Effect of Detergent Attached to Enzyme Molecules on the Activity of Organic-Solvent-soluble Lipases

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In our previous work, the organic-solvent-soluble enzyme (lipase B) was prepared in high yield using a synthetic detergent. Using this method, lipases from various origins have been solubilized in organic solvents with several kinds of detergent and the influence of detergent attached to the enzyme molecule on the reactivity of the organic-solvent-soluble lipase was investigated. The activities of the organic-solvent-soluble lipases have been compared in a homogeneous solvent system, tetrahydrofuran containing 20% buffer. It is shown that the hydrolysis rate of the organic-solvent-soluble lipase is affected by the nature of the surrounding detergent and that the organic-solvent-soluble lipase prepared from a detergent with a large hydrophobic region hydrolyses hydrophobic substrates more rapidly than that prepared from a detergent with a small hydrophobic region.

Recently, several attempts to use the enzyme lipase in organic media have been made by various investigators.[1–5] It has been reported that lipase reactions may be carried out in aqueous solution containing organic solvents in order to dissolve the substrates.[6–8] Alternatively, to avoid the denaturation and inactivation of the enzyme in an organic solvent, the preparation of organic-solvent-soluble enzymes has already been established.[9,10] Some esterifications and hydrolyses have been successfully conducted in organic solvent with these modified enzymes.[11–15]

The structure of the active site in the lipase molecule and the properties of the lipase as a protein have been investigated in detail,[16,17] but information on the enzymatic properties of these organic-solvent-soluble enzymes is limited.[10,18] The relationship between the modification of the lipase and its reactivity needs to be investigated in order to understand the molecular structure of the organic-solvent-soluble lipase.

In our previous work,[19] the method for the preparation of an organic-solvent-soluble lipase in high yield using the synthetic detergent dodecyl glucosylglutamate was reported. In this study organic-solvent-soluble lipases were prepared by the same method using a range of detergents. The activities of these organic-solvent-soluble lipases were compared in organic solvent in order to investigate the effect on reactivity of the detergent attached to the enzyme.

Results

The yield and the relative activity of the organic-solvent-soluble lipases prepared using four different detergents are listed in Table 1. The yield was calculated based on the final weight of lyophilized powder. For the measurement of the activity of the organic-solvent-soluble lipase, 200 μg of the powder containing the organic-solvent-soluble lipase was dissolved in 200 mm^3 of THF and 50 mm^3 of a buffer solution was used for hydrolysis of 4-methylumbelliferone oleate at 0.02 mol dm^{-3}. As shown in Table 1, it was demonstrated that the yield and the specific activity of the organic-solvent-soluble lipase were affected by the detergent used. For the pancreatic lipase, the organic-solvent-soluble enzymes prepared with the commercial detergents were shown to have higher activity than that with the detergent C_{12}. In the cases of lipases P and PN, the organic-solvent-soluble enzyme produced with the detergent C_{12} hydrolysed the substrate more rapidly than those with the commercial detergents. In particular, for lipase PN it was almost impossible to make the organic-solvent-soluble enzyme with the detergent SE L-590. In case of lipase B only slight differences were observed in the activities of the organic-solvent-soluble enzymes prepared with the various detergents.

Table 1 Yield and activity of organic solvent-soluble lipases

Enzyme		Detergent			
		SP-60R	SE S-570	SE L-590	C_{12}
Pancreatic lipase	yield[a]	47.5	31.7	11.7	67.6
	protein content[b]	11	11	11	13
	activity[c]	104	109	116	90
Lipase P	yield	50.7	51.7	11.5	43.7
	protein content	11	11	19	10
	activity	86	65	73	91
Lipase PN	yield	38.0	25.7	3.5	60.8
	protein content	13	18	19	9
	activity	82	67	72	102
Lipase B	yield	45.0	41.7	68.3	62.0
	protein content	10	14	11	14
	activity	92	73	77	84

[a] The yield of the precipitated powder is expressed as a percentage from 10 mg of lipase and 50 mg of detergent as starting materials. [b] The protein content is expressed as a weight percentage of the protein in the precipitated powder. [c] The hydrolysis activity of modified lipase is expressed as a percentage of that of the native lipase, which was taken as 100%.

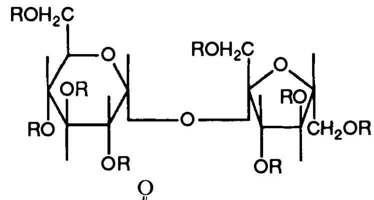
Lipase B was made soluble in organic solvents using five kinds of dialkyl glucosylglutamate with various lengths of the dialkyl chains (C_8–C_{16}). The complex of lipase B and the synthetic detergent was designated as organic-solvent-soluble lipase B. The yield of the powder containing the organic-solvent-soluble lipase B and the protein content of the powder are shown in Table 2. It was demonstrated that both the yield and the protein content are not influenced by the length of the dialkyl chains of the detergent. Furthermore, the number of detergent molecules bound to one enzyme molecule in each organic-solvent-soluble lipase B was investigated. The molecular weight of the organic-solvent-soluble lipase B was determined by a GPC (gel permeation chromatography) method previously (as described in the Experimental section). The molecular weight of the native lipase was known to be 33 kilodaltons. The results showed that one enzyme molecule was attached to 120–200 moledules of the detergent in the organic-

Dialkyl glucosyl glutamate

$HOCH_2CH(OH)CH(OH)CH(OH)CH(OH)OCHNHCHOCO(CH_2)_nCH_3$
$\qquad (CH_2)_2OCO(CH_2)_nCH_3$

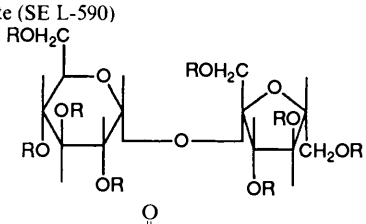
n = 6 dioctyl glucosylglutamate (C_8)
n = 8 didecyl glucosylglutamate (C_{10})
n = 10 didodecyl glucosylglutamate (C_{12})
n = 12 ditetradecyl glucosylglutamate (C_{14})
n = 14 dihexadecyl glucosylglutamate (C_{16})

Sucrose stearate (SE S-570)



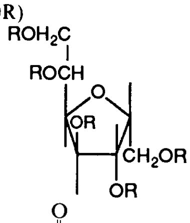
R=H or $\overset{O}{\overset{\|}{C}}CH_2(CH_2)_{15}CH_3$
mono 29%
di 37%
tri 25%
>tetra 10%

Sucrose laurate (SE L-590)



R=H or $\overset{O}{\overset{\|}{C}}CH_2(CH_2)_9CH_3$
mono 28%
di 37%
tri 25%
>tetra 10%

Sorbitan stearate (SP60R)



R=H or $\overset{O}{\overset{\|}{C}}CH_2(CH_2)_{15}CH_3$
mono 30%
di 50%
>tri 20%

Table 2 Yield and protein content of lipase B complexes

	Lengths of detergent side-chains				
	C_8	C_{10}	C_{12}	C_{14}	C_{16}
Yield of powder[a] (%wt)	51.7	61.3	63.3	62.1	57.0
Protein content in the complex (%wt)	13.3	12.8	13.4	12.3	12.9

[a] The weight of the precipitated powder was expressed as a percentage based on the weight of the starting materials.

solvent-soluble lipase. This was calculated from the molecular weights of the complex and the native enzyme (data not shown). The length of dialkyl chains of the detergent did not affect the ratio of detergent to enzyme in the organic-solvent-soluble lipase.

The effect of the length of the dialkyl chains of the detergent on the reactivity of the organic-solvent-soluble lipase was investigated. In the experiment on the hydrolysis reaction further purification of the organic-solvent-soluble lipase by the GPC method was not carried out, because the free excess detergent in reaction mixture did not affect the activity of the organic-solvent-soluble lipase. 4-Methylumbelliferyl esters were hydrolysed by the organic-solvent-soluble lipase B prepared using 5 synthetic detergents, C_8–C_{16}. When 4-methylumbelliferyl oleate and 4-methylumbelliferyl palmitate were used as substrates, the rate of hydrolysis by the organic-solvent-soluble lipase B prepared with the detergent with long dialkyl chains was more rapid than by those prepared with the detergents with short dialkyl chains (Fig. 1). In the case of 4-methylumbelliferyl phosphate as a hydrolysis substrate, the organic-solvent-soluble lipase B with the detergent with short dialkyl chains was found to hydrolyse the substrate more rapidly than that with the detergent with long dialkyl chains.

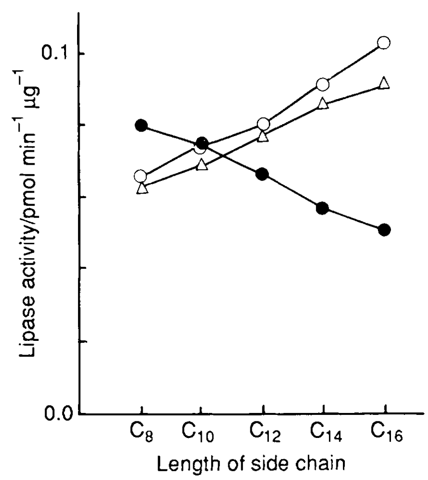


Fig. 1 Hydrolysis rate of acyl 4-methylumbelliferone. 4-Methylumbelliferyl oleate (○), 4-methylumbelliferyl palmitate (△) and 4-methylumbelliferyl phosphate (●) were hydrolysed at 37 °C for 30 min by the organic-solvent-soluble lipase B prepared using synthetic detergents with side chains of various length. The activity was determined by measuring the fluorescence intensity of the 4-methylumbelliferone released.

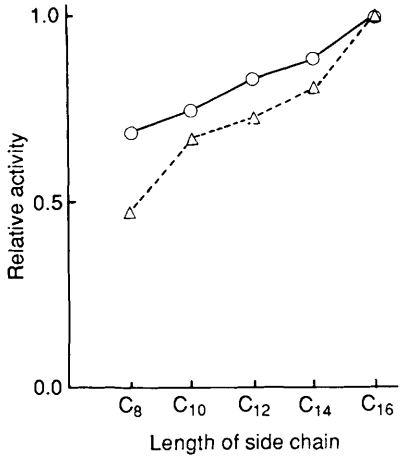


Fig. 2 Relative hydrolysis rate of triglycerides. Tricaprylin (○) and tripalmitin (△) were hydrolysed at 37 °C for 30 min by the organic-solvent-soluble lipase B prepared using synthetic detergents with side chains of various lengths. The activity was determined by the fluorimetric–HPLC method as described previously.[22]

Furthermore, the rate of triglyceride hydrolysis by five kinds of the organic-solvent-soluble lipase B were compared. Tripalmitin and tricaprylin were used as substrates (Fig. 2). It was demonstrated that the rate of hydrolysis of both trigylcerides depended on the length of dialkyl chains of the detergent attached to the organic-solvent-soluble lipase B. The organic-solvent-soluble lipase B prepared from the detergent C_{16} hydrolysed both triglycerides most rapidly of those kinds of organic-solvent-soluble lipase B tested.

Discussion

By the method described previously,[19] the organic-solvent-soluble lipase could be produced in higher yield than the method of Okahata and Ijiro.[9] The advantage of the improved method enabled us to prepare complexes of organic-solvent-soluble enzymes with other lipases and other detergents. The specific activities of lipase P, lipase PN and pancreatic lipase which were used in this experiment were lower by a factor of 20–150 than that of lipase B. Nevertheless, these lipases could be solubilised and their activity in organic solvents detected for the first time. The comparison of the specific activity of the organic-solvent-soluble lipase with that of the native lipase shows that no remarkable inactivation occurred in the organic-solvent-soluble lipases prepared by the modified method.

In our previous work,[19] only the synthetic detergent (C_{12}) was used to make the organic-solvent-soluble lipase. In this experiment, commercially available detergents such as sugar esters were used to modify four kinds of lipase to be solubilized in organic media. The yield and the specific activity of the organic-solvent-soluble enzyme varied with the detergent used. Among the detergents used in this experiment, the molecular structure of the hydrophobic region of C_{12} was the same as that of SE S-570. The difference between them is in the hydrophilic region. The specific activities of the organic-solvent-soluble lipases with C_{12} were higher than those of the complexes with SE S-570, except in the case of pancreatic lipase. Only the hydrophilic region was different between SP-60R and SE L-590. The specific activities of the complexes with SE L-590 were higher than those with SP-60R, except for the complex of pancreatic lipase. These suggest that the molecular structure of the hydrophilic region of the detergent affects the activity of the organic-solvent-soluble lipase. On the other hand, the hydrophilic regions of SE L-590 and SE S-570 are identical. There is a difference in the length of the fatty aid chain of their detergent. The organic-solvent-soluble lipase with SE L-590 hydrolysed the substrate more rapidly than that with SE S-570. This result suggests that the activity of the organic-solvent-soluble lipase is also influenced by the length of fatty acid chains of the detergent. As described above, the properties of the detergent strongly affected the enzymatic hydrolysis activity. It has also been demonstrated that the modification of lipase B with activated polyethyleneglycol influences its esterification activity.[11]

A comparative study of the relationship between the hydrolysis rate of the organic-solvent-soluble lipase and the length of dialkyl chains of the detergent attached to the enzyme molecule suggests that the hydrophobicity of the detergent molecules affects the hydrolysis reactivity of the organic-solvent-soluble enzyme. It is possible that the hydrophobicity of the detergent is related to the ability of the hydrophobic substrates to bind to the complex.

The preparation of organic-solvent-soluble enzymes with detergents is very important to promote enzymatic synthesis in organic media and to modify the substrate specificity of the enzymes. Further studies using various types of hydrophobic substrates will be reported elsewhere.

Experimental

Materials.—Lipase B from Wako Pure Chem. Co. (Osaka Japan), lipase P and lipase PN from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan) and pancreatic lipase from Sigma Chemical Co. (St. Louis, USA) were purchased commercially. Dialkyl glucosyl glutamates (C_8, C_{10}, C_{12}, C_{14}, C_{16}) were synthesized according to the method of Okahata *et al.*[9] Other detergents, sucrose ester of stearic acid (SE S-570), sucrose ester of lauric acid (SE L-590) and sorbitol ester of stearic acid (SP-60R) were kindly supplied from Nippon Oil & Fats Co. Ltd. Several triglycerides and acyl 4-methylumbelliferone were obtained from Sigma Chemical Co. Ltd. 9-Bromomethylacridine, a fluorescent labelling reagent for released fatty acids, and tetraethylammonium carbonate, a catalyst for the labelling reaction, were synthesized by Dr. Akasaka of Tohoku University according to a previous method.[20] All other chemicals of super-pure grade used in this experiment were obtained from Wako Pure Chem. Co. and Kanto Chem. Co. (Tokyo, Japan). The number of detergent molecules bound to one enzyme in each organic-solvent-soluble lipase B was investigated by GPC according to our previous method.[19]

Preparation of the Organic-solvent-soluble Lipase.—The modification for the preparation of the organic-solvent-soluble lipase was as follows. Lipase (10 mg) in water (2 cm^3) was added to the detergent (50 mg) in THF (4 cm^3) and stirred vigorously at 4 °C for 24 h. After evaporation of the solvent, the precipitates were collected by centrifugation, washed with water to eliminate the unmodified lipase and lyophilized overnight. The powder thus obtained, which contained the organic-solvent-soluble lipase and free detergent, was stored at 4 °C until use. The molecular weight of the organic-solvent-soluble lipase was determined by GPC according to our previous method.[19] The apparatus was the EYELA model PLC-5D (Tokyo Rika Kikai Co., Ltd.). The separation column was a TSK-gel $G400H_{LH}$ (Tosoh Manufacturing Co., Tokyo, Japan; 7.8 mm i.d. × 300 mm in length). The sample filtered through a 0.4 μm filter tip was injected and eluted with dichloromethane at a flow rate of 0.4 cm^3 min^{-1}. To estimate the molecular weight of the purified complex, the molecular-weight markers were eluted under the same conditions. The molecular weight of the organic-solvent-soluble lipase was calculated by comparing the elution time of the calibration standards. The number of detergent molecules bound to the enzyme was estimated based on the molecular weight of the complex.

Lipase Assay.—All hydrolysis reactions were conducted in a homogeneous solvent system, tetrahydrofuran (THF) containing 20% of 0.04 mol dm^{-3} Britton–Robinson buffer (pH 7.0). The composition of this buffer is 65.6 cm^3 each of 0.04 mol dm^{-3} phosphoric acid, acetic acid, and boric acid, and 34.4 cm^3 of 0.2 mol dm^{-3} sodium hydroxide. The hydrolysis activity was determined by two kinds of fluorimetric methods confirmed in previous studies. In one method, acyl 4-methylumbelliferone was hydrolysed by lipase and the fluorescence intensity of the liberated 4-methylumbelliferone was measured.[21] In another method, triglyceride was used as a substrate and the released fatty acids were labelled with the fluorescent labelling reagent, 9-bromomethylacridine, and determined with a high performance liquid chromatograph equipped with a fluorescence photometer.[22]

Acknowledgements

The authors are grateful to Mr. Y. Shindo of Tosoh Manufacturing Co. for supplying the HPLC columns. We also wish to thank Mr. T. Ito of Tohoku Fukushi University for his excellent technical assistance and Dr. K. Akasaka of Tohoku University for his kindly gifts of synthetic fluorescent reagents and for his helpful suggestions. This work was partly supported by the Grant of 'Biomedia project', Ministry of Agriculture, Forestry and Fisheries.

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Paper 1/01507I
Received 28*th March* 1991
Accepted 29*th July* 1991