Studies on Applications of Lipolytic Enzyme in Detergency I. Effect of Lipase from *Candida cylindracea* on Removal of Olive Oil from Cotton Fabric

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To investigate the effect of lipolytic enzyme on removal of triglyceride soils in laundry, the removal of olive oil from cotton fabric was examined by washing with an aqueous solution of lipase from *Candida cylindracea* with and without surfactants at various washing temperatures and times.

It was proved that, at optimum conditions, the removal of olive oil with the addition of lipase was 15 to 20% higher than without lipase. Therefore, it might be expected that lipase will be applied in the laundry detergents in practice.

Two major developments since 1970 have facilitated the practical use of enzymes in detergent formulations. One is the reduction of phosphate builder in detergents to solve environmental problems, and the other is the movement toward lower temperature washing to save energy (1-4).

Today, alkaline protease is formulated into most commercially available detergents. It has been observed that protease promotes removal of natural soil which contains protein stains (5). Further, it is expected that lipase, glycerol ester hydrolase (EC 3.1.1.3), will be used in laundry detergents to promote removal of triglyceride soils of human sebum which are difficult to remove under normal washing conditions (6). Lipase hydrolyzes triglycerides to di- and monoglycerides and free fatty acids (FFA). During removal of such glycerides by washing with alkaline solution of surfactant, FFA are easily removed from fabric by formation of water soluble fatty acid soaps, but triglycerides are not saponified by the alkaline solution and remain on the fabric (7).

In this paper, the effect of the lipase from *Candida cylindracea* on removal of olive oil from cotton fabric was examined by washing with an aqueous solution of the lipase with and without surfactants under various washing temperatures and times in order to investigate how the lipase improves removal of triglyceride soils in laundry.

MATERIALS AND METHODS

Lipase. Lipase used in this study was Lipase MY® (25,000 units/g, Lot. No. S5213, Meito Sangyo Co., Ltd.) from Anascosprogenous yeast of *Candida cylindracea.* One unit of lipase activity is defined as the amount of lipase which liberates 1 μmol of fatty acid from olive oil per min at 37 C (8).

Olive oil. Olive oil used was of Japan pharmacopeia grade (Lot. No. 1548, Yamakei Sangyo Co., Ltd.). The composition of fatty acids of olive oil analyzed by gas chromatographic method is shown in Table 1.

Surfactants. Surfactants used were selected from the commercially available products; decaethyleneglycol dodecylether (Emulgen 120, Kao Corporation, abbr. APE) and nonaethyleneglycol nonylphenylether (Emulgen 909, Kao Corporation, abbr. APPE) as nonionic surfactant; sodium dodecylbenzene sulfonate (chemical grade, Wako Pure Chemical Industries Ltd., abbr. LAS), and sodium α-olefin sulfonate (Lion Co. Ltd., abbr. AOS) as anionic surfactant.

Preparation of fabric soiled with olive oil. The cotton fabric (5 × 10 cm²) to be soiled was highly defatted in boiling chloroform for four hr. This treatment was repeated three times. The cotton fabric was soiled by spotting with 0.5 ml of olive oil benzene solution (100 mg/ml conc.) with micropipette two times. Thus, the quantity of the olive oil on the soiled fabric was 100 mg/5 × 10 cm².

Preparation of washing solution. Four kinds of washing solution were prepared; their compositions are shown in Table 2.

Solution B-S-L, which contained the buffer solution, the surfactant solution and the lipase solution, was prepared in the following manner: the buffer solution and the surfactant solution were measured into an Erlenmyer flask with ground stopper and preheated at 37 C for 10 min, followed by the addition of the lipase solution. Solutions B-L, B-S and B were prepared in the same manner. The volume of the final solutions was adjusted to 50 ml by adding distilled water. Then, a piece of the soiled fabric was put into the flask.

Washing procedure. The soiled fabric was washed at 37 C for 20 min by incubator (M-100N type, Taiyo Kagaku Co., Ltd.) with the shaking of 100 s.p.m. and the

TABLE 1

Properties of Olive Oil Used for Experiments

Specific gravity	D_{25}^{25}	0.912	
Acid value		0.33	
Saponification value		188	
Iodine value		85.0	
Composition of fatty acid[a]			(%)
C16:0		11.79	
C16:1		0.50	
C18:0		2.78	
C18:1		75.15	
C18:2		8.62	
Others		0.98	

[a]Analyzed by GLC.

TABLE 2

Composition of Washing Solution

		Washing solution (ml)			
		Solution B-S-L	Solution B-L	Solution B-S	Solution B
0.1M Phosphate buffer solution (pH 7.0)	(B)	20	20	20	20
Surfactant solution	(S)	25	—	25	—
Lipase solution	(L)	5	5	—	—
Distilled water		—	25	5	30
Total volume		50	50	50	50

shaking distance of 4 cm. Then, 5 ml of 2 N H_2SO_4 was added to terminate the activity of the lipase. The fabric was rinsed twice; each rinsing was with 100 ml of distilled water at 37 C for 3 min. Then, the fabric was air-dried. Washing also was done at various temperatures and times, as well as by using the lipase solution and the surfactant solution, both in various concentrations.

Determination of olive oil. Olive oil was extracted from the fabric with ethyl ether for four hr in a Soxhlet extractor. After ethyl ether was completely evaporated from the extract, 1 ml of methanol solution of margaric acid (99% purity, Nakarai Chemical Co., Ltd.) (50 mg/ml conc.) was added to the extract as internal standard. According to the standard fat analytical method (9), triglyceride in the extract was hydrolyzed to fatty acid by addition of 2 ml of 0.5 N NaOH methanol solution. Then, fatty acid methylester was prepared according to the boron trifluoride methanol method. The methylester obtained was separated and determined with gas chromatograph (Shimadzu GC-5A) and digital integrator (Shimadzu ITG-4A) under the following conditions: Column, 3 mm × 2 m of stainless steel packed with 10% diethyleneglycol succinate on 80–100 mesh Chromosorb W (Wako Pure Chemical Industries Ltd.); carrier gas, nitrogen; flow rate of nitrogen gas, 30 ml/min; flow rate of hydrogen gas, 40 ml/min; flow rate of air, 800 ml/min.

Calculation of removal. The removal of olive oil was calculated by equation [1], based on the weight of the total fatty acids on the fabric before and after washing.

$$\text{Removal (\%)} = (F_b - F_a) \times 100/F_b \qquad [1]$$

where F_b = weight of total fatty acids before washing (mg), and F_a = weight of total fatty acids after washing (mg).

RESULTS AND DISCUSSION

Effect of lipase concentration on removal of olive oil. The relation between the concentration of the lipase solution and the removal of the total fatty acids of olive oil from cotton fabric, both with and without nonionic surfactant APPE, is shown in Figure 1.

In both cases, olive oil removal increases as the concentration of the lipase increases. However, it becomes almost constant to reach the equilibrium state at a concentration of higher than 2 units of the lipase. This is explained as follows: the initial rate of hydrolysis of triglyceride by lipase depends on the interface area between the insoluble triglyceride and the aqueous solution of lipase (10); as the surface area of a given amount of olive oil which spreads on the fixed area of the fabric is considered constant, the effect of the lipase on the removal of olive oil will be constant at a certain concentration of the lipase with which the interface is saturated.

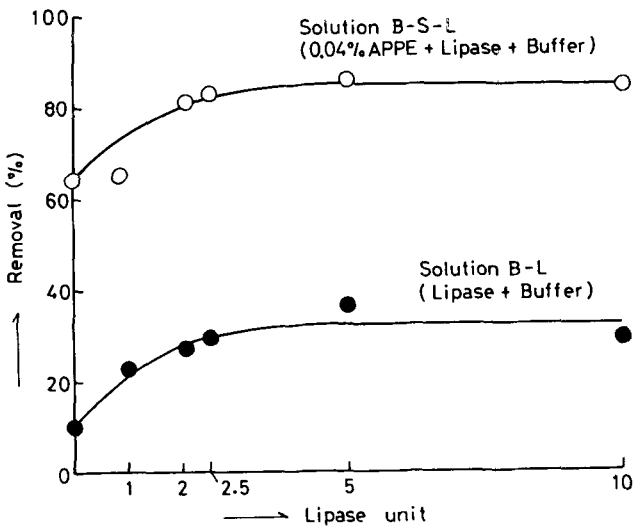

FIG. 1. Effect of concentration of lipase on removal of total fatty acid of olive oil at 37 C for 20 min.

As shown in Figure 1, the addition of the lipase improved the equilibrium removal of olive oil by 15 to 20%. The improvement was 65 to 85% with the surfactant APPE, and from 10 to 30% without the surfactant APPE.

Effect of washing temperature on removal of olive oil with lipase. The relation between the washing temperature and the removal of olive oil with the lipase is shown in Figure 2. With Solution B-L, the removal of olive oil increased to its maximum at the *Candida cylindracea* lipase's optimum temperature of 37 C. On the other hand, with Solution B-S containing APPE, the removal increased continuously as the washing temperature rose. With Solution B-S-L containing APPE and the lipase, the removal was higher at any washing temperature than with any other solution. However, the contribution of the lipase to the removal of olive oil was

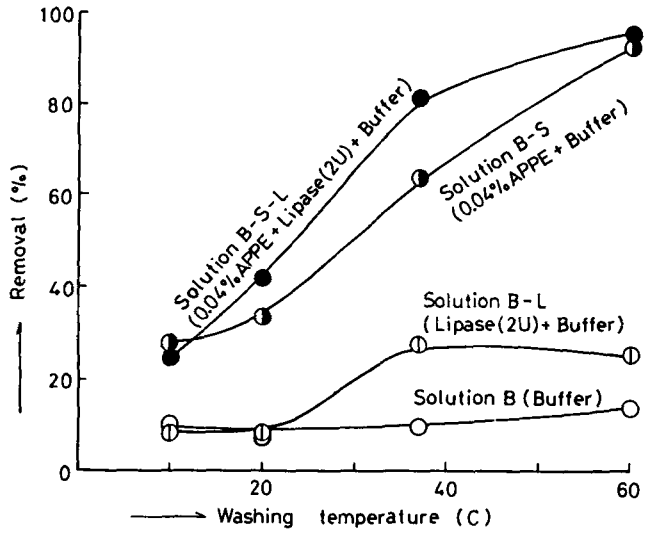

FIG. 2. Effect of washing temperature on removal of total fatty acid of olive oil for 20 min.

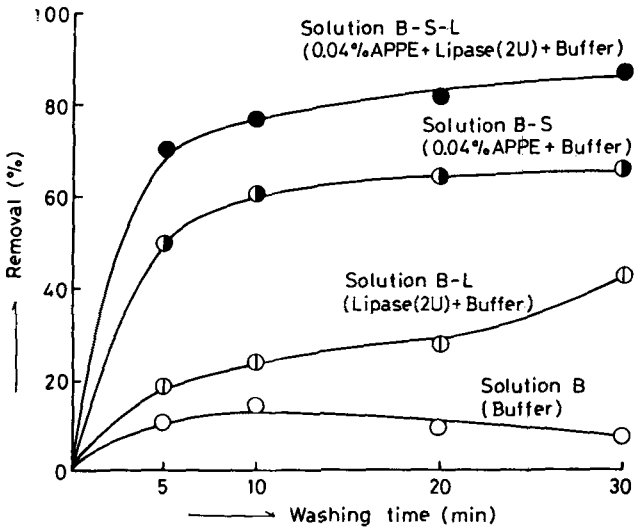

FIG. 3. Effect of washing time on removal of total fatty acid of olive oil at 37 C.

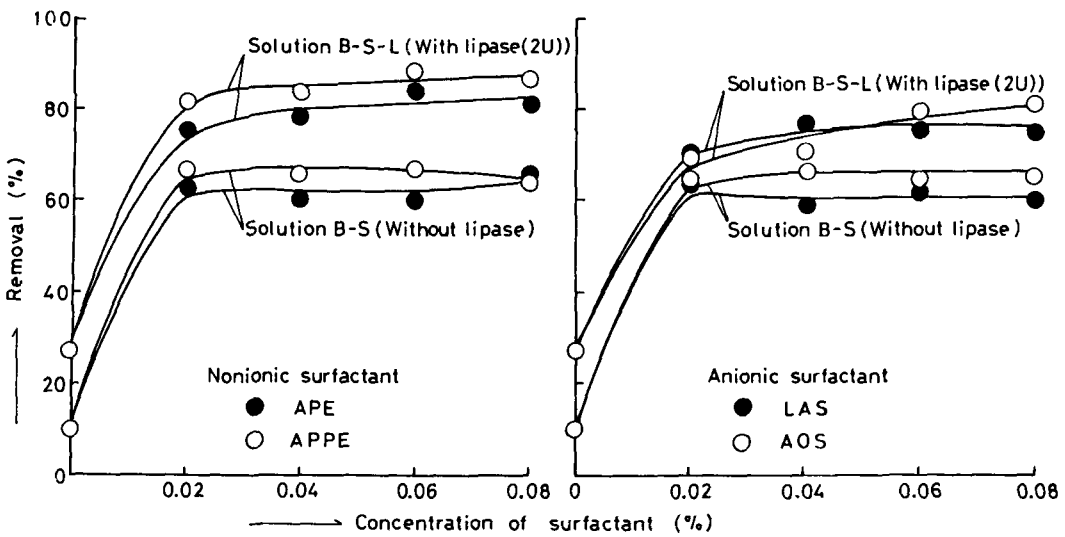

FIG. 4. Removal of total fatty acid of olive oil by various surfactants with or without lipase (2 units) at 37 C for 20 min.

observed most significantly at the optimum temperature of the lipase of 37 C.

Effect of washing time on removal of olive oil with lipase. The relation between the washing time and the removal of olive oil with the lipase is shown in Figure 3. It was observed that removal of olive oil with Solution B-L increased with a longer washing time. On the other hand, the removal with Solution B-S became nearly constant after 10 min. Further, the removal with Solution B-S-L was increasing continuously with the longer washing time; it was the highest at any washing time. Thus, the effect of the lipase on removal of olive oil can be observed significantly as the washing time becomes longer.

Effect of surfactant on removal of olive oil with lipase. The relation between the concentration of surfactant in the presence of the lipase and the removal of olive oil is shown in Figure 4.

At any concentration of any surfactant, whether the nonionic (APPE and APE) or the anionic (LAS and AOS), the removal of olive oil with Solution B-S-L was always higher than with Solution B-S. Thus, it was proven that the lipase was effective with any surfactant system at any concentration. In detail, the lipase was more effective with the nonionics than with the anionics. This is because the activity of the lipase is less inhibited by the nonionics than by the anionics (11).

It is proven, based on the results of this study, that the lipase from *Candida cylindracea* improves the removal of olive oil from cotton fabric by 15 to 20% under the op-

timum conditions of 2 units as the lipase concentration, 37 C as washing temperature and 10 min or longer as washing time.

Further, the effect of the lipase is more significant with the nonionic surfactant than with the anionic surfactant. It can be expected that the lipase will be used for laundry detergents.

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Kinetics of the Thermal Decomposition of Alkyl Hydrogen Sulphates

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First order rate constants and Arrhenius parameters have been obtained for the thermal decomposition of 1-hexadecyl hydrogen sulphate. From published data on thermal decomposition of lauryl hydrogen sulphate and lauryl ether hydrogen sulphate, first order rate constants and Arrhenius parameters have been obtained. The agreement between the two sets of data for the two alkyl hydrogen sulphates is within the 95% confidence limits, a combined Arrhenius plot giving an activation energy of 12.69 ± 1.97 K cal mol^{-1} and pre-exponential factor of $10^{(4.68 \pm 1.24)}$ sec^{-1}. For lauryl ether hydrogen sulphate, a 3-point Arrhenius plot gives an activation energy of 9.4 K cal. mol^{-1} and a pre-exponential factor of 10^2 sec^{-1}.

Thermal decomposition of primary alkyl hydrogen sulphates is a conversion-reducing side reaction which should be minimized in the manufacture of primary alcohol sulphates by sulphonation of primary alcohols. In this paper we present kinetic data on the thermal decomposition of 1-hexadecyl hydrogen sulphate, and compare our findings with those published by Takei, Tsuto, Miyamoto and Wakatsuki (1), for lauryl hydrogen sulphate.

EXPERIMENTAL

1-Hexadecyl hydrogen sulphate was prepared by the reaction of hexadecanol with chlorosulphonic acid, as described by Maurer, Stirton and Weil (2). After recrystallization from 30-40 C petroleum ether, the product was shown by nuclear magnetic resonance (NMR) to be ca 99% pure 1-hexadecyl hydrogen sulphate, containing ca 1% hexadecanol as the only impurity (triplet at 4.03 ppm, $-CH_2OSO_3H$; triplet at 3.75 ppm, $-CH_2OH$; spectra recorded on solutions in chloroform/trifluoroacetic acid).

Di-(1-hexadecyl) sulphate and di-(1-hexadecyl) ether for use as analytical standards were prepared by the methods of Barkenbaus and Owen (3) and Perron and Paquot (4), respectively.

Kinetic runs were carried out as follows. Hexadecyl hydrogen sulphate (ca 10 g) was placed in a 50 ml two-necked flask and, with magnetic stirring, heated at a constant temperature by means of an oil bath. Samples (ca. 0.2 g) were removed at measured times, weighed accurately and quenched immediately with N/10 ethanolic sodium hydroxide (15 ml). The quenched samples were then made up to 50 ml with distilled water, and these solutions were used to estimate the percent of anionic detergent (AD) in the samples by the two phase titration method of Epton, using methylene blue/cetyl dimethyl benzyl ammonium chloride (5).

The data of Takei, Tsuto, Miyamoto and Wakatsuki for lauryl hydrogen sulphate are given in their paper in the form of graphs of AD against time. We estimated numerical values for these data points by reading from the graphs.

The AD values are shown in Tables 1 and 2.

Qualitative analysis of nonionic decomposition products was carried out as follows. After the kinetic run at 104 C had been completed, a sample (3 g) was dissolved in ether (20 ml) and neutralized by addition of 100 ml of an aqueous 2M sodium hydroxide solution. The nonionic products were extracted with hexane (5 × 100 ml), with heating and addition of t-butanol (2 ml) as necessary to break emulsions. After evaporation of the hexane, the nonionic products were analyzed by thin layer chromatography (TLC) on plates of silica gel (0.025 in thickness) impregnated with sulphuric acid. This adsorbent was prepared by slurrying silica (45 g Merck Kieselgel G) with a mixture of water (100 ml) and 98% sulphuric acid (2.25 ml). The TLC runs were carried out at 5 C. Plates were visualized by spraying with 50% aqueous sulphuric acid followed by heating at 180 C overnight. A 1-1.5% diethyl ether solution in hexane was found to be suitable for separation of the less polar components, which were identified by comparison of