Synthesis of O-Acyl-L-Homoserine by Lipase

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Lipases (EC 3.1.1.3) from *Candida cylindracea* and *Rhizopus delemar* were found to catalyze O-acylation of L-homoserine or L-serine with free fatty acids or with fatty acids from triacylglycerol. The active pH range of the reation for O-acylation was from 7.0 to 8.5. Emulsifying activity of O-oleoyl-L-homoserine was higher than those of conventional surfactants.

Lecithin from natural sources and chemicals such as monoglycerides, sorbitan fatty acid esters and sugar esters are permitted as food emulsifiers and used widely in food industries. Chemically synthesized emulsifiers have problems with coloration of products and residual toxic solvents. Hence, the enzymatic production of these emulsifiers is expected to resolve these problems. Lipase has been shown to have a catalytic function in esterifying various alcohols with fatty acids (1-3). Lipase-catalyzed synthesis of sugar esters and monoglycerides has also been reported (4-6).

The strategy of the present study is to develop a new food emulsifier with excellent properties by using the catalytic function of lipase. Esterification of hydroxy amino acids with fatty acids was designed for this purpose. Naturally occurring amino acids and fatty acids were used. Hence, the products are thought to be safe for human health as food emulsifiers after digestion by pancreatic lipase. The products are amphoteric substances and are expected to have some characteristic properties different from those of nonionic food emulsifiers. In this paper, O-acylation of L-homoserine by lipase and the excellent emulsifying activity of the product is described.

MATERIALS AND METHODS

Lipases of *Candida cylindracea* (60U/mg protein) and *Rhizopus delemar* (6,000 U/mg protein) were obtained from Meitou Sangyo Co. Ltd. and Seikagaku Kogyo Co. Ltd., Tokyo. TLC plates (Art. 5721) and casein (Art. 2242) were purchased from Merck, Darmstadt, West Germany. Other chemicals were all reagent grade.

Enzyme reaction. The enzyme reaction was initiated by the addition of 0.1 g fatty acids or oils to the amino acid solution containing 600 U of *Candida cylindracea* lipase. The reaction mixture (1 ml) was placed in an 8-ml vial and shaken at 120 rpm, at 37 C. The reaction was stopped by mixing vigorously with 4 ml of chloroform/methanol (2:1, v/v), and then the chloroform layer was withdrawn. The product was extracted two more times from the aqueous layer with 3 ml of chloroform. The combined chloroform layers were evaporated in a rotary evaporator, dissolved in 20 ml of chloroform/methanol (2:1, v/v) and washed with 5 ml of distilled water. The washed chloroform layer was evaporated in a rotary evaporator and redissolved in 2 ml of chloroform/methanol (2:1, v/v). Then, quantification of the product in the extract was carried out in triplicate by the following procedures. The extract (50 μl) was subjected to thin layer chromatographic (TLC) separation with a developing solvent of chloroform/methanol/acetic acid/water (85:20:10:3.5, v/v/v/v). The ninhydrin-positive band with an Rf value of 0.4-0.5 was scraped off and transmethylated with 0.5 M sodium methoxide/methanol or 2% sulfuric acid/methanol in the presence of methyl pentadecanoic acid (10 μg) as the internal standard. The fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) with a Shimadzu GC-9APTF equipped with a flame ionization detector (FID), on a glass column (2.1 m × 3.2 mm), packed with 10% Silar 10C on 60-80 mesh Neopak 2A. The carrier gas was N_2, at a flow rate of 60 ml/min. The column temperature was programmed at 4 C/min from 160 C to 216 C. The injector and detector temperatures were 260 C.

Purification of product Purification of the reaction product from oleic acid and L-homoserine was carried out in order to identify the product and to estimate its emulsifying activity. Reaction mixture contained 2.5 g of oleic acid, 250 mg of lipase and 25 ml of 3 M L-homoserine in 0.1 M potassium phosphate buffer, pH 7.5. The reaction was carried out for 48 hr. The reaction product was extracted as described above and applied to a silicic acid column packed with chloroform. After washing the column with chloroform and chloroform/methanol (9:1, v/v), elution was carried out with chloroform/methanol (2:1, v/v). This fraction was evaporated in a rotary evaporator, and the resulting white powder was washed with distilled water and subsequently with diethyl ether. The powder was dried in *vacua* over potassium hydroxide pellets. The product purified by this procedure was pure when developed on a TLC plate, because no other ninhydrin-positive or iodine vapor-stained spots were detected.

Analysis of product. The constituents of the reaction product made from oleic acid and L-homoserine were quantitatively estimated in the following way. The purified product (1 mg) was transmethylated with 5% hydrogen chloride/methanol at 70 C, for 2 hr, in the presence of methyl heptadecanoic acid (0.72 mg) and L-alanine (0.223 mg) as internal standards. Fatty acids methyl esters (FAME) were recovered with *n*-hexane and subjected to GLC analysis under the same conditions as above. The aqueous phase was evaporated in a stream of nitrogen, and the residue was hydrolyzed in 0.1 N hydrogen chloride at 100 C for 2 hr. The hydrolysate was subjected to a Hitachi 835 amino acid analyzer. The structure of the product was also confirmed by measurement of an infrared spectrum in a potassium bromide pellet, with a Shimadzu infrared spectrophotometer.

Analysis of emulsifying activity. The emulsifying acitivity of the product was compared with those of casein, sodium oleate and other surfactants. The activity was measured in triplicate according to the turbimetric method of Pearce and Kinsella (7). The 0.2% surfactant solution in 0.025 M potassium phosphate buffer, pH 7.0, was added to soybean oil in a small test tube. The mixture, which had been preincubated at 30 C, was mixed well with a Vortex mixer and sonicated with a Branson Sonifier 250. After a 4-min incubation at 30 C, 20 μl of the

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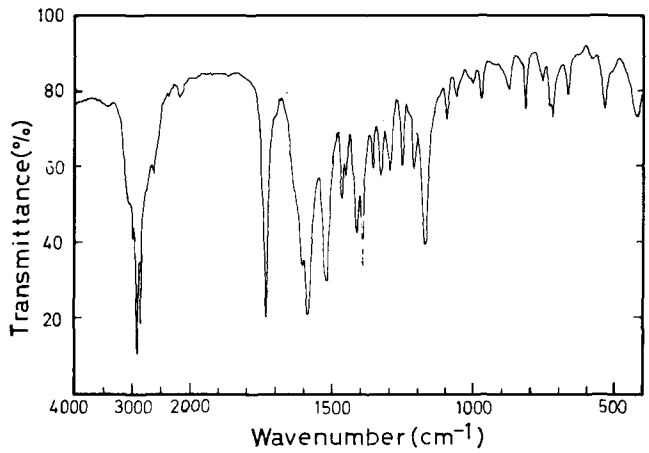

FIG. 1. Infrared absorption spectrum of the purified reaction product between oleic acid and L-homoserine in the presence of *C. cylindracea* lipase.

resulting emulsion was diluted with 0.1% sodium dodecyl sulfate, and absorbance at 500 nm was measured.

RESULTS

Reaction product. The reaction was carried out between oleic acid and L-homoserine in the presence of *Candida cylindracea* lipase. A ninhydrin-positive and iodine vapor-stained fraction (Rf value of 0.4-0.5) was detected when the chloroform extract of the reaction mixture was developed on a TLC plate. The fraction was purified, and the components were analyzed after methanolysis. The purified product (2.78 μmol; this value was calculated from the sample weight of the product on the assumption that the product was O-oleoy-L-homoserine) was composed of 2.97 ± 0.08 μmol of oleic acid and 3.06 ± 0.07 μmol of L-homoserine (triplicate analyses.) These results indicate that the product was composed of an equimolecular amount of oleic acid and L-homoserine. Their linkage was assumed to be an ester bond because of its susceptibility to methanolysis. Figure 1 shows the infrared absorption spectrum of the product. The strong absorptions, due to the ester bond, were observed at 1730 and 1170 cm^{-1}, and absorption around 1580 cm^{-1} showed the presence of $-COO^-$ and $-NH_3^+$ groups. There was no absorption at 1680-1630 cm^{-1} (amide bonds) or at 3300 cm^{-1} (hydroxyl groups of L-homoserine). From these results, the product was identified as O-oleoyl-L-homoserine.

TABLE 1

Specificity of Lipase to Acyl Donors

Acyl donor	Relative activity (%)[a]	
Myristic acid	10.7	2.5[b]
Palmitic acid	0	4.8[b]
Stearic acid	0	2.9[b]
Palmitoleic acid	106.4	
Oleic acid	100	3.6[b]
Linoleic acid	81.7	
Soybean oil	109.8	
Corn oil	108.3	
Olive oil	113.6	

[a]Relative activity was calculated from the weight of fatty acids bound to L-homoserine. The value for oleic acid was regarded as 100%.
Reactive mixture contained 100 mg of the fatty acid or oil, 10 mg of lipase and 3 M L-homoserine in one ml of 0.1M potassium phosphate buffer, pH 7.5. Reaction was carried out for 3 hr.

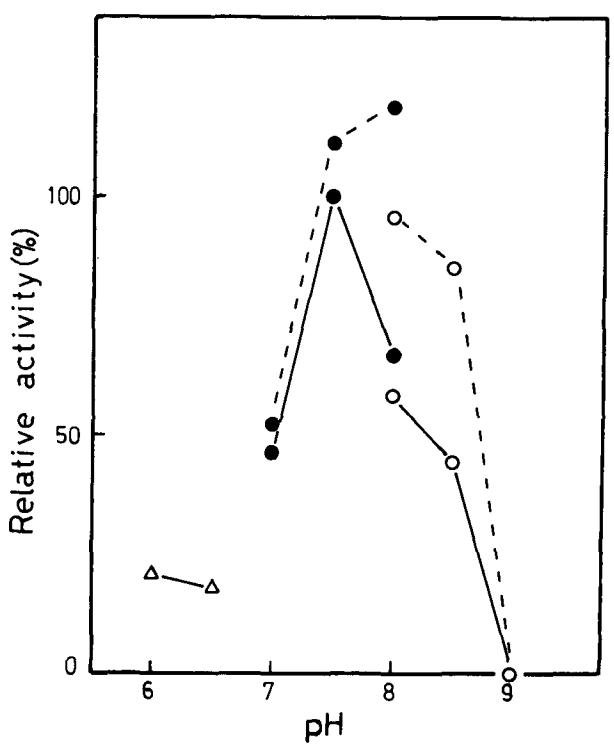

FIG. 2. Effect of pH on the synthesis of O-oleoyl-L-homoserine. Reaction mixture contained 100 mg of oleic acid, 10 mg of lipase and 3 M L-homoserine in one ml of 0.1 M Succinate-KOH buffer (△; 0.1 M potassium phosphate buffer (●), and water (○). ————, 3-hr reaction; – – – – –, 24-hr reaction. Relative activity was calculated from the amount of oleic acid bound to L-homoserine after 3 hr, at pH 7.5, as 100%.

Reaction condition. To determine whether the O-acylation of L-homoserine was catalyzed by *C. cylindracea* lipase, the reaction was carried out with oleic acid and 3 M L-homoserine in the absence of lipase or in the presence of heat-inactivated lipase under the conditions described in Table 1. A small amount of O-oleoyl-L-homoserine was synthesized in these reaction mixtures. However, it was only 0.8% of the product formed in the presence of active lipase. Thus, *C. cylindracea* lipase had a catalytic function on O-acylation of L-homoserine. To examine whether another lipase has the same catalytic function, a reaction was carried out in the presence of *R. delemar* lipase (600 U) instead of *C. cylindracea* lipase under the same conditions as above. The amount of the product via *R. delemar* lipase was 17.9% of that formed using *C. cylindracea* lipase.

Figure 2 shows the effect of pH on the synthetic reaction of O-oleoyl-L-homoserine by *C. cylindracea* lipase. The optimal pH was in the range of 7.5 to 8.0. The reaction proceeded linearly for the initial 4 hr after the reaction

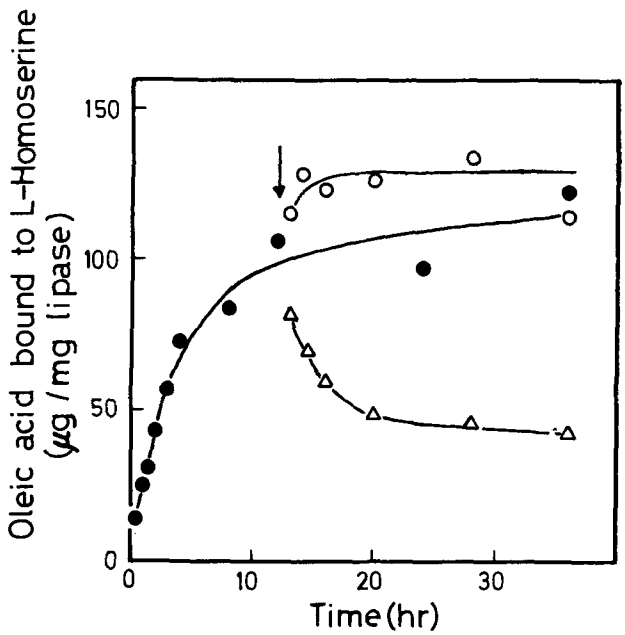

FIG. 3. Time course for the synthetic reaction of O-oleoyl-L-homoserine. Reaction mixture contained 100 mg of oleic acid, 10 mg of lipase and 3 M L-homoserine in one ml of 0.1 M potassium phosphate buffer, pH 7.5 (●). The arrow incidates the time at which 10 mg of fresh lipase (○) or one ml of 0.1 M potassium phosphate buffer, pH 7.5, (△), was added to reaction mixture.

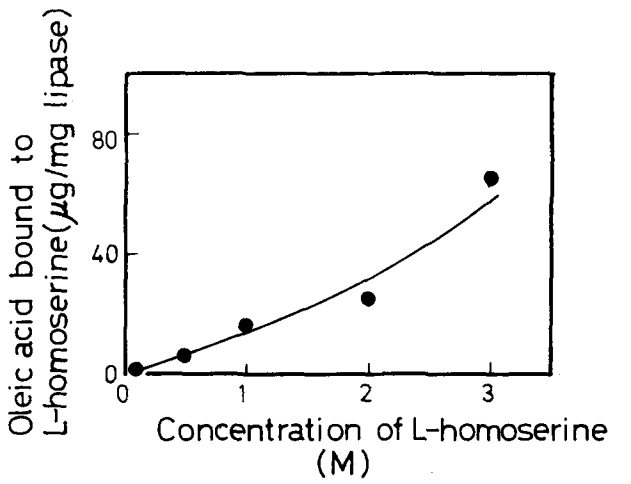

FIG. 4. Effect of L-homoserine concentrations on the synthesis of O-oleoyl-L-homoserine. Reaction mixture contained 100 mg of oleic acid, 10 mg of lipase and L-homoserine in one ml of 0.1 M potassium phosphate buffer, pH 7.5. Reaction was carried out for 3 hr.

started and then gradually reached a plateau. After 36 hr, the amount of oleic acid esterified to L-homoserine was about 0.12 mg/mg protein of lipase (Fig. 3). After 12 hr, fresh lipase (10mg) was added to the reaction mixture to determine whether the reduction in the reaction rate was due to inactivation of lipase. The amount of O-oleoyl-L-homoserine increased slightly and reached a plateau about 2 hr after the addition of lipase. This indicated that lipase was not significantly inactivated during the course of the reaction. After 12 hr, one ml of buffer was added to the reaction mixture. Then, the amount of O-oleoyl-L-homoserine in the reaction mixture rapidly decreased and reached a plateau after 8 hr. These results suggest that the reaction mixture was in a state of equilibrium after a 12-hr reaction. The synthetic reaction was enhanced as the concentration of L-homoserine increased (Fig. 4).

Substrate specificity. To determine the specificity of lipase to hydroxy amino acids, a reaction was carried out with oleic acid as an acyl donor under the same conditions as described in Table 1, except that various amino acid solutions besides 3 M L-homoserine were used. L-Homoserine was the most effective substrate among the four hydroxy amino acids tested. L-Serine was acylated at the same concentration (3 M) as L-homoserine, although the degree of acylation was only 1% that of L-homoserine. L-Threonine and L-tyrosine could not be acylated at the concentrations of 0.8 M and 25mM, respectively.

Substrate specificity to the acyl donor was examined (Table 1). Vegetable oils and unsaturated fatty acids were available substrates. However, solid fatty acids at the reaction temperature, such as palmitic and stearic acids, were not effective acyl donors. Myristic acid was slightly effective, because it was partially dissolved in the reaction mixture. The reactivity of palmitic and stearic acids increased when *n*-hexane was added to the reaction mixture, whereas the reactivity of oleic acid greatly decreased in the presence of *n*-hexane.

Fatty acid compositions of O-acyl-L-homoserines were close to those of the original oils (Table 2). O-Acyl-L-homoserines synthesized from soybean and corn oils contained higher percentages of linoleic acid and lower percentages of oleic acid than the original oils.

TABLE 2

Fatty Acid Composition of O-Acyl-L-Homoserines Synthesized from Vegetable Oils and L-Homoserine

Fatty acid	Fatty acid composition (wt %)					
	Soybean oil		Olive oil		Corn Oil	
	Oil	Product	Oil	Product	Oil	Product
Palmitic acid	10.5	9.2	10.3	9.4	10.8	9.1
Palmitoleic acid			0.6	0.3		
Stearic acid	3.5	2.7			1.6	1.1
Oleic acid	23.3	19.3	2.7	2.0	32.1	27.7
Linoleic acid	55.6	59.2	77.4	78.5	53.8	61.4
Linolenic acid	7.2	9.6	9.2	9.9	1.7	0.7

Emulsifying activity. O-Oleoyl-L-homoserine formed o/w emulsions at oil to water ratios (v/v) of 0.2, 0.5 and 1.0. Its emulsifying acitivty was higher than those of conventional surfactants and casein (Fig. 5). Sodium oleate had little emulsyfing activity in the 0.025 M potassium phosphate buffer, pH 7.0

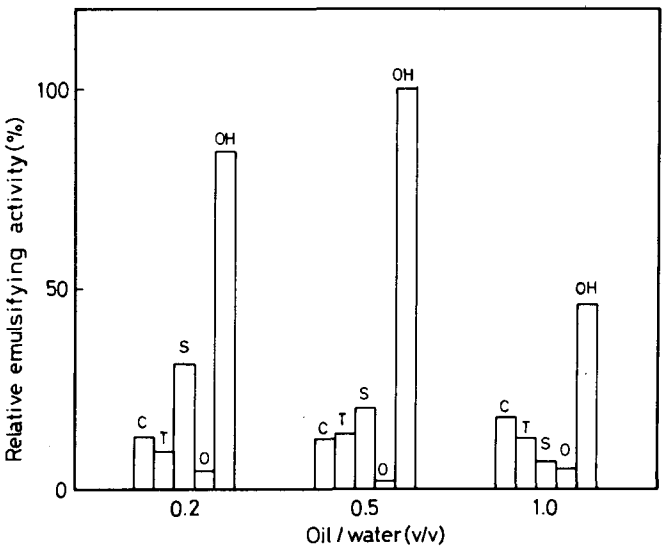

FIG. 5. Relative emulsifying activity of O-oleoyl-L-homoserine and surfactants. Relative emulsifying activity was calculated by regarding the activity of O-oleoyl-L-homoserine to the mixture of the oil to water ratio of 0.5 as 100%. C, Casein; T, Tween 40; S, span 80; 0, sodium oleate; OH, O-oleoyl-L-homoserine.

DISCUSSION

Lipase has been shown to have a catalytic function in the synthesis of fatty acid esters of various alcohols, including polyol, terpene alcohols and sugars (1-6). In addition to these functions, it was shown in the present study that a hydroxy amino acid was acylated by lipase. *R. delemar* lipase as well as *C. cylindracea* lipase had a catalytic function.

It has been reported that the optimal pH for the hydrolysis of olive oil by *C. cylindracea* lipase was 7.2, and that activity (more than 50% of the maximum) was seen in the range of pH 3.0 to 9.0(8). However, in the present study, the synthetic activity of O-oleoyl-L-homoserine was restricted to a narrow pH range. This difference may be caused by a pH-dependent change in the ionic state of L-homoserine, of which the electric charge may have some effect on its affinity for lipase.

The synthetic reaction of fatty acid esters by lipase is known to be reversible and to reach a state of equilibrium after a prolonged reaction. In the current study, the synthesis of O-oleoyl-L-homoserine reached a plateau after 12 hr. This may be due to the formation of an equilibrium state. The addition of buffer to the reaction mixture enhanced the hydrolysis of O-oleoyl-L-homoserine. It is likely that the equilibrium state shifted in the direction of hydrolysis by the addition of the buffer. Dependence of the reaction rate on the L-homoserine concentration did not follow Michaelis-Menten's equation, and the reaction was enhanced as the concentration increased. This suggests that hydrolysis was reduced under higher concentrations of L-homoserine, because the water concentration in the reaction mixture decreased. Therefore, it is possible to accumulate the product by removing O-oleoyl-L-homoserine from the reaction system.

L-Serine was acylated, but its reactivity was very small. It is likely that its affinity to lipase is low because of steric hindrance and electric charge, because the hydroxyl group of L-serine is spatially closer to the amino and carboxyl groups than is the case with L-homoserine. *C. cylindracea* lipase was active to the secondary hydroxyl group, suggesting a tolerance to steric hindrance. Thus, electric charge may have a larger influence on the affinity of amino acids to lipase than does steric hindrance.

Unsaturated fatty acids and vegetable oils, which were in a liquid state at the reaction temperature, were good acyl donors. Solid fatty acids such as palmitic and stearic acids were not reactive. However, they became reactive by improving contact between the fatty acids and lipase by the addition of *n*-hexane to the reaction mixture. Oleic acid became less reactive by adding *n*-hexane. The effect may be caused by the dilution of oleic acid.

There is a possibility that O-oleoyl-L-homoserine is produced by direct transesterification between vegetable oils and L-homoserine. However, it remains unknown which is a true substrate, esterified fatty acids or free fatty acids, because almost all of the vegetable oils were hydrolyzed to fatty acids under the conditions described in Table 1.

O-Oleoyl-L-homoserine was found to form o/w emulsions and showed the highest emulsyfing acitivity among the tested surfactants and casein. O-Oleoyl-L-homoserine is an amphoteric surfactant and is expected to have some characteristic properties different from those of nonionic food emulsifiers, such as monoglycerides and sugar esters. It may be safe for humans after digestion by pancreatic lipase for the following reasons. O-Acyl-L-homoserine can be synthesized by enzymatic process and hydrolyzed to fatty acids and L-homoserine in the digestive organs. L-Homoserine is a naturally occurring amino acid and is distributed in plants, especially in germinating pea seeds (9).

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