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STUDIES ON THE MECHANISM OF THE LIPASE REACTION

II. COMPARATIVE STUDIES ON THE ADSORPTION OF LIPASES AND VARIOUS PROTEINS AT THE AIR-WATER INTERFACE*

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Summary

Adsorption of lipases (EC 3.1.1.3) and various proteins at the air-water interface has been investigated in relation to the mechanism of lipase reaction.

Aqueous solutions of lipases and denaturated proteins show surface activity as strong as that of synthetic detergents. However, the surface activity of esterases and various other proteins is little or none.

By foam fractionation it was shown that lipases were adsorbed at the air-water interface and the adsorption followed the equation of Langmuir's adsorption isotherm.

The properties of lipase at the interface are discussed in relation to the mechanism of lipase reaction and the differences from the esterase reaction.

Introduction

The reaction of lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) is different from the reaction of carboxyl esterase (carboxylic-ester hydrolase, EC 3.1.1.1) in the physical state of the substrate, as pointed out by Desnuelle et al. [1]. The former reaction takes place in a heterogeneous system and the latter reaction proceeds in a homogeneous system. Benzonana et al. [2] reported kinetic studies showing that pancreatic lipase was adsorbed on the substrate according to the Langmuir isotherm and the reaction rate depended upon the area at the interface but not directly upon the weight of the insoluble substrate.

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Little work has been done on the molecular properties which lead to the affinity of lipase for the substrate interface. Okuda et al. [3] suggested that the lipid component in liver lipase has an important role in lipase activity and that the loss of lipid in the lipase, caused the conversion of the enzyme to a esterase. As the hydrophobic amino acid content in various lipases [4,5,6] is higher than that of other enzymes, it has also been postulated that the hydrophobic amino acid residues must play a role in lipase reaction. Brockerhoff [7] proposed a model for the interfacial orientation of the lipase molecule which had a hydrophobic head and hydrophilic tails, but this speculation was not supported by experiment.

In the previous paper [8] of this series, we reported that the lipase reaction was inhibited by emulsion of various organic solvents and concluded that lipases are adsorbed on the oil-water interface independent of the molecular structure of the oil phase.

The purpose of this work was to investigate the adsorption of lipase at the oil-water interface and elucidate the mechanism of the lipase reaction.

Experimental

Enzyme and protein

Lipase of *Chromobacterium* and *Pseudomonas* were purified as reported previously [9,10] and the homogeneity was confirmed by disc electrophoresis. Pancreatic lipase was prepared in the laboratory according to the method of Verger et al. [4] and the fraction of lipase L_B was used. *Candida* lipase was purified by the technique of Tomizuka et al. [11]. *Aspergillus* lipase was obtained from Amano Pharmaceutical Co., Ltd and the crude preparation used without further purification. Carboxylesterase of chicken liver was purified by procedures similar to those of Horgan et al. [12]. Trypsin, chymotrypsin and lysozyme were obtained from Eizai Co., Ltd and α-amylase from *Bacillus subtilis* (Liquefying type) was the preparation of Seikagaku Kogyo Co., Ltd. Cytochrome *c* (horse heart, type IV) and bovine serum albumin were obtained from Sigma Chemical Co. Pepsin, hemoglobin (human) and γ-globulin (bovine serum, Fr. II) were the preparations of Pentex, Nutritional Biochemicals and Armour Pharmaceutical Co., Ltd, respectively.

Assay of the activity of enzymes

The activity of lipase was assayed in accordance with the previous report [9] using olive oil emulsion as a substrate. The activity of carboxylesterase was determined spectrophotometrically [13] using *p*-nitrophenyl acetate as a substrate. The activity of protease and amylase were assayed by Casein-Folin method [14] and blue value method [15] respectively.

Assay of protein

The protein content was determined in accordance with the method of Lowry et al. [16].

Estimation of surface tension

Various proteins were dissolved in distilled water and the surface tension was measured with du Noüy's tensiometer at 4°C.

Foam fractionation

Protein was dissolved in 10 mM phosphate buffer at pH 7.0 and was fractionated with the apparatus shown in Fig. 1. Nitrogen gas was introduced into the bottom of the foaming tower through a glass filter at a constant flow rate of 10 ml/min. The surface area of the bubbles was calculated from the average diameter of the bubbles.

Results and Discussion

Surface tension of aqueous solution of lipases and various proteins

The effects of varying concentrations of lipases and esterase on the surface tension of water are shown in Fig. 2.

At a very low concentration, lipases from *Chromobacterium*, *Pseudomonas* and porcine pancreas caused a decrease in surface tension, and gave a surface tension-concentration curve with a minimum. The reason for the existence of the minimum value was considered to be either a change in the conformation of the enzyme proteins arising in the process of adsorption on the interface, or the presence of a trace amount of contaminant which is highly surface active. By contrast, carboxylesterase from chicken liver caused only a weak decrease in surface tension. The same effects were obtained with lipases and esterase on the interfacial tension of water-heptane (data not shown). From this result, it is apparent that lipases have an affinity for adsorption on the interface and are different from the carboxylesterase in this property.

In order to study whether the property of adsorption on the interface is specific for lipase, the surface tension of aqueous solutions of various proteins was measured. Results from native and denatured lysozyme are shown in Fig. 3.

Other proteins were also examined. Native lysozyme, which is in a folded and compact conformation, did not cause lowering of the surface tension of water. Pepsin, cytochrome *c* and γ-globulin were also surface inactive sub-

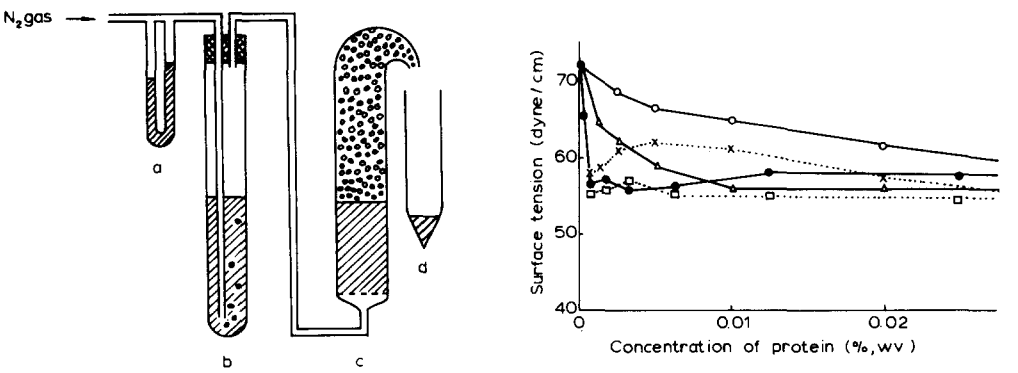

Fig. 1. Apparatus for foam fractionation. N_2 gas was introduced through a flow meter (a) and reservoir with water (b) at a constant flow rate of 10 ml/min. Various protein solutions were placed in a foaming tower (c; 1.5 × 15 cm) with a bottom of glass filter and formed bubble was collected in a tube (d).

Fig. 2. Surface tension of aqueous solution of esterase and lipases. Surface tension was measured by du Noüy's tensiometer at 4°C. ○———○, chicken liver esterase; △———△, *Candida* lipase, □·····□, *Chromobacterium* lipase; ×·····×, pancreatic lipase; ●———●, *Pseudomonas* lipase.

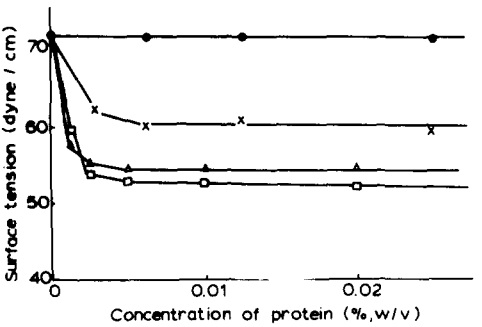

Fig. 3. Surface tension of lysozyme and denaturated enzyme. Surface tension was measured by du Noüy's tensiometer at 4°C. ●———●, native lysozyme in water; X———X, native enzyme in 50 mM NaOH; △———△, denaturated enzyme with performic acid; □———□, denaturated enzyme with 8 M urea.

stances. On the other hand, lysozyme dissolved in 50 mM NaOH as well as proteins denaturated with perfolmic acid or 8 M urea caused very great lowering of the surface tension. Trypsin, chymotrypsin, amylase from *Bacillus*, hemoglobin and bovine serum albumin showed a weak lowering effect of the surface tension similar to that of esterase from chicken liver. If a minute amount of denaturated protein is present, lowering of the surface tension occurs. In this method for the assay of surface tension the influence of any contamination of denatured protein cannot be ruled out so it is not clear whether the surface activity of the esterase is a property of the native protein. The surface activity of lipases, however, was obviously different from the other proteins.

TABLE I

FOAM FRACTIONATION OF *CHROMOBACTERIUM* LIPASE AND CHICKEN LIVER ESTERASE

Enzyme solutions, 40 μg of *Chromobacterium* lipase B or 50 μg of chicken liver esterase was dissolved in 10 ml of 10 mM phosphate buffer at pH 7.0, were fractionated at a constant flow rate of nitrogen gas 10 ml/min.

	Volume (ml)	Total activity (unit)	Activity/ml
Chromobacterium lipase			
Initial solution	9.00	292	32.4
Foamate, Fr. 1	0.30	43.9	146
Fr. 2	0.10	49.2	492
Fr. 3	0.20	86.7	434
Fr. 4	0.23	44.4	193
Residual solution	8.00	24.5	3.4
Total recovered	8.83 = 98%	248.7 = 85%	
Chicken liver esterase			
Initial solution	8.00	1.39	0.154
Foamate, Fr. 1	0.23	0.03	0.128
Fr. 2	0.21	0.02	0.096
Fr. 3	0.21	0.02	0.096
Fr. 4	0.26	0.02	0.077
Residual solution	7.05	1.13	0.160
Total recovered	7.96 = 99%	1.22 = 87%	

Foam fractionation of lipase and various proteins

Foam fractionation was applied to lipases, esterase and the other proteins, and the property of adsorption on air bubbles was observed. The result for the lipase of *Chromobacterium* is shown in Table I.

Most of the activity of the lipase contained in the initial solution was separated out in the foamate and only a part of the activity was found in the residual solution. The concentration of the lipase in the foamate is 5—15 times that of the initial solution. From these results, it can be seen that the lipase from *Chromobacterium* can absorb to the air-water interface without denaturation. The same result was obtained with the other lipases from *Pseudomonas*, *Candida*, *Aspergillus* and porcine pancreas.

Esterase of chicken liver was also applied on foam fractionation and the result is shown in Table I. The esterase was not separated out in the foamate and almost all of the activity of the initial solution was in the residual solution. This result indicates that the esterase has a low affinity of adsorption on the air-water interface. The same experiments were carried out with various proteins. Trypsin, chymotrypsin and amylase from *Bacillus* showed a weak surface activity in the experiment of surface tension, but they did not become adsorbed onto air bubbles like the esterase. Hemoglobin, γ-globulin and bovine serum albumin were coagulated in the foaming tower and a part of them were fractionated out into the foamate. Although these proteins denaturated in the air-water interface and became weakly surface active, the native protein can be considered as surface inactive.

Relationship between the amount of the adsorbed lipase at air-water interface and the surface area of air bubbles was investigated, and it was attempted to apply the adsorption on the Langmuir's adsorption isotherm. The result of lipase from *Chromobacterium* was shown in Fig. 4. The lipase should be adsorbed on air-water interface as a monomolecular film and the property was similar to those of synthetic detergents.

From the results in these experiments, it was found that lipase has a specific affinity of adsorption on the interface whereas the esterase is surface inactive. These results agree with the previous paper of this series [8] and it is confirmed that the affinity of lipase for the interface is independent of the

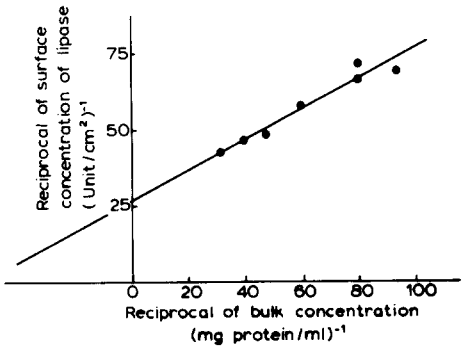

Fig. 4. Reciprocal plots of Langmuir's adsorption isotherm of *Chromobacterium* lipase B. *Chromobacterium* lipase B was dissolved in 10 mM phosphate buffer (pH 7.0) at various concentrations and was fractionated by foaming. Activity of lipase in an amount of foam was assayed.

molecular structures which constitute the interface. The substrates of lipase are fatty acid esters in a state of emulsion, micelle or monomolecular films. The first step of the reaction before the formation of enzyme-substrate complex is adsorption of the lipase onto the surface of the substrate. The subsequent steps of catalytic action proceed on the interface and finally the enzyme is regenerated with the liberation of the products. This idea was previously postulated by Verger et al. in the case of phospholipase [17]. By adsorption on the interface of the substrate and the medium, the lipase molecule is concentrated near to the substrate molecule and the catalytic action will thus accelerate. Lipase does not have any specific property of adsorption on dispersed substrates and water-soluble esters were hydrolyzed little or not at all. Esterase, however, is surface inactive and does not adsorb onto the interface. Therefore, the reaction of esterase takes place in aqueous solution and esters dispersed in water are good substrates but water-insoluble esters are poor substrates. From the results of the present experiments, it is shown that the differences in adsorption on the oil-water interface cause the difference in the rate of reaction between lipase and esterase.

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