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Technical

Studies on Applications of Lypolytic Enzymes in Detergency II. Evaluation of Adaptability of Various Kinds of Lipases in Practical Laundry Conditions

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ABSTRACT

Kinetic parameter of hydrolysis, degree of hydrolysis, pH and temperature characteristics and positional specificity of hydrolysis of seven kinds of lipases from various microorganisms and a pancreatic lipase were examined for triolein or olive oil emulsion as a substrate and the adaptability of lipases for laundry systems was evaluated on the basis of these properties.

As a result, it was found that lipases from yeast of *Candida cylindracea* (Lipase MY® and OF®) were excellent in kinetic parameter and degree of hydrolysis and temperature characteristics and had no positional specificity, and that lipases from mold of *Mucor* (Lipase M-AP® and SP®) were excellent in pH and temperature characteristics and had positional specificity. On the viewpoint of the present laundry practice under alkaline conditions and the tendency toward low temperature washing, lipases from *Candida* and *Mucor* seem to be more suitable for laundry systems in comparison with lipases from other microorganisms and a pancreatic lipase.

The effect of positional specificity in hydrolysis of lipase will be discussed on the basis of examination on the removal of triglyceride and its hydrolysates by surfactant solution in a subsequent paper.

INTRODUCTION

The importance of enzyme applications in laundry detergents has been enhanced due to a decrease in detergency performance caused by lower levels of phosphate builder because of environmental control and a tendency toward lower temperature washing to save energy (1,2).

The authors suggested that lipase, glycerol-ester hydrolase (EC 3.1.1.3) would be favorably applicable in removal of oily soil based on the fact that lipase from *Candida cylindracea* improved the removal of olive oil from cotton fabric by 15-20% at optimum conditions (3).

On the other hand, various kinds of stable lipases of high activity from microorganisms have become available as a result of the recent development of the enzyme industry. Although the properties of these lipases have been reported (4-7), the characteristics of lipases in hydrolysis of oil depend largely on the kind and condition of substrate as well as on the hydrolysis conditions such as pH and temperature, etc.

This study is to evaluate the characteristics of the lipase suitable for laundry through investigation of kinetic parameter of hydrolysis, degree of hydrolysis, pH and temperature characteristics and positional specificity of hydrolysis for the same substrate under the same condition. It was found there were remarkable differences in characteristics of various lipases.

TABLE I

Lipases Used for Experiments

	Origin	Activity (u/g)	Trade name	Maker	Abbrev.
Yeast lipase	*Candida cylindracea*	24500	Lipase MY	Meito	Can-1
	Candida cylindracea	24200	Lipase OF	Meito	Can-2
Mold lipase	*Mucor sp.*	7300	Lipase M-AP	Amano	Mu-1
	Mucor sp.	3000	Lipase SP	Novo	Mu-2
	Aspergillus var.	5700	Lipase AP	Amano	Asp
	Rhizopus arrhizus	90000		Gist-Brocades	Rhi
Bacterial lipase	*Pseudomonas*	29600	LP-Amano	Amano	Pse
Pancreatic lipase	Porcine pancrea	2800		Sigma	Pan

MATERIALS AND METHODS

Lipases

Seven kinds of lipases from various microorganisms and a pancreatic lipase shown in Table I were used in this study. One lipase unit was defined as the amount of the lipase which liberated 1μ mol of fatty acid from olive oil/min at 37 C, according to Yamada et al. (8).

Substrates

As a substrate of hydrolysis by lipase, triolein (extra pure grade, Tokyo Kasei Kogyo Co., Tokyo, Japan) and olive oil (Japan pharmacopeia grade, Yamakei Sangyo Co., Osaka, Japan) were used as received.

Preparation of Emulsion

The emulsion was prepared by twice homogenizing a mixture of 25ml of triolein or olive oil and 75ml of 2% polyvinylalcohol solution consisting of 1.85% PVA 117 (polymerization degree 1725±25, saponification value 98.5±0.5 mole%, Kuraray Co. Ltd., Osaka) and 0.15% PVA 205 (550±50, 88.0±1.5 mole%, respectively, Kuraray) with Emulation (Teraoka Co., Osaka) at 11,000 rpm for 5 min each at 5-10 C.

Procedures of Hydrolysis by Lipase and Determination of Hydrolysates

Hydrolysis of triolein emulsion. Triolein emulsion prepared as described above was diluted with distilled water (9) to obtain emulsions of various concentrations (2, 4, 10 and 50%). These emulsions were hydrolyzed by 8 kinds of lipases in the following procedure: A mixture of 5ml triolein emulsion, 3ml phosphate buffer (0.1M, pH 7.0) and 1ml distilled water was preheated at 37 C for 10 min and hydrolyzed for 60 min by the addition of 1ml lipase solution (2.5u/ml). Then, 20ml ethyl ether containing internal standard (p-hydroxybenzoic acid, m.p. of 215.0-215.8 C, obtained from Ueno Fine Chemical Ind. Ltd., Osaka) was added to the reaction mixture to stop hydrolysis and to extract triolein and its hydrolysates simultaneously. At the same time, a similar procedure without addition of lipase was used as control.

The extracts were concentrated and dried over sodium sulfate. The remaining triolein and its hydrolysates in the extracts were separated and quantitated by TLC-FID using Iatroscan TH-10 (Iatron Lab. Inc., Tokyo), Hitachi 056 2-pen recorder (Hitachi Ltd., Tokyo) and Chromatopack R-1B (Shimadzu Scientific Instrument & Equipment, Kyoto, Japan) under the following conditions (10): Stationary phase, Chromarod S-II (Iatron) impregnated with 3% boric acid; mobile phase; hexane/ethyl ether/acetic acid (70:30:1, v/v/v); flow rate of hydrogen gas, 160ml/min; flow rate of air, 2000ml/min; scanning speed, 40 sec/scan; range of recorder, 50-100mV, and chart-driving speed, 120mm/min.

Triolein and each component of hydrolysates (1,3- and 1,2-diolein, monoolein and free oleic acid) in the reaction mixture and in the control mixture were determined by the use of calibration curves as previously reported (10). A difference in the amount of oleic acid in the reaction mixture and in the control mixture was judged to be the amount of oleic acid liberated by lipase.

Hydrolysis of olive oil emulsion. A mixture of 5ml olive oil emulsion, 3ml phosphate buffer (0.1M, pH 7.0) and one ml distilled water was preheated at a given temperature for 10 min and hydrolyzed for 20 min by the addition of one ml lipase solution (2.5u/ml). Then, 20ml of a mixture of acetone/ethanol (1:1, v/v) was added to the reaction mixture to stop hydrolysis. As control, the above procedure was repeated with the exception that the lipase was added only after the addition of a mixture of acetone/ethanol.

Free fatty acids both in the reaction mixture and in the control mixture were titrated with 0.05N NaOH with phenophthalein as indicator. The amount of free fatty acid liberated by lipase was obtained as the difference of the amount of the free fatty acids in the reaction mixture and in the control mixture.

Furthermore, the effect of pH on hydrolysis by lipase was investigated by changing pH of the above reaction system using the buffer solution of Clark-Lubs (pH 6.0-10.0).

RESULTS AND DISCUSSION

Kinetic Parameter for Hydrolysis of Triolein by Lipases

Hydrolysis of triolein emulsion of various concentrations by eight kinds of lipases was carried out to determine kinetic parameter and degree of hydrolysis. As shown in Figure 1, the relation between the concentration of triolein emulsion and the reaction rate was described as typical Michaelis-Menten curves for all the lipases except Mu-2. By converting on reciprocal scale according to Lineweaver-Burk, they gave straight lines (Fig. 2). Maximum reaction rate and Michaelis constant as kinetic parameters of hydrolysis by lipase were obtained from the intercepts and slopes of these lines by the least squares method. The results are shown in Table II. The maximum rates of Mu-1, Can-1 and Can-2 were larger and those of Asp, Rhi, Pse and Pan were about one-half the former values. The values of Michaelis constant were in the range of about 5-15% with the exception of Asp, which had a very large value.

Using Can-1, kinetic parameters for hydrolysis of olive

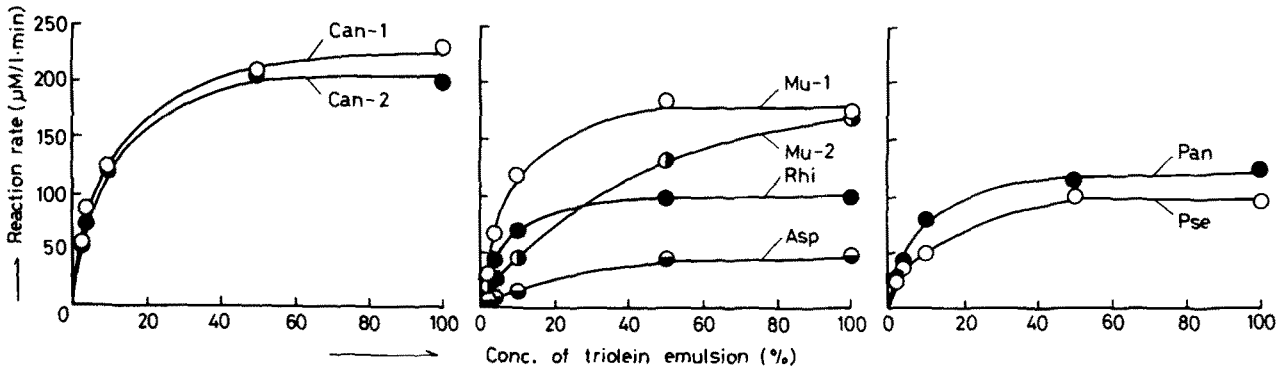

FIG. 1. Relation between reaction rate of triolein hydrolysis by various lipases and concentration of triolein emulsion at 37 C and pH 7.0.

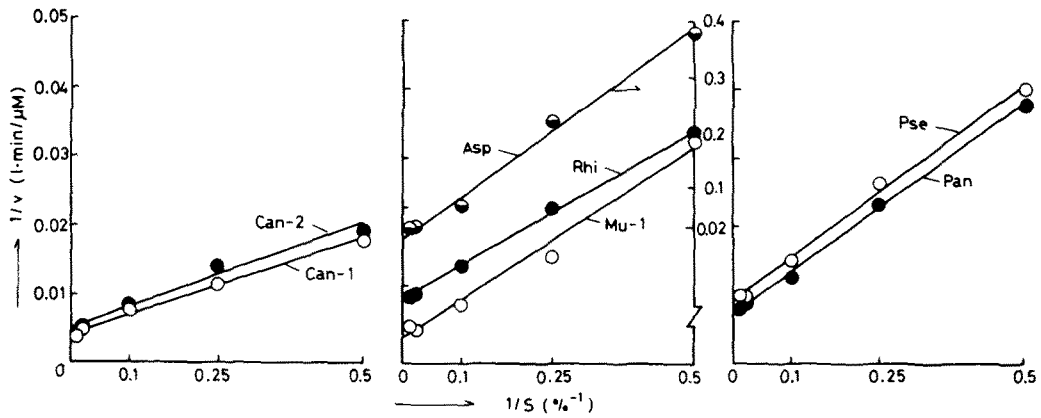

FIG. 2. Reciprocal plot of reaction rate of hydrolysis vs concentration of triolein emulsion according to Lineweaver-Burk.

oil emulsion also were determined by the alkaline titration method in the same procedure. Both maximum reaction rate and Michaelis constant for hydrolysis of olive oil emulsion, shown in Table II, were similar to those for triolein emulsion.

The degree of hydrolysis of triolein emulsion at each concentration was calculated by equation [1] (11) and listed in Table III.

$$\text{Hydrolysis (\%)} = \frac{\text{amount of oleic acid liberated by lipase}}{\text{total amount of the constitutive oleoyl group of triolein}} \times 100 \quad [1]$$

The lower the concentration of emulsion is, the higher the degree of hydrolysis is. Hydrolysis of triolein emulsion by Can-1 and Can-2 proceeds most rapidly while hydrolysis by Asp is substantially slower.

Effect of pH on Hydrolysis by Lipase

The amount of liberated fatty acid from olive oil emulsion by lipases was measured at various pH. The pH of the hydrolysis system varied from 6 to 10 in the application of lipase in the practical laundry condition. As shown in Figure 3, the effect of pH on the amount of liberated fatty acid is classified roughly into three groups, depending on

TABLE II

Kinetic Parameter of Hydrolysis of Triolein Emulsion by Various Lipases

	Lipase						
Kinetic parameter	Can-1	Can-2	Mu-1	Asp	Rhi	Pse	Pan
Maximum rate (μ mol/l · min)	221 (260)[a]	200	259	95	104	103	139
Michaelis constant (%)	5.9 (3.6)	5.8	14.1	71.4	5.1	6.5	8.4

[a]Parameter of hydrolysis of olive oil emulsion.

TABLE III

The Degree of Hydrolysis of Triolein Emulsion by Various Lipases

Conc. of emulsion (%)	Degree of hydrolysis (%)								
	Lipase								
	Can-1	Can-2	Mu-1	Mu-2	Asp	Rhi	Pse	Pan	Control
100	5.5	5.0	4.7	4.0	2.7	3.6	3.4	3.8	1.9
50	8.0	8.2	7.6	5.1	3.0	5.4	4.8	5.3	1.7
10	21.7	20.6	20.4	7.6	4.3	14.5	12.3	14.8	1.7
4	37.0	31.5	28.8	12.5	5.8	18.8	17.2	20.7	1.6
2	47.4	44.9	29.0	18.4	6.4	25.0	24.2	25.4	1.3

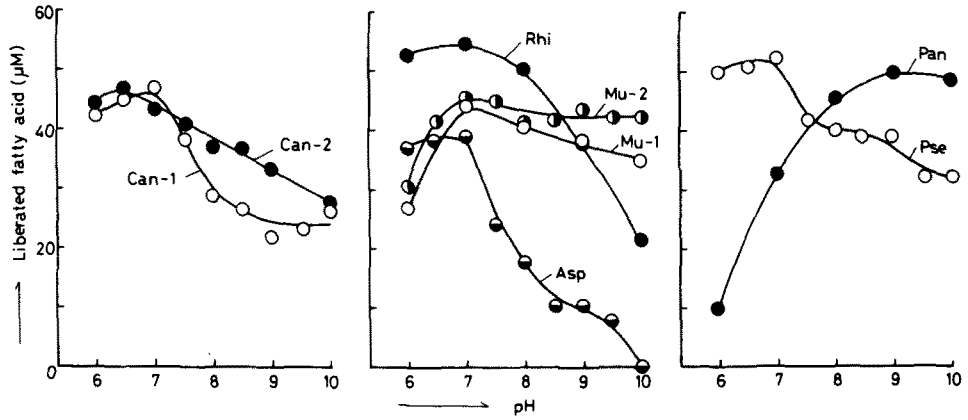

FIG. 3. Relation between amount of liberated fatty acid from olive oil emulsion by various lipases and pH of buffer solution at 37 C.

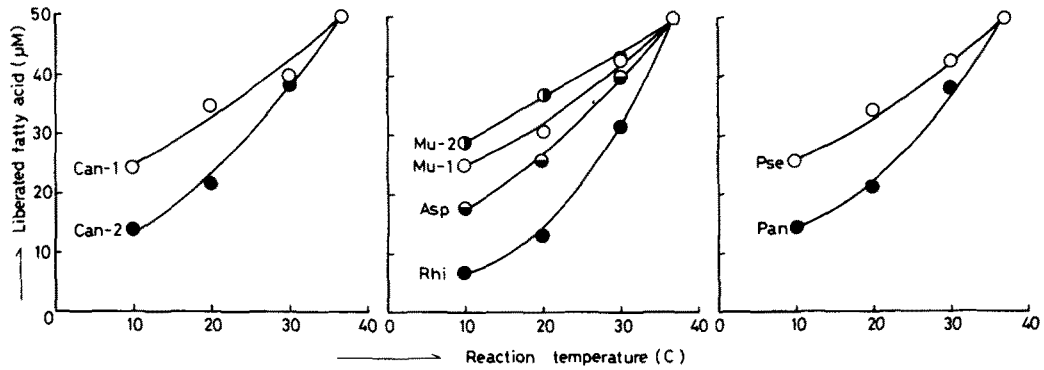

FIG. 4. Relation between amount of liberated fatty acid from olive oil emulsion by various lipases and temperature at pH 7.0.

the kind of lipase. Mu-1, Mu-2 and Pan belong to the group in which the amount of liberated fatty acid is almost constant and free from the effect of pH. Can-1, Can-2 and Pse belong to the group in which the amount of liberated fatty acid decreases as pH increases (the amount of liberated fatty acid at pH 10 is about one-half that at pH 7). Rhi and Asp are in the group in which the amount of fatty acid decreases substantially as pH increases (the amount of the fatty acid at pH 10 is smaller than one-third that at pH 7).

Effect of Temperature on Hydrolysis by Lipase

The amount of liberated fatty acid from olive oil emulsion by lipases was measured at 10 C to 37 C. As shown in Figure 4, for all lipases the amount of liberated fatty acid increased with an increase in temperature. Hydrolysis by Can-1, Mu-1, Mu-2 or Pse was less dependent on temperature than that by Can-2, Rhi or Pan.

Positional Specificity of Hydrolysis by Lipase

Hydrolysates of triolein by lipases were analyzed by TLC-FID. The chromatogram of the triolein hydrolysate by Mu-2 is shown in Figure 5b as an example, while that of the model of the hydrolysates in which all the possible components are contained is shown in Figure 5a in comparison. In Figure 5a, the chromatogram peaks are identified as 1-monoolein, internal standard (p-hydroxybenzoic acid), 1,2-diolein, 1,3-diolein, oleic acid and triolein, respectively, in order from the original point. In Figure 5b, the chromatogram identified as 1,3-diolein is missing and the peak for monoolein is very small. In this manner each component of the triolein hydrolysates by each lipase was determined quantitatively with high accuracy by TLC-FID using internal standard.

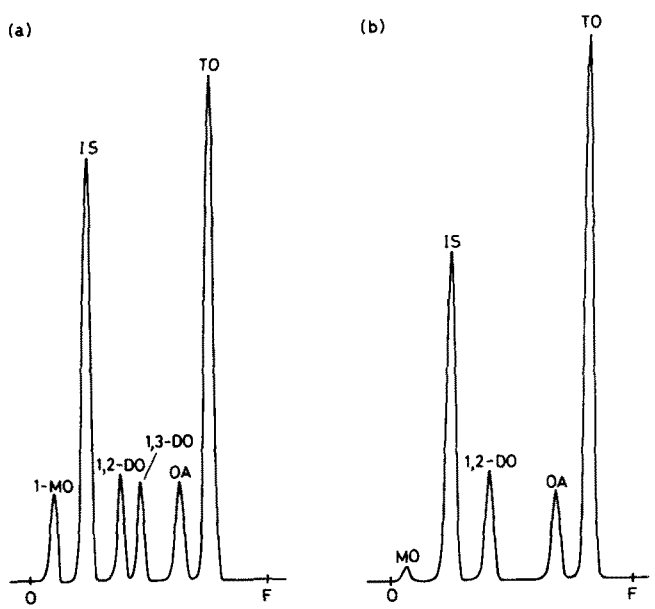

FIG. 5. Chromatograms by TLC-FID analyzer on Chromarod S-II. (a) A mixture of glyceryl oleates and internal standard. (b) The reaction mixture of 4% triolein emulsion hydrolyzed by lipase SP® (Mu-2) in addition to internal standard. TO, triolein; DO, diolein; MO, monoolein; OA, oleic acid; IS, internal standard (p-hydroxybenzoic acid).

The relation between components of hydrolysates in the reaction mixture and the degree of hydrolysis is shown in Figure 6. In the reaction mixture by lipase from yeast of *Candida cylindracea* (Can-1 and Can-2), only oleic acid is

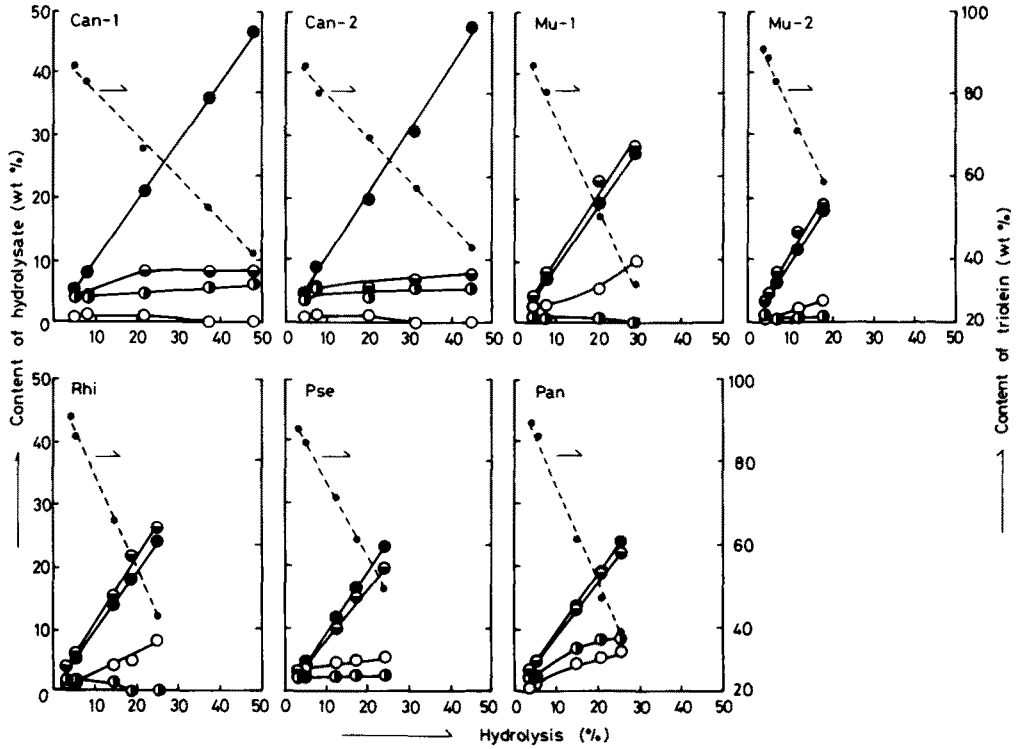

FIG. 6. Proportion of hydrolysates in the reaction mixture of triolein emulsion by various lipases at 37 C and pH 7.0. Triolein, - - ●- - -; hydrolysates, ◑, 1,3-diolein; ◒, 1,2-diolein; ◐, monoolein, and ●, oleic acid.

TABLE IV

Evaluation of Various Lipases for Application to Laundry System

Properties	Lipase							
	Can-1	Can-2	Mu-1	Mu-2	Asp	Rhi	Pse	Pan
Kinetic parameter	◎	◎	○					
Degree of hydrolysis	◎	○	○			○	○	○
pH Characteristics	○	○	◎	◎			○	◎
Temperature characteristics	◎	◎	◎	◎			◎	○
Positional specificity	N	N	S	S	(S)	S	S	S

◎: excellent adaptability, and ○: good adaptability.
Positional specificity: N, nonspecific; S, specific, and ( ), unable to be determined accurately because of lower degree of hydrolysis.

increased significantly as hydrolysis proceeds while the amount of 1,3- and 1,2-diolein are very small at the similar ratio and monoolein is hardly present. On the other hand, in the reaction mixture by lipase from molds of *Mucor* and *Rhizopus* (Mu-1, Mu-2 and Rhi), not only oleic acid but also 1,2-diolein are increased at a similar ratio while monoolein is increased less and 1,3-diolein is hardly present. The mode of hydrolysis by Pse and Pan is similar to that by mold lipase. Based on these results it was proved that lipase from yeast has no positional specificity of hydrolysis (12) and is able to attack all the ester bonds of triolein regardless of their position to produce oleic acid without occurrence of di- and monooleins and that other lipases have positional specificity (13,14) and prefer to attack $\alpha(\alpha')$-ester bonds prior to β-bond to produce oleic acid together with 1,2-di- and monooleins.

The characteristics of eight kinds of lipases discussed above are summarized in Table IV, and the adaptability of these lipases for laundry systems was evaluated. Can-1 and Can-2 are excellent in kinetic parameters and the degree of hydrolysis and temperature characteristics and have no positional specificity. Mu-1 and Mu-2 are excellent in pH and temperature characteristics and have positional specificity. Asp, Rhi, Pse and Pan seem to be less applicable in laundry systems in comparison with lipases from *Candida* and *Mucor*, in spite of their inherent characteristics. On the viewpoint of the present laundry practice under alkaline conditions and the tendency toward low temperature wash-

ing, it seems that lipases from *Candida cylindracea* are the most suitable because they have a higher rate of hydrolysis and higher productivity of fatty acid and are less dependent on temperature. Lipase from *Mucor* is also effective due to excellent pH and temperature characteristics.

The effect of positional specificity in hydrolysis of lipase on the removal of triglyceride soil should be discussed on the basis of examination of the removal of triglyceride and its hydrolysates by surfactant solution. Adaptability of lipase for laundry will be discussed based on the results. This will be reported in the next paper.

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Comparison of Commercially Available Quaternary Ammonium Structures for Phase Transfer Catalysis

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ABSTRACT

A variety of commercially available tetralkyl ($R_1R_2R_3R_4N^+$) ammonium chlorides and methyl sulfate salts were examined under phase transfer conditions. For conversion of benzyl chloride to benzyl acetate with aqueous potassium acetate, tri C_{8-10} methyl ammonium chloride was the most efficient, with tri C_{16-18} methyl ammonium chloride was next. The alkyl trimethyl ammonium chlorides (particularly C_{12-14} trimethyl) performed well for the oxidation of benzyl alcohol to benzaldehyde with sodium hypochlorite. Trimethyl tallow, C_{16-18} partially unsaturated, ammonium chloride was the catalyst of choice for the dichlorocarbene addition to cyclohexene.

INTRODUCTION

Phase transfer catalysis (PTC) is a rapidly growing field. The industrial applications are limited at present, but most assuredly will increase in the future. One of the earliest applications of phase transfer catalysis by quaternary ammonium salts (quats) is the synthesis of polycarbonates (1). Typically, 2,2-(4,4'-di-hydroxydiphenyl)propane (bisphenol A) is dissolved in concentrated aqueous sodium hydroxide and exposed to a dichloromethane solution of phosgene. Salts such as benzyltriethylammonium chloride or tertiary amines catalyze the condensation polymerization.

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Various linear polymers can be crosslinked with PTC agents also (2). Displacement of halogens by cyanide is common in the manufacture of insecticides (3).

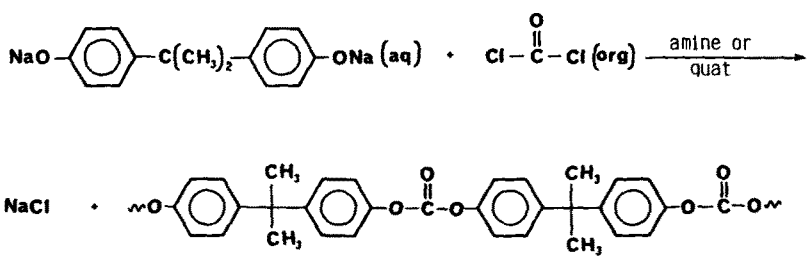
The literature on phase transfer catalysts (4,5) is becoming enormous. However, most PTC work involving quats has centered on:

- Examination of one quaternary in one particular reaction on a variety of organic substrates with the same functional group.
- Investigation using one quat in a wide variety of reactions.

A lesser amount of work has occurred on the mechanism of the reactions, solvent choices and effects, and the effect of quat structure. Recent work has involved immobilizing the catalyst on a polymer backbone (6-8).

Certain structures dominate the literature: tetrabutyl ammonium salts, benzyl triethyl ammonium, hexadecyl trimethyl ammonium chloride, or Adogen® 464 (tri C_{8-10} methyl ammonium chloride), and some generalities regarding quat structure have been developed (9-17).

Since certain catalyst structures are more readily available, less expensive and current materials of commerce, we decided to examine various commercially available quaternary ammonium salts (most of which have not been investi-



SCHEME 1