Lipase-Catalyzed Synthesis of Kojic Acid Esters in Organic Solvents

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ABSTRACT: Kojic acid is an inhibitor of bacteria, viruses, and fungi. It is used for inhibiting the browning effect of tyrosinase in the food and cosmetic industries. To improve its lipophilic properties, *Pseudomonas cepacia* lipase and *Penicillium camembertii* lipase were used for catalyzing the esterification of kojic acid to synthesize kojic acid monolaurate and kojic acid monooleate. These products showed a 69.5% inhibitory effect on tyrosinase in hydrophobic organic solvent. The yields of kojic acid esters were affected by enzymes, substrates, organic solvent, and temperature. Lauric and oleic acids were the best substrates for esterification among various fatty acids tested. $CaCl_2$ and $MnCl_2$ stimulate *Pseudomonas cepacia* lipase-catalyzed esterification by 7.0%. On the contrary, $MgCl_2$, $SrCl_2$, and $ZnCl_2$ inhibited the reaction. The best pH of buffer for lipase pretreatment was pH 6.0. *Pseudomonas* and *Penicillium* lipases can be reused for the synthesis of kojic acid esters. After reaction at 40°C for 10 d, the *Penicillium* and *Pseudomonas* lipases still retained 57.0% and 92.0% of their initial activities, respectively.

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KEY WORDS: Esterification, kojic acid, kojic acid ester, lipase, organic solvent.

Kojic acid [5-hydroxy-2-(hydroxymethyl)1,4-pyrone], a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (1), has been recognized for its inhibitory effect on mushroom polyphenol oxidase (PPO) (2). Recently, Chen *et al.* (3) showed the inhibitory effect of kojic acid on mushroom, plant (potato and apple), and crustacean (white shrimp, grass prawn, and Florida spiny lobster) PPO. Kojic acid is mixed with ascorbic acid and citric acid to constitute a Japanese product for inhibiting tyrosinase in foods. Kojic acid inhibits tyrosinase activity by chelation and acts as an antioxidant (4). However, kojic acid is water-soluble, and its instability has been a problem in cosmetic use. Synthesis of kojic acid ester by chemical methods, e.g., esterification of kojic acid with fatty acid in the presence of acid or alkaline catalysts, usually results in a complex mixture. Enzymatic synthesis of kojic acid ester in organic solvent is still an unexplored area of research.

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To improve the lipophilicity of kojic acid, esterification may be a suitable method, since the ester residue is well-characterized as a nontoxic carrier moiety with a high affinity for cell membranes and a great hydrophobicity to prevent degradation.

In the present work, we found that kojic acid esters could be efficiently synthesized by esterification with lipase from *Pseudomonas cepacia* (Amano PS) or *Penicillium camembertii* (Amano G) as biocatalyst in organic media. The effects of acyl donors, organic solvents, temperature, water content, lipase, acyl donors, pH memory, and metal ions on tyrosinase activity were investigated.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Aspergillus niger*, *Mucor* sp. (Amano MAP-10), *P. camembertii* (Amano G), *P. cepacia* (Amano PS), and *Rhizopus* sp. (Amano N-conc.) were purchased from Amano International Enzyme Co. (Nagoya, Japan). Lipase from *Candida cylindracea* Type was purchased from Sigma Chemical Co. (St. Louis, MO). Lipase from *Chromobacterium viscosum* (LP-101-S) was purchased from Toyo Jozo Co. (Shizuoka, Japan). Immobilized lipase IM from *Mucor miehei* was supplied by Novo Nordisk Inc. (Danbury, CT). Acetone, acetonitrile, chloroform, cyclohexanol, diethyl ether, ethyl acetate, *tert*-butyl alcohol, and toluene were obtained from Merck Chemical Co. (Darmstadt, Germany). Kojic acid, lauric acid, ethyl laurate, lauric anhydride, trilaurin, ethyl oleate, oleic acid, and oleic anhydride were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

Instrumentation. Instruments used in this investigation were: Bomem MB-100FT infrared spectrophotometer; Bruker nuclear magnetic resonance spectrometer (model AMX-500; Bruker, Karlsruhe, Germany).

Methods. For the standard reaction, the commercial lipase powder (0.15 g) was added to a reaction mixture (1 mL) containing 90 mM fatty acid and 360 mM kojic acid in acetonitrile. The reaction mixture was incubated in an orbital shaker with a speed of 250 rpm at 40 and 50°C for 48 h, respectively. At various time intervals, 8 μL of the reaction mixture was withdrawn and analyzed by gas chromatography (Hitachi model G-3000; Hitachi, Tokyo, Japan). An Rtx®-65TG fused-silica capillary column (30 m × 0.25 mm; Restek Corpora-

tion, Bellefonte, PA) was used. Hydrogen gas was the carrier gas at a flow rate of 1.2 kg/cm^2. The injection port and flame-ionization detector temperatures were both 300°C. The temperature program was: 155°C, hold 1 min, 4°C/min to 180°C, then 55°C/min to 350°C and hold 7 min. The product compositions were quantitated by an integrator with γ-phenyl-*n*-propyl *n*-butyrate as internal standard.

To study the effect of water content on the lipase-catalyzed synthesis, the enzyme was lyophilized by a Savant Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, NY) under 50 millitor for 24 h. Water was removed from organic media by 3 Å molecular sieve (Merck). To study the pH effect on synthesis, the lipase was dissolved in 10 mM Good's buffer solution {10 mM each of *N,N*-bis(2-hydroxyethyl)-glycine (BICINE), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), sodium acetate, and 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BIS-TRIS propane)}. Various pH values were obtained by adding either concentrated HCl or NaOH, and the mixture was then lyophilized as described above.

Measurement of lipase. The lipase hydrolytic activities were measured according to the method described by R'ua *et al.* (5).

Analysis by thin-layer chromatography (TLC). The method of Fink and Fink (6) was employed with a silica gel 60 plate. The sample was applied with a Hamilton syringe 2 cm from the lower edge of the plates. The plate was then developed with solvent (hexane/ethyl acetate, 75:30, vol/vol). Upon drying, the plate was sprayed with a 2% hydrochloric acid solution of ferric chloride, which permitted detection of the monoesters by color spots.

RESULTS AND DISCUSSION

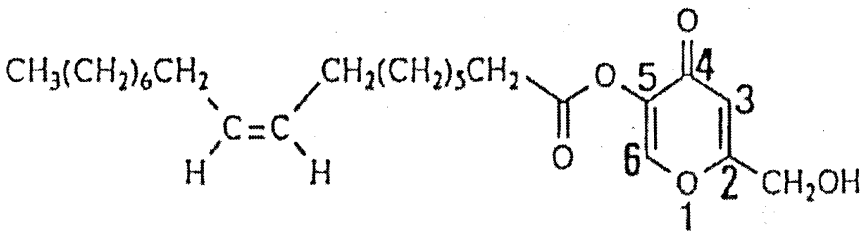
Among nine commercial lipases tested, *P. cepacia* lipase PS and *P. camembertii* lipase G showed the best catalytic efficiency and specificity for enzymatic synthesis of kojic acid monolaurate (KAML) and kojic acid monooleate (KAMO), respectively (Table 1). The yields of KAML and KAMO were 26.0 and 36.5%, respectively, from 90 mM of lauric acid or oleic acid reacting with 360 mM kojic acid, catalyzed by 0.15 g lipase in 1 mL acetonitrile at 50°C (Table 1). The specific activity measured by the hydrolysis of *p*-nitrophenyl butyrate of each lipase is also listed in Table 1.

The yield of monoesters was affected by acyl donors, reaction temperature, organic solvents, kojic acid/fatty acid ratio, metal ions, water content, and pH. The structures of KAML and KAMO were identified as shown in the following spectral data: KAML/infrared/ester absorption at 1732 cm^{-1}; nuclear magnetic resonance (NMR) spectra with tetramethylsilane as an internal standard in $CDCl_3$, ^{1}H NMR, 0.84–0.88 (3H, *t,* J = 10.6 Hz, CH_3), 1.25–1.29 [22H, *m*, $(CH_2)_n$], 1.98–2.0 (4H, *m*, $H_2C{=}CCH_2$), 2.35–2.40 (2H, *t,* J = 12.4 Hz, $COCH_2$), 4.91 (2H, *s*, α-CH_2), 5.31–5.35 (2H, *m*, HC=CH), 6.46 (1H, *s*, 3-CH), 7.81 (1H, *s*, 6-CH). The chemical structure of KAMO is illustrated in Scheme 1. The R_f values of the KAML, KAMO, kojic acid, lauric acid, and oleic acid determined by TLC were 0.14, 0.17, 0, 0.34, and 0.36, respectively. The effects of kojic acid and its ester (KAMO) on mushroom tyrosinase activity by kojic acid and KAMO at 20 mM concentration were 70.2 and 69.5%, respectively. There-

TABLE 1
Kojic Acid Monoester Formation by Lipase from Different Sources

Source	Trade name or brand[a]	Yields (%) KAML	Yields (%) KAMO	Hydrolysis[b] activity (unit/g)
Aspergillus niger	Amano AP-6	0.2	0.0	908.6
Candida antartica	Sigma	1.0	0.6	762.8
C. antarctica	Novo 435	18.9	12.5	14.5
Chromobacterium viscosum LP101-S	Toyo Jozo	9.5	12.0	1195.8
Mucor sp.	Amano MAP-10	11.9	10.4	45.7
M. miehei	Novo IM	9.2	8.4	7.1
Penicillium camembertii	Amano G	18.0	36.5	479.1
Pseudomonas cepacia	Amano PS	26.0	22.0	925.9
Rhizopus sp.	Amano N-conc.	0.3	0.1	71.9

[a]Amano AP-6, MAP-10, G, PS, and N-conc. (Amano International Enzyme Co., Nagoya, Japan; Sigma (Sigma Chemical Co., St. Louis, MO); Novo 435 and IM (Novo Nordisk, Danbury, CT); Toyo Jozo (Toyo Jozo Co., Shizuoka, Japan).
[b]Hydrolysis activity was measured by the hydrolysis of *p*-nitrophenyl butyrate as substrate. One unit of enzyme is defined as the amount of enzyme which produced 1 μmol of *p*-nitrophenol per min. KAML, kojic acid monolaurate; KAMO, kojic acid monooleate.



SCHEME 1

TABLE 2
Lipase-Catalyzed Esterification of Kojic Acid with Lauric Acid and Oleic Acid at Various Temperatures[a]

Reaction temperature (°C)	Formation of kojic acid monoester (%)	
	Lipase PS	Lipase G
	KAML	KAMO
10	4.8	14.3
20	18.3	15.7
30	19.7	17.2
40	28.3	23.4
50	26.0	36.5
60	24.1	27.7

[a]The lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM kojic acid and 90 mM fatty acid in acetonitrile at various temperatures for 48 h. See Table 1 for abbreviations.

TABLE 4
Effect of Kojic Acid/Fatty Acid Ratio on the Lipase-Catalyzed Synthesis of Kojic Acid Monoester[a]

Molar ratio (kojic acid/fatty acid)	Yield (%)	
	KAML	KAMO
1.0	33.0	17.13
0.5	16.5	7.34
0.3	6.1	2.94
0.1	5.1	1.32
2.0	29.3	33.56
4.0	28.3	36.66
10.0	19.0	22.60

[a]The lipase (0.15 g) was added to a reaction mixture (1 mL) containing various ratios of kojic acid/fatty acid in acetonitrile at 40 and 50°C for 24 h, respectively. See Table 1 for abbreviations.

fore, the esterification of kojic acid increased its lipophilic properties for use in cosmetics and the food industry without affecting its inhibitory effect on tyrosinase activity.

The temperature effect is shown in Table 2. The optimal temperatures for the production of KAML and KAMO were about 40 and 50°C, respectively. Organic solvents greatly affected the yield of monoesters. As shown in Table 3, the best yields of KAML were produced in the presence of acetonitrile. The yields were not satisfactory when the reaction was carried out in other organic solvents. In contrast, the yields of KAMO were equally good in either chloroform or acetonitrile. The effects of the kojic acid/fatty acid ratio in the reaction mixture on the lipase-catalyzed esterification are shown in Table 4. The yields of both KAML and KAMO increased when the kojic acid/fatty acid ratio was increased to 4. Higher ratios decreased the yields of both monoesters.

Ethyl laurate and triolein appeared to be poor substrates for lipase-catalyzed synthesis of KAML and KAMO (Table 5). Lauric acid and oleic acid appeared to be the best acyl donors for the lipase-catalyzed synthesis of KAML and KAMO, respectively. The effect of metal ions on the rate of enzyme reaction was examined by adding different metal ions to the reaction media. $CaCl_2$, $CuCl_2$, and $MnCl_2$ slightly increased the yield, while $MgCl_2$, $SrCl_2$, and $ZnCl_2$ decreased the yield of KAML by the catalysis of Amano PS in acetonitrile (Table 6).

Kinetic constants of lipase-catalyzed esterification of kojic acid with various fatty acids are shown in Figure 1. The best substrate for the lipase-catalyzed synthesis of monoesters of kojic acid was oleic acid. The reaction of the lipase-catalyzed synthesis of kojic acid monoesters is shown as follows:

$$\text{kojic acid} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{kojic acid monoester} \qquad [1]$$

TABLE 3
Effect of Organic Solvents on the Lipase-Catalyzed Esterification of Kojic Acid with Lauric Acid and Oleic Acid at 50°C[a]

System	Yield (%)	
	KAML	KAMO
Acetone	3.8	22.8
Acetonitrile	15.2	33.4
Chloroform	3.3	37.5
Cyclohexanol	0.3	1.0
Diethyl ether	2.6	1.0
Ethyl acetate	0.7	1.0
t-Butyl alcohol	2.5	3.9
Toluene	3.7	9.4

[a]The lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM kojic acid and 90 mM fatty acid in various organic solvents at 50°C for 24 h. See Table 1 for abbreviations.

TABLE 5
Effect of Acyl Donors on the Lipase-Catalyzed Synthesis of Kojic Acid Monoester[a]

Acyl donor	Yields (%)
KAML by lipase PS	
Lauric anhydride	18.5
Ethyl laurate	3.0
Trilaurin	15.5
Lauric acid	33.0
KAMO by lipase G	
Oleic anhydride	12.4
Ethyl oleate	10.7
Triolein	9.0
Oleic acid	36.7

[a]The lipase (0.15 g) was added to a reaction mixture (1 mL) containing 360 mM kojic acid and 90 mM fatty acid in acetonitrile at 50°C for 48 h. See Table 1 for abbreviations.

TABLE 6
Effect of Various Metal Salts on the Lipase-Catalyzed Synthesis of KAML[a]

Metal salt	Yields (%) of KAML
None	33.0
$CaCl_2$	38.0
$CuCl_2$	40.3
$MgCl_2$	24.2
$MnCl_2$	38.2
$SrCl_2$	27.6
$ZnCl_2$	23.9

[a]The lipase PS (0.15 g) was added to a reaction mixture (1 mL) containing 90 mM fatty acid, 90 mM kojic acid, and 2 mM metal salts in acetonitrile at 40°C for 48 h. See Table 1 for abbreviations and manufacturer.

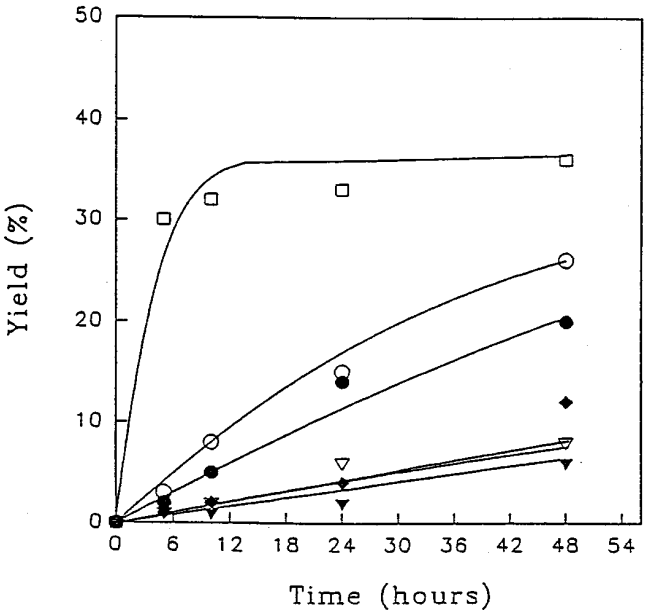


FIG. 1. Time course of esterification between various fatty acids and kojic acid in acetonitrile. The lipase (0.3 g) was added to a reaction mixture (2 mL) containing 90 mM fatty acid and 360 mM kojic acid in acetonitrile at 50°C and 250 rpm for 48 h. Symbols: (◆) hexanoic acid; (○) lauric acid; (●) myristic acid ; (▽) palmitic acid ; (▼) stearic acid ; (□) oleic acid.

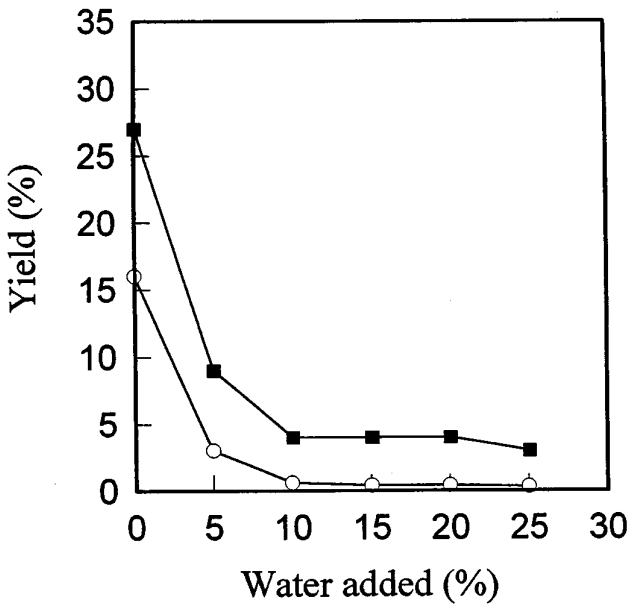


FIG. 2. Effect of water on the synthesis of kojic acid monolaurate (KAML) (○) and kojic acid monooleate (KAMO) (■) by lipases PS and G (Amano International Enzyme Co., Nagoya, Japan). For the synthesis of KAML, lipase PS (0.2 g) was added to a reaction mixture (1 mL) containing 90 mM lauric acid and 90 mM kojic acid in acetonitrile with various amounts of added water at 40°C for 48 h. For the synthesis of KAMO, lipase G (0.2 g) was added to a reaction mixture (1 mL) containing 90 mM oleic acid and 360 mM kojic acid in acetonitrile with various amounts of added water at 50°C for 48 h.

Oleic acid could change enzyme specificity which leads to decreased k_{-1}/k_1 ratios and selective accumulation of monoesters (Table 7). The effect of water content on the synthesis of KAML and KAMO is shown in Figure 2. Apparently, Amano PS and Amano G perform best under anhydrous conditions for the esterification of kojic acid with either lauric or oleic acid. This is similar to our previous observation that *Pseudomonas* lipase performed best under lyophilized conditions for the esterification of propylene glycol with eicosapentaenoic acid and docosahexaenoic acid (7). It is possible that monoesters are easily hydrolyzed by lipase, even in the presence of small amounts of water. Takami *et al.* (8) found that the yield of 1,2-dipalmitoyl-3-*sn*-phosphatidyl kojic acid was poor by phospholipase D-catalyzed reaction in a biphasic system with high water content.

TABLE 7
Kinetic Constants of Lipase-Catalyzed Esterification of Kojic Acid with Various Fatty Acids at 50°C[a]

Carbon number	k_1	k_{-1}	k_{-1}/k_1
$C_{6:0}$	0.005	0.251	48.928
$C_{12:0}$	0.024	0.580	24.298
$C_{14:0}$	0.014	0.162	11.234
$C_{16:0}$	0.005	0.752	149.801
$C_{18:0}$	0.004	0.191	48.232
$C_{18:1}$	0.21	3.658	17.419

[a]The lipase (0.3 g) was added to a reaction mixture (2 mL) containing 360 mM kojic acid and 90 mM fatty acid in acetonitrile at 50°C for 48 h. Abbreviations: $C_{6:0}$, hexanoic acid; $C_{12:0}$, lauric acid; $C_{14:0}$, myristic acid; $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; k_1, positive rate constant; k_{-1}, reverse rate constant.

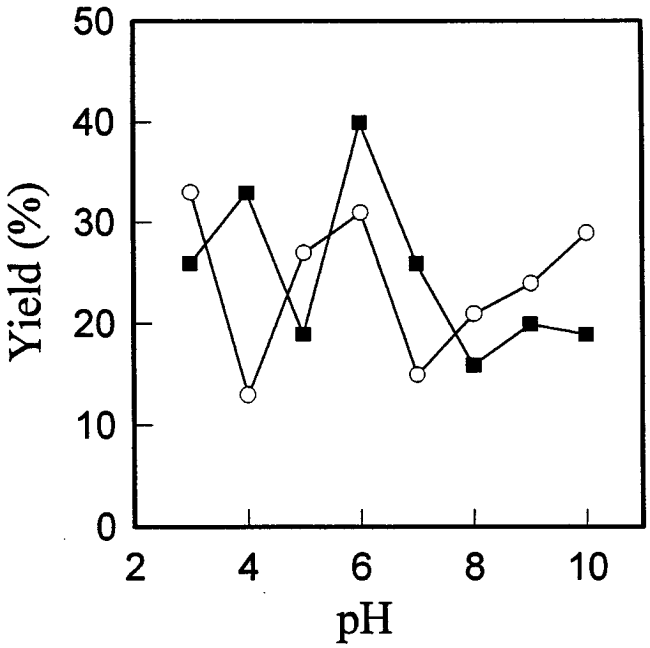


FIG. 3. Dependence of KAML (○) and KAMO (■)formation on the pH of the aqueous solution from which lipases PS and G were lyophilized. For the synthesis of KAML, lipase PS (0.2 g) was added to a reaction mixture (1 mL) containing 90 mM lauric acid and 90 mM kojic acid in acetonitrile; the suspension was shaken at 40°C and 250 rpm for 48 h. For the synthesis of KAMO, lipase G (0.2 g) was added to a reaction mixture (1 mL) containing 90 mM oleic acid and 360 mM kojic acid in acetonitrile; the suspension was shaken at 50°C and 250 rpm for 48 h. For abbreviations and enzyme manufacturer see Figure 2.

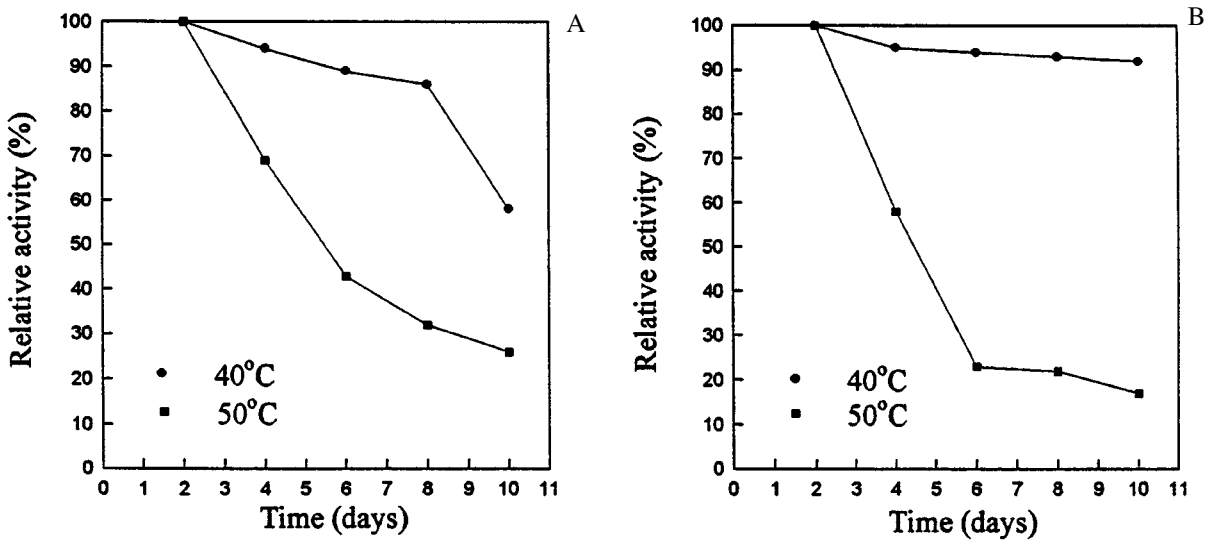


FIG. 4. Operational stability of lipase G- and lipase PS-catalyzed synthesis of (A) KAMO and (B) KAML. The reaction cycle time was 2 d, and the relative activity was assayed immediately after each change. The reaction conditions are the same as described in Figure 3. For abbreviations and manufacturer see Figure 2.

The yields of both monoesters also were affected by the pH of the aqueous solution from which the lipase was lyophilized, a phenomenon named pH memory by Klibanov (9). As shown in Figure 3, the optimal pH for the pretreatment of enzyme was 6 for lyophilized lipase-catalyzed synthesis of KAMO. In contrast, the yield of KAML was less sensitive to pH change in the pretreatment of enzyme. It is possible that small changes in enzyme conformation, which resulted from pH changes in the pretreatment of enzyme, affect enzyme binding more for the longer-chain substrate oleic acid ($C_{18:1}$) than for the shorter-chain substrate lauric acid ($C_{12:0}$).

The operational stabilities of Amano lipase PS- and G-catalyzed esterification of kojic acid with lauric acid and oleic acid as substrates, respectively, are shown in Figure 5. Both lipases PS and G can be reused for the synthesis of kojic acid esters. The lipases G and PS still retained 57.0 and 92.0% of their initial activities for the synthesis of KAMO and KAML, respectively, after 10 d at 40°C. However, the relative activities of Amano PS and G decreased rapidly when the reaction was carried out at 50°C. This could be due to the effect of temperature on the stability of lipases.

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