Preparation of Organic-Solvent-Soluble Enzyme (Lipase B) and Characterization by Gel Permeation Chromatography

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To analyse lipase-catalysed reactions in organic media, a product was developed to aid both solubilization of lipase B and the preservation of its activity in organic solvents using a synthetic detergent, didodecyl glucosylglutamate, according to Okahata's modification method. By this improved method, >50% of the lipase B could be converted into a solvent-soluble enzyme, which was called organic-solvent-soluble lipase. The organic-solvent-soluble lipase was successfully separated by gel permeation chromatography (GPC) from the excess of free detergent. The lipase activity was eluted at the fraction corresponding to a molecular weight of 130 kD.† The composition of the purified solvent-soluble lipase was also investigated. It was estimated that 153 ± 25 molecules of the synthetic detergent were attached to one enzyme molecule, and the molecular weight of the complex was calculated to be 131 kD ± 16 kD based on the composition ratio. This value agreed with the molecular weight determined by GPC. These results confirmed that the organic-solvent-soluble lipase could be chromatographed by the GPC method and that its activity was preserved, suggesting that the GPC might be a very useful technique for the purification of organic-solvent-soluble enzymes.

Lipase, which catalyses the hydrolysis and esterification of fatty acid esters, has been studied for biochemical purposes and industrial applications by many investigators. Previously, most of the studies on the lipase reaction had been carried out using aq. solutions. Owing to the insolubility of the substrates, including fatty acids and lipids, it became necessary to add emulsifying agents or mechanical emulsifiers to the mixture of the lipase assay system. Regarding the complex kinetics,[1] little information is available about the reactive properties of the enzyme, and purification of the lipase has been more difficult than that of other soluble enzymes. If the reactions could take place in a homogeneous solvent, it would become possible to study the kinetic properties of lipase,[2,3] and to use the lipase as a transesterification catalyst for lipophilic substances in organic solvents. Moreover it is considered that the organic-solvent-soluble lipase may be important for industrial applications. Therefore, attempts to use the lipase in organic solvents have been made recently by some investigators and the usefulness of the method has already been confirmed.[4–7] One of the organic-solvent-soluble enzymes was prepared with a synthetic detergent by Okahata.[8] The modified lipase exhibited an activity for the synthesis of triglycerides in most organic solvents, and was used as a transesterification catalyst in organic media,[8] though the yield of the organic-solvent-soluble lipase was relatively low. Owing to the small amount of the complex available, the enzymatic properties of the complex have seldom been analysed. In our study, an improved method for the preparation of the organic-solvent-soluble lipase in very high yield was developed. Furthermore, the molecular weight of the organic-solvent-soluble lipase was determined by chemical analysis and column chromatography. Generally, size-exclusion chromatography in an organic solvent, which is designated gel permeation chromatography (GPC), has been employed mainly for the purification or separation of synthetic organic polymers.[9] We were interested in determining whether the degradation of the complex would take place during the purification by GPC. If the complex was not broken down, this procedure could become essential for the purification of enzymes in the biotechnical field. This is the first report which demonstrates that an organic-solvent-soluble enzyme can be separated by GPC without loss of activity.

† kD = kilodalton.

Table 1 Preparation of the solvent-soluble lipase

Trial	Starting materials: Lipase B (mg)	Starting materials: Detergent (mg)	Yield of complex (mg)	Protein content in complex (mg)	Yield of protein (%)
1	10.0	50.0	41.9	5.08	51
2	10.0	50.0	37.7	5.24	52
3	10.0	50.0	40.1	5.11	51

Results

Organic-solvent-soluble Lipase.—Yields of the complex prepared according to the improved method are listed in Table 1. More than half of the native lipase was estimated to be transformed into the organic-solvent-soluble lipase. When the complex was prepared according to the previous method, only *ca.* 10% of the native lipase could become soluble in organic media.[8] It was suggested that the preparative efficiency of the organic-solvent-soluble lipase would be increased remarkably by use of the improved method described in the Experimental section. The modified lipase in tetrahydrofuran (THF)–water exhibited >80% of the enzymic activity compared with the original lipase in aq. solution, which indicates that considerable inactivation of the organic-solvent-soluble lipase did not occur.

Purification by GPC.—HPLC was successfully used to separate the enzyme complex from excess of free detergent. In the column chromatogram, two peaks corresponding to the

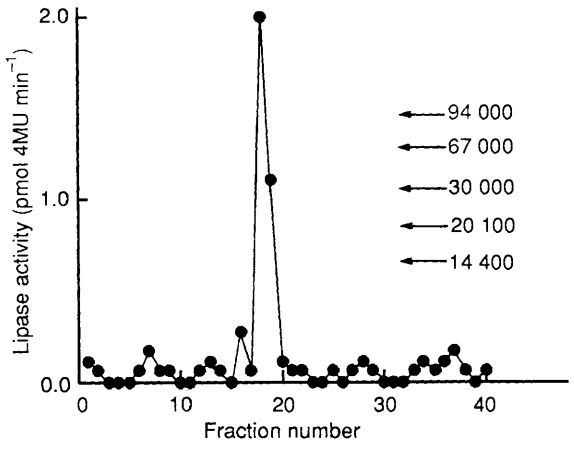

Fig. 1 Elution of lipase activity on the GPC column. Chromatography was conducted as described in the Experimental section. An aliquot (0.1 cm^3) from each fraction (0.4 cm^3) was used for the determination of lipase activity. The fractions containing the lipase activity (No. 18 and 19) were collected, and the molecular weight of the protein was estimated by SDS–PAGE (gel shown in the print). The values beside the gel indicate the molecular weight of the calibration proteins.

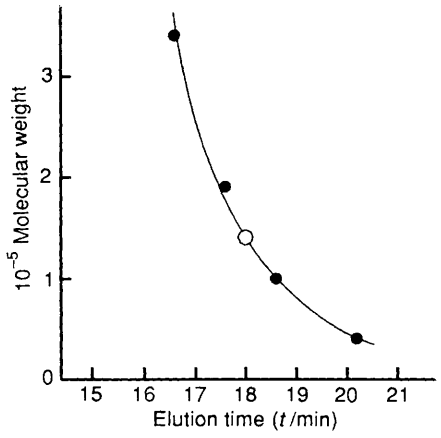

Fig. 2 Estimation of molecular weight of the purified organic-solvent-soluble lipase by GPC. Here, the molecular weights of standard compounds (●) were 4.39×10^4, 9.64×10^4, 1.90×10^5 and 3.55×10^5, respectively, and the modified lipase is indicated as (○).

modified lipase and free didodecyl glucosylglutamate were detected by monitoring at 280 nm. Owing to the high background of the stabilizer in dichloromethane, the chromatogram was not clear (data not shown). Among the fractions eluted each minute (0.4 cm^3), both the 18th and 19th fractions exhibited some hydrolysis activity for 4-methylumbelliferyl oleate as shown in Fig. 1. In addition, sodium dodecyl sulphate–polyacylamide gel electrophoresis (SDS–PAGE) demonstrated that these fractions contained a protein with the same molecular weight as that of lipase B (Fig. 1). An aliquot (4 μg) of the purified organic-solvent-soluble lipase was collected in each fractionation. The specific activity of the purified complex was compared with that of the total complex before purification, and there was no loss of activity. It was thus confirmed that the organic-solvent-soluble lipase could be successfully separated by GPC without loss of activity.

Determination of the Molecular Weight of the Organic-solvent-soluble Lipase.—In order to estimate the molecular weight of the purified complex, four kinds of molecular-weight markers were eluted under the same conditions. As shown in Fig. 2, the molecular weight of the organic-solvent-soluble lipase was calculated to be 130 kD by comparison of the elution times of the calibration standards. On the other hand, the molecular weight of the modified lipase was also determined on the basis of the composition ratio of the enzyme to didodecyl glucosylglutamate as listed in Table 2. The protein content in the purified organic-solvent-soluble lipase was first determined using a protein-assay kit. After the hydrolysis of the purified organic-solvent-soluble lipase by the conventional method, the content of glutamic acid liberated from didodecyl glucosylglutamate and that of protein were determined. In these determinations, it was estimated that 153 ± 25 molecules of didodecyl glucosylglutamate were attached to one lipase molecule. In this case, the molecular weight of the complex was calculated to be 131 ± 16 kD. This value agreed with the molecular weight estimated by GPC.

Discussion

To avoid the loss of activity and the denaturation of an enzyme in organic solvents, it is necessary to develop a method for the solubilization of the enzyme and the preservation of its activity in hydrophobic environments. To achieve this purpose, two procedures were developed in this study. One of them involved the preparation of the organic-solvent-soluble enzyme, which resulted in the production of a modified enzyme in high yield. In case of lipase B, a *ca.* 4-fold increase of the organic-solvent-soluble enzyme could be prepared by the improved method compared with the original method. It was also confirmed that several lipases from different origins could be solubilized in organic solvents by this method (data not shown). This improved method may be useful for the preparation of the organic-solvent-soluble enzyme. The other emphasis of our study was placed on the purification of the organic-solvent-soluble lipase in order to avoid degradation of the lipase–detergent bond or inactivation when using GPC. Although the molecular weight of the lipase B was 33 kD, hydrolysis activity was detected in the region corresponding to a molecular weight of 130 kD by GPC. This finding indicated that the breakdown of the bond between the lipase and didodecyl glycosylglutamate did not occur during GPC purification. In this study, since lipase B is homogeneous under electrophoresis, its complex was separated from the free detergent by GPC, and the molecular weight of the complex was estimated. This result suggested that GPC may be very effective not only for the determination of the molecular weight of the organic-solvent-soluble enzyme but also for the purification of a specific enzyme from other substances or contaminants, based on the molecular weight of the organic-solvent-soluble product. We have been able to purify a crude lipase by this method (manuscript in preparation).

In our studies, the modified enzyme fraction could not be detected on the chromatogram when using a normal UV-detector, due to the extremely low concentration of the enzyme. Although we used the analytical column of the GPC system for the purification of the organic-solvent-soluble lipase in this experiment, the introduction of a preparative column may enable us to obtain a larger amount of modified protein.

Recently, it has been reported that the enzymatic properties and substrate specificity of lipase in organic solvents were different from those of the intact type in aq. solution due to the change in the environment surrounding the enzyme molecule.[10,11] We have also observed that the specificity of the modified lipase was different from that of the native lipase. Further studies on the kinetics and the properties of the organic-solvent-soluble enzyme in organic media are currently in progress in our laboratory.

Table 2 Components of purified organic-solvent-soluble lipase

Trial	Protein content in purified complex (μg) (mol)	Total glutamate content after hydrolysis[a] (μg) (mol)	Number of molecules of detergent attached to one lipase molecule
1	8.55 (2.59×10^{-10})	7.56 (5.13×10^{-8})	188
2	8.48 (2.57×10^{-10})	5.35 (3.77×10^{-8})	137
3	4.24 (1.28×10^{-10})	2.57 (1.81×10^{-8})	131
4	7.56 (2.29×10^{-10})	4.85 (3.41×10^{-8})	139
5	4.07 (1.23×10^{-10})	3.20 (2.26×10^{-8})	173

[a] The value was the sum of the glutamate content derived from both the detergent and protein. It was estimated that there were 9.93 glutamate residues in one lipase molecule by the same method.

Experimental

Reagents.—Lipase B from *Pseudomonas fragi* 22. 39B was purchased from Wako Pure Chemical Co. (Osaka, Japan). Didodecyl glucosylglutamate used for the preparation of the organic-solvent-soluble lipase was synthesized according to the method[8] previously described. THF without stabilizer, and dichloromethane, were purchased from Wako Pure Chemical Co. 4-Methylumbelliferyl oleate was purchased from Sigma Chemical Co. (St. Louis, USA). Four kinds of polystyrene compounds for molecular-weight calibration of the HPLC system were purchased from Tosoh Manufacturing Co. (Tokyo, Japan). All the other chemicals of analytical grade, were purchased from Wako Pure Chemical Co. or Kanto Chemical Co. (Tokyo, Japan).

Preparation of Organic-solvent-soluble Lipase.—The modification for the preparation of the organic-solvent-soluble lipase was as follows. A mixture of lipase (10 mg) in water (2 cm^3) was added to a solution of didodecyl glucosylglutamate (50 mg) in THF (4 cm^3) and the mixture was stirred vigorously at 4 °C for 24 h. After evaporation of the solvent, the precipitate was 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 didodecyl glucosylglutamate, was stored at 4 °C until use.

Purification of Organic-solvent-soluble Lipase by GPC.—The apparatus used was the EYELA model PLC-5D equipped with a loop-type sample injector (Tokyo Rika Kikai Co., Ltd). The separation column was a TSK-gel $G4000H_{LX}$ (Tosoh Manufacturing Co., Tokyo, Japan; 7.8 mm i.d. × 30 cm in length). The aliquot (20 mm^3) which was filtered through a 0.4 μm filter tip was injected into the HPLC system and eluted with dichloromethane at the flow rate of 0.4 $cm^3\ min^{-1}$.

Lipase Assay.—The activity of the organic-solvent-soluble lipase was determined according to the method of Shimura *et al.*[12] 4-Methylumbelliferyl oleate was used as a substrate in the solvent mixture of THF and water. The activity was determined by measuring the fluorescence intensity at 450 nm (excitation at 320 nm) with a JASCO FP-770 spectrofluorometer. Changes in the fluorescence intensity induced during the assay of the lipase were converted into units of pmol of 4-methylumbelliferone generated per minute by using a standard solution of 4-methylumbelliferone in the concentration range 20–1000 nmol dm^{-3}.

Quantitative Determination of Protein and Didodecyl Glucosylglutamate.—The content of protein in the organic-solvent-soluble lipase and native lipase was determined using a protein assay kit (Bio-Rad Laboratories Co., Ltd). For the modified lipase, the protein content was determined after separation of the detergent from the complex by the addition of methanol. The quantitative determination of didodecyl glucosylglutamate was based on the measurement of amino acids by the ninhydrin method[13] or the measurement of glutamic acid using the Yamasa L-glutamate measurement kit (Yamasashoyu Co., Ltd, Chiba, Japan).

Polyacrylamide Gel Electrophoresis.—The presence of the organic-solvent-soluble lipase was also detected by SDS polyacrylamide gel electrophoresis (PAGE) using the Phast System (Pharmacia Fine Chemicals Co., Ltd). The molecular weight was estimated by SDS–PAGE using a series of proteins with known molecular weights as calibration standards.

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