.33	7.1	9.6	1.87	0.58
Palmitic acid	0.55	4.8	10.8	6.48	0.86
Stearic acid	1.24	3.0	15.2	24.5	2.10
Behenic acid	0.81	4.1	13.6	1.12	0.16
Oleic acid	0.89	4.0	14.7	2.45	1.03
Linoleic acid	0.59	3.7	8.9	1.40	0.52
Linolenic acid	0.40	4.2	6.8	0.29	0.30
Mono-stearin	1.35	2.8	15.4	18.7	1.05
Di-stearin	1.79	3.1	22.8	5.36	1.06
Tri-stearin	0.62	3.1	7.9	0.00	0.45
Tri-stearin*	4.82	2.9	57.2	0.00	3.90
Stearyl alcohol	0.59	2.8	6.8	0.00	0.33
Methyl stearate	0.59	3.4	8.2	19.7	0.84

* Membrane separation; Ppt: precipitate formed after lipid addition to lipase solution.
Lipids were added during modified lipase preparation. See Fig. 2-3-1 for reaction conditions.

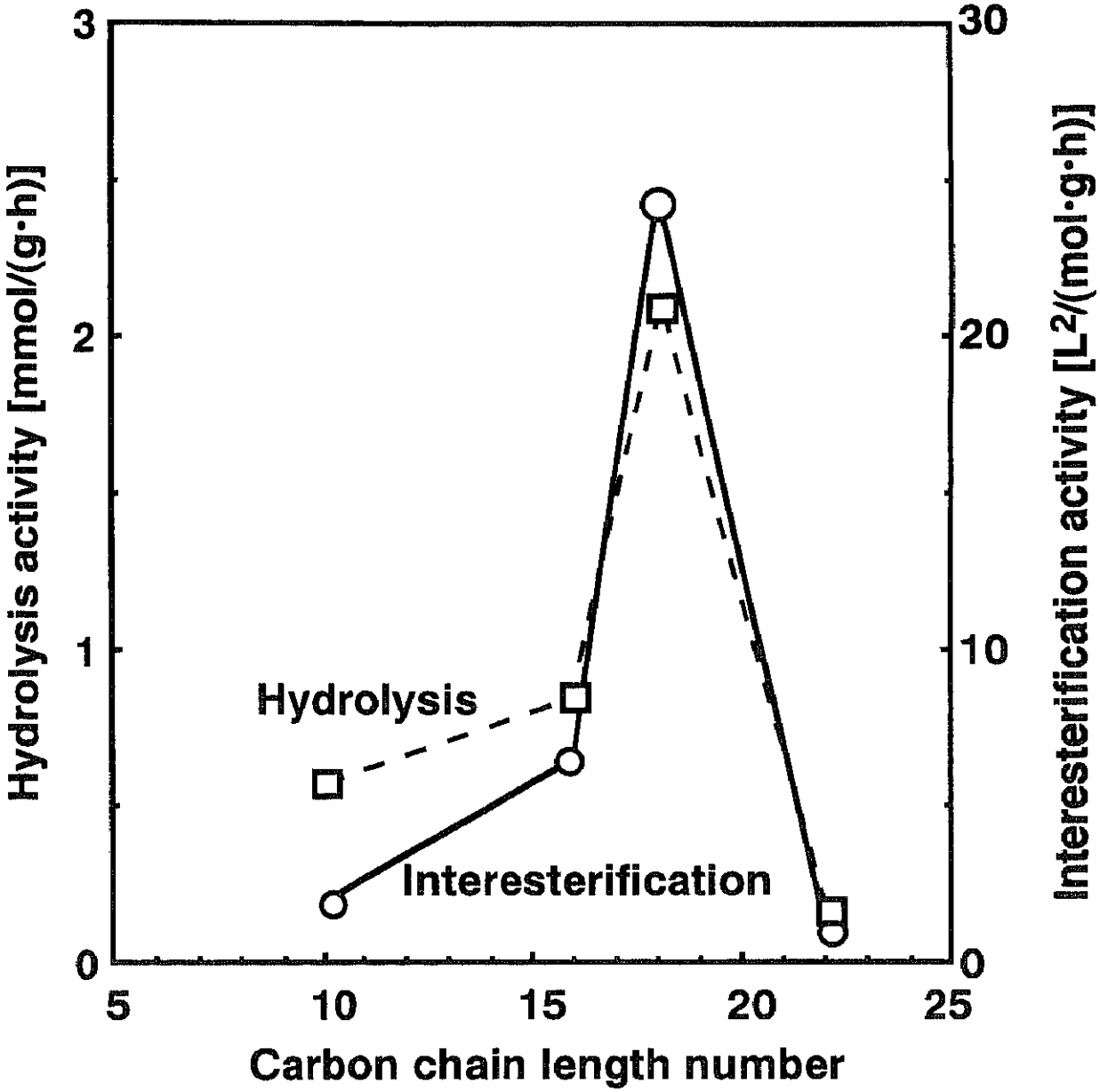

Fig. 2-3-1 Effect of fatty acid carbon chain length on modified Lipase Saiken 100 interesterification ○ and hydrolysis □ activities. Fatty acid was added during modified lipase preparation. Interesterification reaction was between tripalmitin and stearic acid (both 250 mg) in 25 mL *n*-hexane containing 0.5 mL (20 mg/L) water and 20 mg modified lipase. Hydrolysis activity was determined by the tributyrin assay.

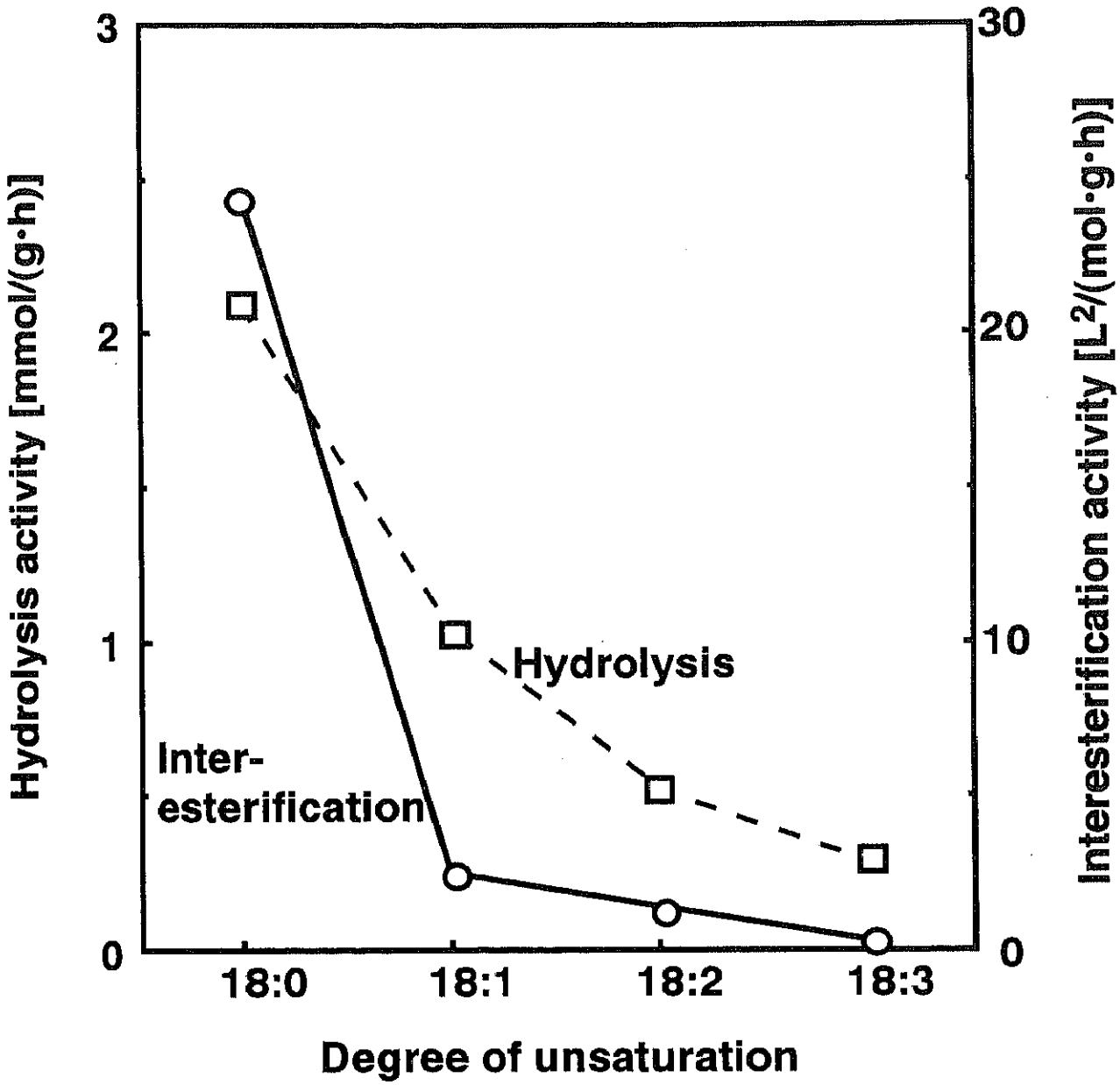

Fig. 2-3-2 Influence of degree of fatty acid unsaturation on modified Lipase Saiken 100 interesterification and hydrolysis activities. Fatty acid was added during modified lipase preparation. See Figure 2-3-1 for reaction conditions.

recovered by centrifugation (7.9%) or membrane separation (57%), no interesterification activity resulted in either case; however, hydrolysis activity was greatly improved with membrane separation (Table 2-3-1). Stearic acid, mono-stearin and methyl stearate-modified lipases displayed the highest activities (Table 2-3-1), while long chain primary alcohols such as stearyl alcohol showed no activity. This implies that the presence of a stearic acid group in each molecule has an important influence on activity. From these results, stearic acid was selected for all subsequent lipase modifications having good overall activity (interesterification and hydrolysis). Interesterification was also monitored using tripalmitin and oleic acid as substrates. In this experiment, stearic acid also proved to be the best fatty acid for lipase modification activity.

It is estimated that 170-200 lipid molecules are required to coat the surface of a lipase molecule as a monolayer[28,81]. The surface of a lipase molecule is slightly negatively charged with non ionic surfactant hydrogen binding probably having the most beneficial effect on lipase activity[55]. In my previous study (Chapter 2-1), it was found that the hydrophile-lipophile balance (HLB) value which describes the ratio of hydrophilic radicals in a surfactant was of some influence in determining activity and yield. Surfactants with HLB values below four were not useful for modifying lipases. Goto *et al.* [29] suggested that surfactants with large amounts of hydrophobic groups were better due to their enhanced solubility in organic solvents with the presence of branching or a double bond showing higher activity than those without. In addition to improved solvent dispersibility, lipase modification may also help to retain water and allow for conformational flexibility[30,108].

Stearic acid modification of Lipase Saiken 100: preparation conditions

Stearic acid used to modify Lipase Saiken 100 was initiated by the dropwise addition of stearic acid (ethanol) to a stirred lipase solution. The weight ratio of stearic acid addition to lipase (R-St value) was investigated with the lipase weight kept

constant (90 mg). Increasing the R-St value from 0 to 1 produced a linear response in the amount of modified precipitate recovered (Fig. 2-3-3). The percentage of lipase protein in the precipitate peaked at an R-St value of 0.55 after which the percentage decreased. The protein recovered reached a maximum (*ca.* 20 %) with an R-St value of 0.55 with no further increase thereafter (Fig. 2-3-3). As expected, the mmol free fatty acid (FFA)/g modified ppt after 24-h hydrolysis reaction mirrored the precipitate protein content with the highest activity between R-St values 0.2 and 0.6 (Fig. 2-3-3). An R-St value of 0.55 was subsequently selected for future experiments because it produced a high precipitate yield with the highest protein content and good hydrolysis activity. It also represented the point after which no more lipase was recovered by further addition of stearic acid.

Lipase Saiken 100 was modified using stearic acid (as previously described) with 25 % of the lipase protein recovered. The supernatant was decanted and 50 mg of stearic acid was added to it resulting in a further 16 % of protein recovered. Two further stearic acid additions (50 mg) to the decanted supernatants recovered a further 9 and 8 % respectively. In total therefore, 58 % (5.8 mg) of the initial protein (10.1 mg) was recovered by a four-step addition of stearic acid. This far exceeds the 25 % or so that was recoverable even at very high R-St values after one-step addition. The presence of the modified lipase may alter the HLB producing unfavorable binding dynamics for any further interaction. Only when the modified protein is removed (centrifugation) and the balance shifted does any further modification proceed.

Protein recovery after stearic acid modification was also affected by the pH of the aqueous preparation phase with pH 5 proving to be the optimal value. Values above pH 5 produced a decrease in the amount of modified lipase precipitate recovered and activity (Table 2-3-2). A tris solution (5 mmol/L) was also found to be more effective than distilled water (pH 7) for protein recovery over a range of pH values. This indicates that ionic interactions between stearic acid and lipase are important. As a result, lipase was dissolved in tris solution (pH 5) prior to modification.

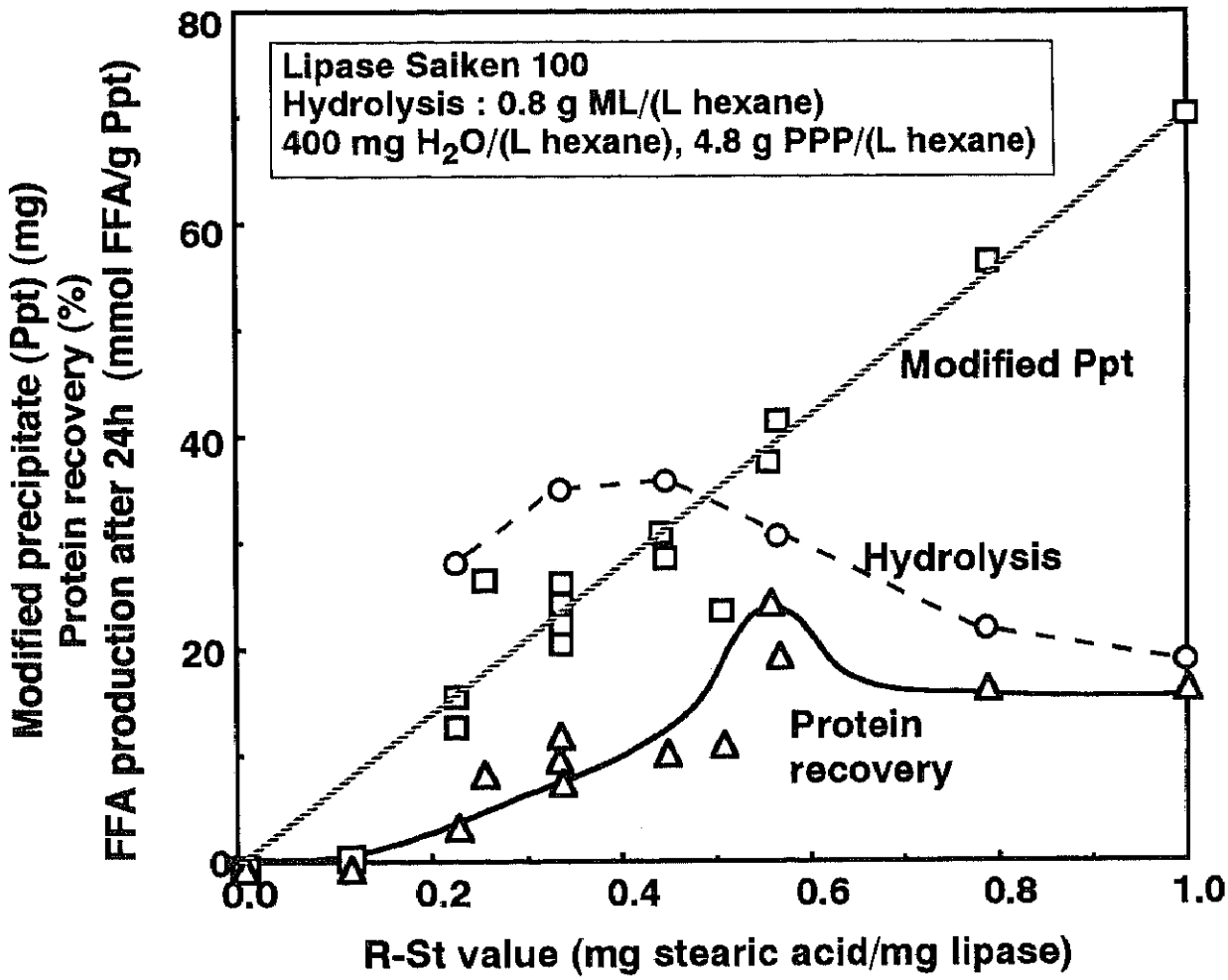

Fig. 2-3-3 The effect of the stearic acid: Lipase Saiken 100 weight ratio (R-St value) on modified precipitate yield □ (mg), protein recovery △ (%) and free fatty acid (FFA) production ○ after 24 hours hydrolysis reaction in *n*-hexane. Stearic acid was added during modified lipase preparation. Hydrolysis reaction conditions: 20 mg modified enzyme, 25 mL *n*-hexane, 10 mL water, 121 mg tripalmitin.

Table 2-3-2 Effect of Tris (5 mM) solution pH on stearic acid modified lipase precipitate (Ppt) yield, Ppt protein content, protein recovery interesterification and hydrolysis activities

Buffer pH	Ppt yield (g/L)	Ppt protein content (%)	Protein recovery (%)	Interesteri-fication [L^2/(mol•g•h)]	Hydrolysis [mmol/(g•h)]
5	0.867	11.9	13.7	23.3	2.89
6	0.491	10.3	6.7	15.7	3.04
7	0.364	7.15	3.5	11.6	2.27
8	0.159	7.95	1.7	12.3	2.38

Ppt: Precipitate formed after lipid addition to lipase solution.
pH: Modified lipase preparation.

Effect of modification on crude lipases

Six lipases were selected and their crude and modified forms compared. Modification was undertaken as previously described with stearic acid. Both interesterification and hydrolysis activities were monitored. In all cases, the interesterification activity of the crude lipases were zero or very low. Modification resulted in a dramatic increase in interesterification activity (Table 2-3-3). Of the lipases tested, modified Lipase Saiken 100, Lipase Asahi, Lipase Kurita and Lipolase showed the best interesterification activities. In contrast, modification did not result in enhanced hydrolysis activity for all lipases with only Lipase Saiken 100, Lipase Asahi and Lipase Kurita preparations having improved performance. Overall, the modified lipases Lipase Asahi and Lipase Kurita were best for interesterification and hydrolysis respectively. However, the high cost of Lipase Asahi and availability of Lipase Kurita may prohibit commercial use. In addition, the proportion of hydrolytic diglyceride byproducts produced during interesterification is also important. It may be beneficial therefore to select a particular lipase for interesterification or hydrolysis or to mix two lipases exhibiting these different properties; indeed it may also be beneficial to mix on the basis of activity and cost.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Modified lipase protein was characterized using SDS-PAGE electrophoresis (Fig. 2-3-4). Sample 1 is crude Lipase Saiken 100 which contained a range of proteins with differing molecular weights. Sample 4 shows the molecular weights of standard proteins. Stearic acid modification (samples 2 and 3) caused a selective separation of lipase protein corresponding to a molecular weight of around 30,000. As previously mentioned, crude lipase has little activity in *n*-hexane. This selective adsorption of "active" protein after stearic acid modification may also help to explain why modified lipases are much more active than their crude counterparts.

Table 2-3-3 Crude and modified lipases, precipitate (Ppt) yield, Ppt protein content, interesterification and hydrolysis activities

Lipase	Ppt yield (mg)	Ppt protein content (%)	Interesterification [mmol/(g•h)]	Hydrolysis [mmol/(g•h)]
Saiken			0.0	0.30
Saiken	**130**	**14.6**	**12.2**	**3.60**
Asahi			0.0	1.3
Asahi	**125**	**12.3**	**5.6**	**10.5**
Kurita			44.4	62.2
Kurita	**133**	**1.2**	**76.2**	**113**
Lipolase			0.0	12.7
Lipolase	**122**	**2.7**	**24.8**	**22.8**
Talipase			0.0	3.2
Talipase	**134**	**7.1**	**0.0**	**5.9**

Lipases: Lipase Saiken 100 (*Rhizopus japonicus*), Lipase Asahi (*Chromobacterium viscosum*), Lipase Kurita (*Pseudomonas* sp.), Lipolase (*Aspergillus oryzae*) and Talipase (*Rhizopus delemar*).
Ppt: Precipitate after lipid addition to lipase solution.
Modification: 90 mg crude lipase and 50 mg stearic acid.

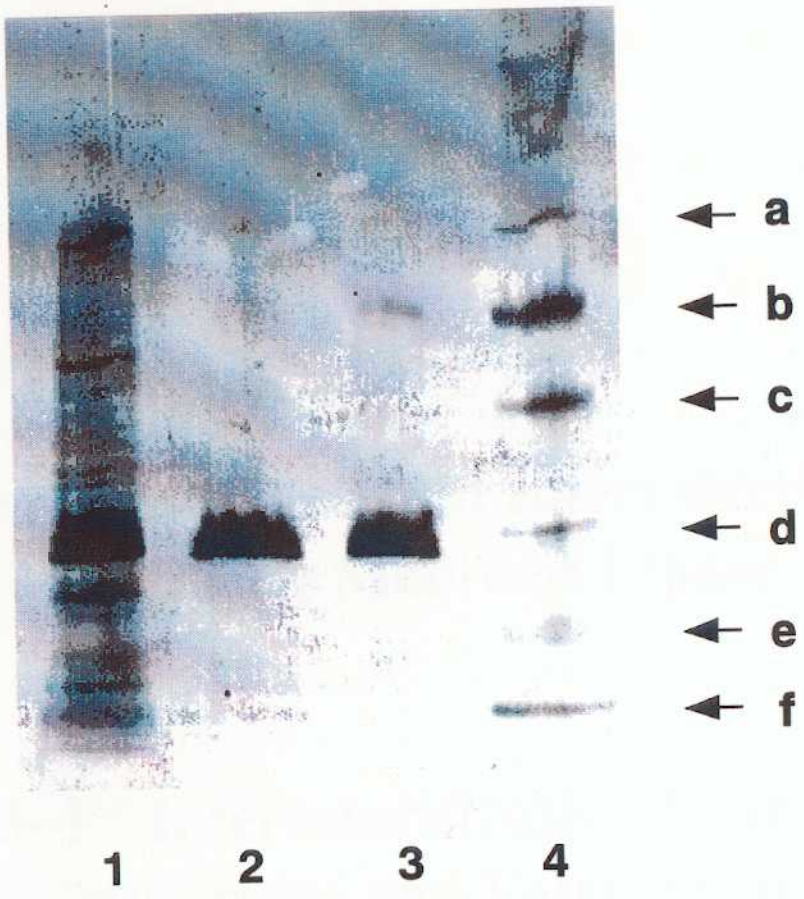

Fig. 2-3-4 SDS-PAGE electrophoresis. Sample 1 is crude lipase Saiken standard (30 mg/mL water). Samples 2 and 3 are stearic acid modified precipitates (10 and 11 mg modified lipase/mL water respectively). Sample 4 is molecular weight standards: a, phosphorylase b (M.W. 94,000); b, albumin (M.W. 67,000); c, ovalbumin (M.W. 43,000); d, carbonic anhydrase (M.W. 30,000); e, trypsin inhibitor (M.W. 20,100); f, α - lactalbumin (M.W 14,400).