Structural Characteristics of Lipid–Lipase Aggregates for Enantioselective Hydrolysis in Organic Solvents

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An ether-linked lipid–lipase aggregate was found to function as an immobilized biocatalyst. Three kinds of aggregate, I, III, and IV, obtained by sonication treatment were considered to have stacked bilayer structure of the lipid in the crystalline phase based on X-ray diffraction analysis. On the other hand, aggregate II obtained by the stirring method appeared to have an inverted micellar structure.

Keywords enantioselective hydrolysis; α-acyloxy ester; lipid–lipase aggregate; diltiazem hydrochloride; X-ray diffraction

In the previous paper,[2,3)] we reported that an ether-linked lipid–lipase aggregate, which was obtained by the sonication treatment of synthetic phospholipid A and the lipase Amano P from *Pseudomonas* sp., is an effective biocatalyst for enantioselective hydrolysis of α-acyloxy ester in organic solvents. By using these aggregates, a chiral intermediate (2*S*,3*S*)-**1** for the synthesis of pharmacologically and optically active diltiazem hydrochloride was obtained from the corresponding (±)-α-acyloxy ester **1**, which is a typical water-insoluble substrate, in water-saturated organic media.[2,3)]

We now report that the lipid–lipase aggregates function as an immobilized biocatalyst suitable for repeated use. The structural characteristics of the aggregates were examined by X-ray diffraction analysis.

Preparation of the Lipid–Lipase Aggregates For the preparation of aggregates, I, II and III, a crude lipase Amano P, which is commercially available, was used. A mixture of 100 mg of crude Amano P in water (5 ml) and 50 mg of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonocholine (P2NM3)[3)] in benzene (40 ml) was sonicated for 30 min at 0 °C. The resulting precipitate was centrifugated at 4000 × *g* and the solvent was removed by decantation. The residual precipitate was lyophilized to obtain an amorphous powder (aggregate I). The aggregate III of Amano P and 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphonoxy ethyl *N*-methyl morphonium (inner salt; P2NMMO)[3)] was prepared in the same way. When a mixture of Amano P and P2NM3 was stirred for 24 h at room temperature, the yield of the aggregate II was found to decrease. On the other hand, the purified crystalline lipase[4)] from *Pseudomonas* sp. was used for the preparation of aggregate IV. A mixture of 50 mg of the purified emzyme in water (5 ml) and 50 mg of P2NM3 in benzene (40 ml) was sonicated for 30 min at 0 °C and treated in the same way as described for the preparation of aggregates, I, II and III. The results are shown in Table I. The amount of the respective dry aggregates depended on the dialkyl ether-type phospholipid analogues used and the aggregation method.

Enantioselective Hydrolysis by Using Lipid–Lipase Aggregates When (±)-**1** was exposed to the enzymatic reaction using the presently prepared aggregates in water-saturated isopropyl ether, enantioselective hydrolysis smoothly occurred. The results by a high-performance liquid chromatographic (HPLC) analysis are given in Table II. The absolute structure and optical purity of the respective products were determined by the method previously reported.[3)]

It was found that the enantioselective hydrolysis was completed in 2 d, and the optical purities of the products ((2*R*,3*R*)-**2** and the desired (2*S*,3*S*)-**1**) depended on the phospholipid analogues used and the purity of the lipase. Namely, the aggregate III (entry 3) and the aggregate IV

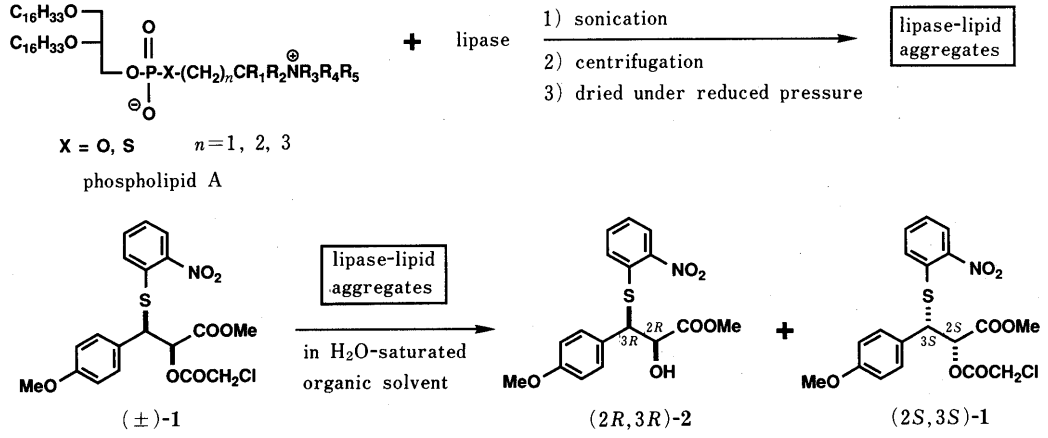

Chart 1

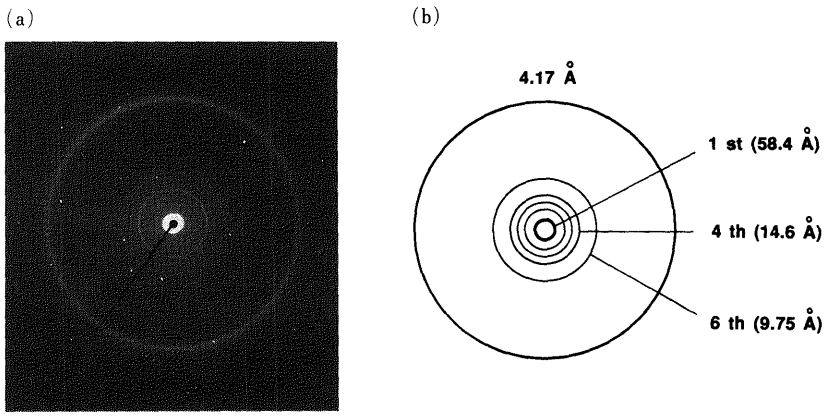

Fig. 1. X-Ray Diffraction Pattern of Aggregate I (a) and Its Schematic Representation (b)

TABLE I. Yield of Dry Aggregates

Entry	Lipase (mg)	Method	Lipid (mg)[a]	Aggregate (mg)
1	Crude Amano P (100)	Sonication	P2NM3 (50)	Aggregate I (46)
2	Crude Amano P (100)	Stirring	P2NM3 (50)	Aggregate II (14)
3	Crude Amano P (100)	Sonication	P2NMMO (50)	Aggregate III (54)
4	Purified Amano P (50)	Sonication	P2NM3 (50)	Aggregate IV (45)

Amano P; lipase from *Pseudomonas* sp. *a*) Phospholipid:

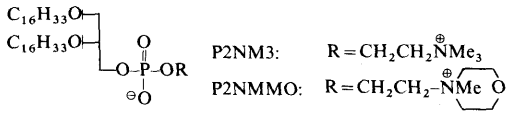

TABLE II. Enzymatic Reactions with 10 mg of (±)-**1**

Entry	Aggregate	(2*R*,3*R*)-**2**	(2*S*,3*S*)-**1**
		Yield (%)(Optical purity (% ee))	
1	Aggregate I	44.4 (98)	55.3 (79)
2	Aggregate II	47.5 (99)	52.3 (90)
3	Aggregate III	50.5 (97)	49.4 (99)
4	Aggregate IV	50.7 (96)	49.2 (99)
5[a]	Aggregate IV	50.0 (99)	49.9 (≥99)
6[b]	Aggregate IV	49.9 (99)	49.9 (≥99)

a) The same aggregate used in entry 4 was employed again after centrifugation and subsequent separation of the organic layer. *b*) The same aggregate used in entry 5 was employed again after centrifugation and subsequent separation of the organic layer.

(entry 4) were found to give products having high optical purity in high yield. When the recovered aggregate IV was used repeatedly, the yield and optical purity of the reaction products were maintained at extremely high levels (entries 5 and 6). This observation indicates that the lipid–lipase aggregate can function as an immobilized biocatalyst.

Analysis of Structural Characteristics of the Aggregates I, II, III and IV In order to determine the number of phospholipid molecules in the aggregate, elemental analysis of phosphorus in the aggregate IV was carried out; An average value of 2.84% was obtained. The molecular weight of the purified lipase from *Pseudomonas* sp. was reported to be 32000 by gel filtration.[4] As the molecular weight of P2NM3 was calculated to be 706 or 760 (with 3 moles of water), the number of phospholipids (P2NM3) per protein molecule was calculated to be 90 on average. Next, we carried out X-ray diffraction measurements for

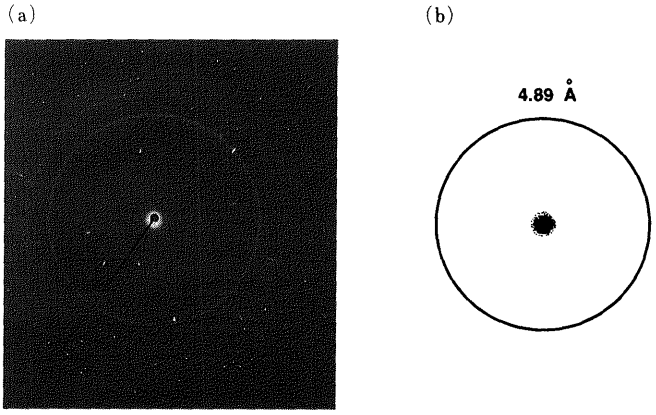

Fig. 2. X-Ray Diffraction Pattern of Aggregate II (a) and Its Schematic Representation (b)

the above four specimens. The X-ray power diagram for each specimen was obtained at room temperature with nickel-filtered CuK_α radiation (37.5 kV, 20 mA) using a flat plate camera with a sample-to-film distance of 6.31 cm. The X-ray diffraction patterns obtained for the four specimens are shown in Figs. 1 to 4 together with schematic drawings. Since the relative intensity of diffraction in the small angle region is very strong compared with that in the wide angle region, it is difficult to reproduce the intensity profile adequately in a printed photograph.

The results of X-ray measurements can be summarized as follows. In the X-ray diffraction pattern of aggregate I, a ring in the small angle region with high intensity at $(58.4\,\text{Å})^{-1}$ together with higher-order reflections including a 4th-order one with high intensity and a reflection at $(4.17\,\text{Å})^{-1}$ in the wide angle region were observed (Fig. 1).

For aggregate II, no ring in the small angle region was observed, but a strong reflection was seen at $(4.89\,\text{Å})^{-1}$ (Fig. 2). A few very weak reflections were also observed in a wider angle region than $(4.89\,\text{Å})^{-1}$. However, these reflections seemed to arise from components other than lipids in this specimen.

For aggregate III, a ring with high intensity at $(57.6\,\text{Å})^{-1}$ together with its 4th- and 6th-order reflections in the small angle region and a reflection at $(4.17\,\text{Å})^{-1}$ in the wide angle region were observed, as shown in Fig. 3.

For aggregate IV, a ring with high intensity at $(60.3\,\text{Å})^{-1}$ together with its 4th-order reflection in the small angle

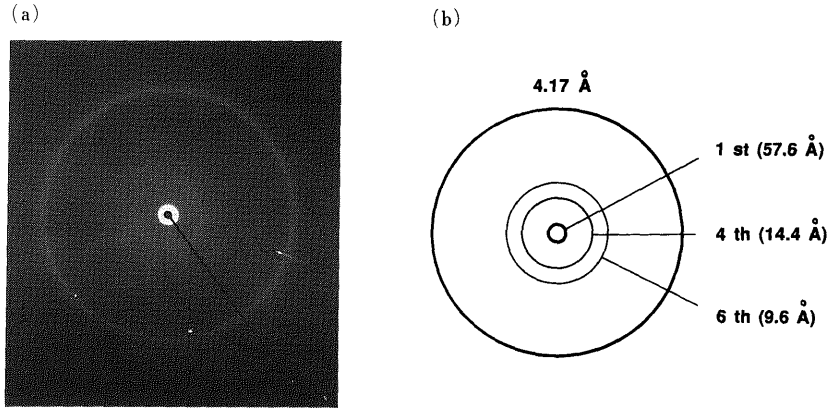

Fig. 3. X-Ray Diffraction Pattern of Aggregate III (a) and Its Schematic Representation (b)

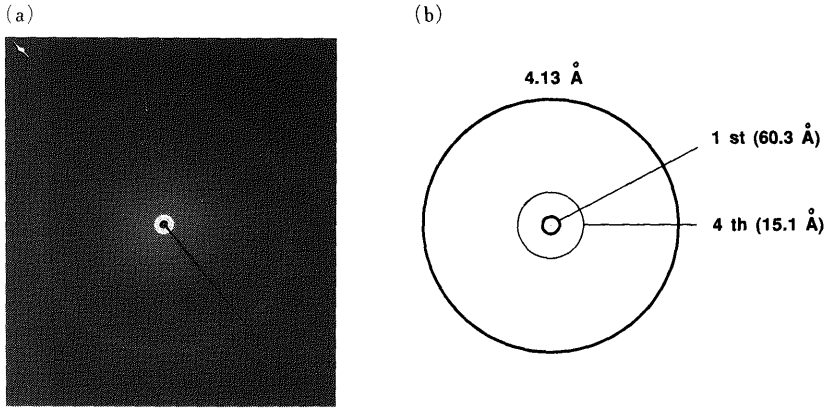

Fig. 4. X-Ray Diffraction Pattern of Aggregate IV (a) and Its Schematic Representation (b)

region and a reflection at $(4.13\,\text{Å})^{-1}$ in the wide angle region were observed (Fig. 4).

The X-ray diffraction patterns for aggregates I, III and IV each showed a reflection at about $(60\,\text{Å})^{-1}$ and its higher-order reflections including a strong 4th-order one in the small angle region and also a reflection at about $(4.2\,\text{Å})^{-1}$ in the wide angle region. The value of about 60 Å corresponds to the thickness of a bilayer observed in a phospholipid/water system.[5] The existence of the reflection with a dimension of about 60 Å together with its higher-order reflections including a strong 4th-order one for lipid systems has previously been observed and interpreted to suggest a stacked bilayer structure of phospholipids.[6-8] The reflection with the dimension about 4.2 Å implies a side-by-side packing of planar zig-zag hydrocarbon chains of lipid in the crystalline state.[9] Consequently, these X-ray diffraction patterns for aggregates I, III and IV suggest the existence of a stacked bilayer structure of lipid in the crystalline phase of these specimens.

In the X-ray diffraction pattern of aggregate II, the reflection with the dimension of 4.89 Å seems to arise from an arrangement of polar head groups of phospholipids, for the following two reasons. (1) If this reflection come from a hydrocarbon chain arrangement, it would not be a sharp reflection as shown in Fig. 2 but rather a diffuse scattering owing to the melting states of hydrocarbon chains.[9] (2) A sharp reflection from polar head group packing with a dimension of about 4.89 Å was observed in X-ray diffraction patterns of phosphatidylcholines (*i.e.* 4.85 Å[7]). There were no reflections corresponding to the thickness of a bilayer in multi-lamellar structure or to the dimension of about 4.2 Å. Therefore, we propose the existence of an inverted micellar structure for P2NM3 in the specimen of aggregate II.

From the standpoint of the present X-ray diffraction analyses of aggregates I, III and IV together with the number of phospholipids per protein molecule based on the elemental analysis of phosphorus in the aggregate IV (*i.e.* 90 phospholipid molecules per protein molecule), lipase was considered to be aggregated in the stacked lipid bilayer structure in the crystalline phases in these aggregates. This is supported by the fact that the ratio of lipid molecule/protein molecule (bacteriorhodopsin) is 10:1 in the bilayer structure (purple membrane) of *Halobacterium halobium*.[10] In this case, the molecular weight (about 26000) of bacteriorhodopsin is of the same order as that (about 32000) of the lipase Amano P from *Pseudomonas* sp. The number of phospholipid molecules per protein molecule is not enough to construct a multi-lamellar vesicular structure with the protein inside the vesicle.

It is most interesting to have obtained almost the same result in 4 kinds of enzymatic reactions despite the fine structures of the aggregates being different. Further development of amphiphiles having different types of hydrophilic portion as well as a long-chain ether linkage might lead to still more effective aggregates. Investigations

along this line and application of our aggregation method to a wide variety of enzymes are in progress.

Experimental

The HPLC system was composed of two SSC instruments (ultraviolet (UV) detector 3000B and flow system 3100).

Preparation of Lipid–Lipase Aggregate The procedure was described in the text.

General Procedure of Enantioselective Hydrolysis A mixture of (±)-**1** (10 mg) and lipid–lipase aggregate (5 mg) in water-saturated (iso-$Pr)_2O$ (2 ml) was shaken at 33 °C for 2 d. The reaction mixture was dried over $MgSO_4$ and evaporated to provide a crude product, which was analyzed by HPLC (a chiral column; Chiracel OD (4.6 × 250 mm)) under the following analytical conditions: eluent, hexane–EtOH (85 : 15) + AcOH (0.1%); detection, UV at 254 nm; flow rate, 1.5 ml/min.

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References and Notes

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