Studies on Applications of Lipolytic Enzymes in Detergency. III.

Removal of Triolein and its Hydrolysates from Cotton Fabric

Takako HASHIMOTO, Tomiko FUJII, Tokuzo KAWASE,
and Motoi MINAGAWA

Faculty of the Science of Living, Osaka City University
(3-3-138, Sugimoto, Sumiyoshi-ku, Osaka)

The removal of unitary and the mixed oily soils of triolein and its hydrolysates such as diolein, monoolein and oleic acid from cotton fabric were examined by changing the surfactant and pH of the washing solution. Using various lipases having different positional specificities of hydrolysis, triolein soiled fabrics were washed by a surfactant solution containing lipase.

By a nonionic surfactant, monoalkyl deca(oxyethylene) ether (APE), unitary and the mixed soils of triolein and its hydrolysates could all be removed to nearly the same extent regardless of the kind of glyceride. By an anionic surfactant, sodium lauryl sulfate (SDS), diolein and monoolein were removed in preference to triolein. In alkaline solution, oleic acid could be removed most easily by formation of oleic acid soap, which promoted the removal of other glycerides present.

In the removal of triolein by SDS containing lipase at pH 7.0, lipases with positional specificity and which accumulated di- and monoglycerides in the reaction mixture were more effective than those without positional specificity producing considerable amounts of free fatty acid. In the removal of triolein by APE containing lipase, no significant difference in the extent of removal depending on the particular kind of lipase could be observed. These results agree well with those for the removal of triolein and its hydrolysates by a surfactant solution.

1 Introduction

Development of enzyme for laundry detergents has been highly promoted due to lower level of phosphate builder in detergents to eliminate the environmental problem and due to the movement toward lower temperature washing to save energy.^{1)~4)} Nowadays alkaline protease is formulated in most of the commercial heavy duty detergents.

The authors have made the fundamental studies on possible application of lipolytic enzyme in deterative system for the purpose of improvement of triglycerides of human sebum which are difficult to be removed under the normal washing conditions. Based on the fact that it improved the removal of olive oil from cotton fabric by 15 to 20% at the optimum conditions⁵⁾, it was confirmed

that the lipase from *Candida cylindracea* was practically effective. In the previous paper⁶⁾, adaptability of various lipases from various origins as detergent enzyme was evaluated on kinetic parameter of hydrolysis, temperature and pH characteristics, and positional specificities were also examined. Lipases are different from each other in positional specificity⁷⁾, but are roughly classified into two groups: One has no positional specificity which means that it hydrolyzes equally three ester bonds of triglyceride to produce free fatty acid without accumulation of di- and monoglycerides⁸⁾ and other has positional specificity which means that it hydrolyzes preferentially the ester bond of $\alpha(\alpha')$ -position to produce free fatty acid with accumulation of di- and monoglycerides.⁹⁾

When lipase is applied in deterative system, it is considered that the removals of triglyceride

ride soils are influenced by hydrolysates produced by lipase.^{10), 11)} In this study, the removals of unitary and mixed soils of triolein and its hydrolysates, that is, diolein, monoolein, and oleic acid from cotton fabric were examined by changing the surfactant and pH of the washing solution to clarify the influence of coexisting hydrolysates on the removal of triolein. And further using various lipases different in positional specificity the removal of triolein with surfactant solution containing lipase was examined to discuss the effect of lipase on the removal.

2 Experimental

2.1 Materials

2.1.1 Lipases

Five kinds of fungal lipases, a bacterial lipase, and a pancreatic lipase shown in **Table-1** were used in this study. One lipase unit was defined as the amount of the lipase which liberated 1 μ mol of fatty acid from olive oil per 1 min at 37°C¹²⁾ according to Yamada *et al.*¹³⁾

2.1.2 Glycerides

Oleic acid (>99%) was obtained from Nippon Oils & Fats Co., Ltd.. 1-Monoolein was synthesized almost quantitatively as described by Daubert *et al.*¹⁴⁾ and purified (>99%) by several fractional recrystallization from *n*-hexane. 1, 3-Diolein was synthesized by monooleoylification of 1-monoolein synthesized as above and purified (>98%) by column chromatography on silica with benzene. Triolein was purchased from Tokyo Kasei Kogyo Co. and purified (>99%) by column chromatography on silica with benzene followed by distillation.

The purities were determined by TLC-FID method.

2.1.3 Surfactants

Surfactants used here were monoalkyl decyl (oxyethylene)ether (R : C₁₆/C₁₈=56 : 44, Nikkol BC-7, Nikko Chemicals Co., namely APE) and sodium lauryl sulfate (for biochemical, Wako Pure Chemical Industries Ltd., namely SDS).

2.2 Preparation of Soiled Fabric

The cotton fabric to be soiled was purified with amylase, cut into 5 cm $\times$ 10 cm, and defatted by a Soxhlet extraction with chloroform for 6 h. Each fabric was soiled by spotting twice 0.5 ml of benzene solution of various glycerides (30 mg/ml) with micropipette. Unitary soils were prepared with triolein, 1, 3-diolein, 1-monoolein, and oleic acid respectively. Binary mixed soils were prepared as equiweight mixtures of triolein/1, 3-diolein, triolein/1-monoolein, and triolein/oleic acid. Quaternary mixed soil was equiweight mixture of triolein/1, 3-diolein/1-monoolein/oleic acid.

2.3 Washing of Various Glycerides by Surfactant Solution

2.3.1 Washing Procedures by Surfactant Solution

Washing solution consisting of 15 ml of surfactant solution (0.2%APE or 2.5%SDS), 25 ml of 0.1 M phosphate buffer (pH 7.0), and 35 ml of distilled water was placed in 100 ml Erlenmyer flask with ground stopper and kept in incubator (M-100 N, Taiyo Kagaku Co., Ltd.) at 37°C for 10 min. Washing was held by adding of 3 pieces of soiled fabric and shaking for 60 min at 100 spm and with shaking distance of 4 cm. Washed fabric were twice

Table-1 Lipases used for experiments.

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

rinsed with 75 ml of distilled water at 37°C for 5 min and then air dried.

2.3.2 Washing Procedures at Various pH

Washing solution was prepared by mixing 25 ml of buffer solution (pH 7~10) and 50 ml of distilled water. The buffer solution was prepared by addition of 0.1 N NaOH to 0.1 M phosphate buffer. Washing was carried out in the same manner as explained in 2.3.1.

2.3.3 Determination of Glycerides

Residual glycerides on fabric after washing were extracted by a Soxhlet extraction with ethyl ether for 4 h. Ethyl ether was completely evaporated and 10 ml of chloroform solution of *p*-methoxybenzyl alcohol (extra pure, Tokyo Kasei Kogyo Co.) purified by redistillation was added to the extracts as internal standard.

Glycerides were separated and quantitated by TLC-FID method using Iatroscan TH-10 (Iatron Lab. Inc.), Hitachi 2 pen recorder (Hitachi Ltd.), and Chromatopack R-1 B (Shimadzu Scientific Instrument & Equipment) under the following conditions¹⁵⁾: Stationary phase, Chromarod S-II rods (Iatron) impregnated with 3% boric acid; mobile phase, hexane/ethyl ether/acetic acid (70 : 30 : 1, vol/vol/vol); flow rate of hydrogen, 160 ml/min; flow rate of air, 2000 ml/min; scan speed, 40 s/scan; chart-drive speed, 120 mm/min; and voltage of recorder, 100~200 mV.

2.4 Washing of Triolein by Surfactant with Lipase

2.4.1 Washing Procedures

Washing solution consisting of 15 ml of surfactant solution (0.2% APE or 2.5% SDS), 25 ml of buffer solution (pH 7.0 or 10.0), and 25 ml of distilled water was kept at 37°C for 10 min. Washing was held by adding of 10 ml of lipase solution (2.5 u) and a piece of soiled fabric and shaking for 60 min in the same manner as explained in 2.3.1. After 5 ml of 2 N H₂SO₄ was added to terminate the lipase activity, the washed fabric was rinsed.

2.4.2 Determination of Triolein

Residual triolein and its hydrolysates hydrolyzed by lipase on fabric were extracted by a Soxhlet extraction with ethyl ether for 4 h. Ethyl ether was completely evaporated and 5 ml of benzene solution (6 mg/ml) of heptadecanoic acid (99%, Nakarai Chemical Co., Ltd.)

was added to the extracts as internal standard.

Based on the standard fat analytical method¹⁶⁾, triolein in the extract was hydrolyzed to oleic acid by addition of 2 ml of 0.5 N NaOH-methanol solution. Then, oleic acid was methylated according to boron trifluoride methanol method. Methyl ester obtained was separated and quantitated by gas chromatograph (Shimadzu GC-7 AG) and Chromatopack R-1 B 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, 50 ml/min; flow rate of hydrogen gas, 40 ml/min; flow rate of air, 500 ml/min; column temperature, 180°C; and injection port temperature, 230°C.

2.5 Calculation of Removal

Removal of glycerides and triolein was calculated overall and individually by each component by the equation (1) based on the number of constitutive oleoyl group on the fabric before and after washing.

$$\text{Removal (\%)} = (N_b - N_a) / N_b \times 100 \quad \dots\dots\dots (1)$$

where, N_b is number of oleoyl group on the fabric before washing and N_a is that after washing.

Washing experiments were all repeated 6 times with an error in removal of $\pm 3.1\%$ by TLC-FID method and of $\pm 1.3\%$ by GLC method.

3 Results and Discussions

3.1 Removal of Triolein and Its Hydrolysates

3.1.1 Removal of Unitary Soil of Triolein and Its Hydrolysates

The removal of each unitary soil of triolein and its hydrolysates, 1, 3-diolein, 1-monoolein, and oleic acid from cotton fabric by surfactant solution is shown in **Table-2**. The removal of these glycerides by APE, nonionic surfactant, were all low and no significant difference were observed relating to the kind of glycerides. On the otherhand, the removals of 1, 3-diolein, 1-monoolein, and oleic acid by SDS, anionic surfactant, were significantly high, while that of triolein was low. The removal by SDS de-

Table-2 Removal of unitary soil of triolein and its hydrolysates.

Surfactant	Removal (%)			
	Triolein	1, 3-Diolein	1-Monoolein	Oleic acid
APE	20.8	19.3	15.1	18.2
SDS	14.1	85.1	92.5	82.8

pended on the kind of glycerides.

3.1.2 Removal of Mixed Soil of Triolein with Its Hydrolysates

The overall removal and the individual removal of each component of binary and quaternary mixed soils of triolein with its hydrolysates from cotton fabric by surfactant solution are shown in **Table-3**, where, binary mixed soil of triolein/oleic acid was used as the model of composition of reaction mixture by lipase having no positional specificity, and other binary mixed soils of triolein/diolein and triolein/monoolein and quaternary mixed soil of triolein/diolein/monoolein/oleic acid were used as the one by lipase having positional specificity.

In case of SDS, there observed statistically significant difference between the overall removal of quaternary mixed soil and that of any binary soil according to analysis of variance at reliability of 95%, where effect of multicomponent of hydrolysates on the removal was observed.

The removal by APE of each component of both binary and quaternary mixed soils was

almost same and independent of the kind of glycerides. Therefore, it was considered that these mixed soils were removed as a homogeneous single phase droplet by APE. By contrast, the removals by SDS of 1-monoolein, 1, 3-diolein, and oleic acid were remarkably high in comparison with

that of triolein. Hydrolysates in the mixture seemed to tend to be removed in preference to triolein by SDS. Further, based on the fact that the removal of triolein in mixed soil was higher than that of triolein unitary soil, it was evident that the presence of hydrolysates in the mixture contributed to removal of triolein.

3.1.3 Effect of pH on Removal of Triolein and Its Hydrolysates

Effect of pH on the removal of triolein and its hydrolysates, 1, 3-diolein, 1-monoolein, and oleic acid from cotton fabric was examined in

Fig.-1 Effect of pH on removal of unitary soil of triolein and its hydrolysates.**Table-3** Overall removal and individual removal of each component of mixed soil of triolein with its hydrolysates.

Component of soil	Surfactant	Removal (%)				
		Triolein	1, 3-Diolein	1-Monoolein	Oleic acid	Total
Triolein	APE	22.4			19.4	17.7
Oleic acid	SDS	41.9			79.4	60.4
Triolein	APE	20.3	19.9			16.7
1, 3-Diolein	SDS	42.4	63.7			51.0
Triolein	APE	19.7		18.1		18.6
1-Monoolein	SDS	38.8		92.1		61.2
Triolein	APE	26.6	20.0	13.9	23.4	21.4
1, 3-Diolein						
1-Monoolein						
Oleic acid	SDS	48.2	85.0	91.2	85.9	75.7

Fig.-2 Effect of pH on removal of binary mixed soil of triolein with its hydrolysates.

Fig.-3 Effect of pH on removal of quaternary mixed soil of triolein with its hydrolysates.

the pH range from 7 to 10. The results are shown in **Fig.-1**, **Fig.-2(a)(b)(c)**, and **Fig.-3** for unitary soil, binary soil and quaternary soil, respectively.

In case of unitary soil (**Fig.-1**) the removal of oleic acid was increased with increase of pH while those of 1-monoolein, 1,3-diolein, and triolein remained unchanged. In case of binary soil of triolein/oleic acid [**Fig.-2(a)**], the removal of oleic acid was increased with increase of pH and followed by increase of removal of triolein. In case of binary soils of triolein/1,3-diolein [**Fig.-2(b)**] and triolein/1-monoolein [**Fig.-2(c)**], the removals remained unchanged in spite of change of pH. In case of quaternary soil (**Fig.-3**), increase of the removal of oleic acid with a rise of pH promoted the removals of 1-monoolein, 1,3-diolein followed by slight increase of the removal of triolein.

As a result, it was found that in alkaline solution, oleic acid was removed most easily of all the hydrolysates by formation of water-soluble oleic acid soap¹⁷⁾ and presence of oleic acid in the mixed soils promoted the removals of coexisting other components.¹⁸⁾

The results mentioned above suggested that removal of triolein

was influenced by coexisting hydrolysates.

a) In case of using nonionic surfactant, triolein and its hydrolysates are removed as a homogeneous single phase droplet independently of kind of glycerides. The removal of triolein would not be so influenced by the composition of hydrolysates.

b) In case of using anionic surfactant, the hydrolysates, especially 1-monoolein and 1,3-diolein are preferentially removed by the interaction between these glycerides and anionic surfactants,¹⁹⁾ which promotes the removal of triolein.

c) In case of alkaline solution, oleic acid is the easiest to be removed and the oleic acid soap produced from oleic acid and alkali promotes the removal of coexisting monoolein, diolein, and triolein.

3.2 Effect of Lipase on Removal of Triolein

The triolein soiled fabric was washed by the surfactant solution with various lipases. Of the lipases used in this study Can-1 and Can-2 did not exhibit positional specificity, while others have positional specificity, especially Mu-1, Mu-2 and Rhi have strong specificity.⁶⁾ The results obtained at pH 7.0 and 10.0 are shown in **Figs.-4** and **5**, respectively. In these figures, the broken lines indicate the removal of triolein by surfactant solution alone. The column graphs show the removal of triolein by surfactant plus lipase solution and the parts marked by slash lines show the removal of

Fig. 4 Effect of various lipases on removal of triolein by APE or SDS at pH 7.0.

Fig. 5 Effect of various lipases on removal of triolein by APE or SDS at pH 10.0.

triolein by lipase alone (calculated by deducting the removal by buffer solution alone from that by lipase buffer solution).

At pH 7.0, the addition of lipase to surfactant solution was effective to increase the removal of triolein. In case of APE there were no significant differences in removal by kind of lipases. In addition, the removal by the combination system of APE and lipase is found as the sum of the removal by surfactant alone and by lipase alone. It means the effect of lipase is directly additive. On the other hand, in case of SDS the removals by Mu-1, Rhi and Pan were higher than those by Can-1 and Can-2. Further, the removal by the combination system of SDS and the former lipases is higher than the sum of the removal by SDS alone and that by lipase alone. Here lipase having positional specificity seems to be effective synergistically with SDS.

At pH 10.0, the removal by surfactant alone, either APE or SDS, is higher than that at pH 7.0. However, at pH 10.0 lipase are not so effective compared to the results at pH 7.0, particularly the effects of Can-1 and Can-2 are rarely observed.

From these results, effect of lipase in the practical deterative system was discussed on the view point of the positional specificity of lipase. At pH 7.0, the effect of lipase with nonionic surfactant was independent of the positional specificity. In contrast, with anionic surfactant lipases having positional specificity showed superior effects, which would be caused by the interaction between di-, monooleins produced by lipase and anionic surfactant. At pH 10.0, the removal of triolein was not improved by Can-1 and Can-2 which have no positional specificity, although it was expected such lipase would be more effective in alkaline solution from the results in 3.1. Consequently, the effect of lipase in alkaline condition seems to be influenced not only by its positional specificity but also by stability of lipase in alkaline solution.^{6), 20)}

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脂質分解酵素の油脂汚れの洗浄への
応用に関する研究 (第3報)
——トリオレイン及びその加水分解
生成物の洗浄性——

橋本尊子・藤井富美子・川瀬徳三・皆川 基

大阪市立大学生活科学部 (大阪市住吉区杉本 3-3-138)

脂質分解酵素リパーゼによるトリグリセリドの洗浄機構を明らかにするため、トリオレインとその加水分解生成物であるジオレイン、モノオレイン及びオレイン酸のそれぞれ単一及び混合汚れの綿布からの除去を、界面活性剤の種類及び洗液の pH を変えて調べた。また、位置特異性の異なる各種リパーゼを含む界面活性剤水溶液によりトリオレインを洗浄した。

非イオン界面活性剤、モノアルキル=デカ (オキシエチレン) エーテル (APE) では、トリオレイン及びその加水分解生成物のそれぞれ単一及び混合汚れはグリセリドの種類によらずすべて同じ程度で除去されたが、アニオン界面活性剤、硫酸ラウリル=ナトリウム (SDS) では、ジオレイン、モノオレインがトリオレインに優先して除去された。また、アルカリ水溶液では、オレイン酸はオレイン酸セッケンの生成により最も容易に除去され、それが共存する他のグリセリドの除去を促進した。

リパーゼを含む SDS 水溶液によるトリオレインの除去において、pH 7.0 では、トリグリセリドの加水分解反応系中にジ及びモノグリセリドを蓄積する位置特異性を有するリパーゼの方が、脂肪酸生成量の多い位置特異性を持たないリパーゼよりも効果的であった。リパーゼを含む APE 水溶液によるトリオレインの除去において、リパーゼの効果は位置特異性の有無による有意差はみられなかった。これらの結果は、界面活性剤によるトリオレインとその加水分解生成物の除去の結果とよい対応を示した。

書 評

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化粧品やトイレタリー製品はわれわれの日常生活と密接な関係を持つ製品の一つであり、これらの大部分は界面活性剤の助けをかりなければ作ることができないと言っても過言ではない。化粧品やトイレタリー製品における界面活性剤の使われ方や役割は製品の剤形や使用目的により種々異なっており、界面活性剤のもつ性質である乳化、可溶性、浸透、ぬれ、分散、洗浄、保温、殺菌、潤滑、帯電防止、柔軟、起泡等の性質をうまく利用して用いられており、その目的によって非常に多くの種類の界面活性剤が用いられている。また、これらの製品が直接、人間の皮膚に用いられることから、皮膚刺激や肌荒れに対する界面活性剤が及ぼす影響についても注意を払う必要がある。

本書は、実際に化粧品やトイレタリー製品を研究する人々によって書かれており、界面活性剤の利用のしかたや役割を豊富な引用文献を用い多方面から解説している。すなわち、化粧品やトイレタリー製品に用いられる界面活性剤の構造や概説に始まり、乳化や可溶性といった界面化学を基礎から専門的な分野に至るまで、また界面活性剤の分析法や界面活性剤の防腐殺菌作用や化粧品に要求される対微生物安定性等について述べられており、さらに界面活性剤と皮膚との相互作用をまず皮膚の構成や役割から始まり界面活性剤と皮膚との生化学的及び物理化学的な相互作用をわかりやすく解説している。次に化粧品やトイレタリー製品の各論として、シャンプー、歯磨などの口内衛生用品、皮膚洗浄剤、エアゾール製品及び粉末分散系の化粧品をとりあげ、それぞれの製品における界面活性剤の役割や、用いられる界面活性剤の種類や注意点等に関して詳述している。

このように、本書は界面化学はもとより、皮膚科学、薬学、毒物学、微生物学、分析化学などの非常に広範囲な分野を取り扱っているにもかかわらず、それぞれが基礎からわかりやすく書かれているため、専門外の方でも興味を持って読むことができる。

したがって、化粧品技術者はもとより、化粧品やトイレタリー製品における界面活性剤の使われ方や問題点等に興味を持つ人々には大いに役立つものであると確信する。
(富田健一)