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PII: S0308-8146(96)00044-1

Food Chemistry, Vol. 57, No. 4, pp. 515–521, 1996

0308-8146/96 $15.00+0.00

Lipase from neon flying squid hepatopancreas: purification and properties

Sukarno,[a] Koretaro Takahashi,[a]* Mutsuo Hatano[a] & Yasunori Sakurai[b]

[a]*Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido, 041, Japan*
[b]*Laboratory of Marine Ecology, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido, 041, Japan*

(Received 25 September 1995; accepted 18 December 1995)

Lipase from hepatopancreas of the neon flying squid (*Ommastrephes bartramii*) has been partially purified. The molecular weight determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 33 000. Optimum pH was around 7.0 and the enzyme was relatively stable between pH 6.0 and 9.0 for 6 h at 25 °C. Optimum temperature of the enzyme reaction was around 25 °C. The enzyme was tolerably stable up to 37 °C. Several triglyceride (TG) molecular species were used as substrates for investigating the positional and fatty acid specificities of the enzyme. With respect to the hydrolysate of TG, the enzyme examined was not an *sn*-1,3 or an *sn*-2 positionally specific lipase. The enzyme appeared to have specific activity on monounsaturated and saturated chain TGs, most likely oleic and/or palmitic acid. In this study, colipase was also separated from the enzyme.

INTRODUCTION

Lipase, a glycerol ester hydrolase, is widely distributed in animals, plants and microorganisms. Many reports on its purification and application have been published. The purified lipases show wide variations of properties depending on their sources with respect to substrate and/or positional specificity, thermostability, pH optimum and stability, activator requirements, etc. (Verger, 1984; Huang, 1984; Iwai & Tsujisaka, 1984; Sugiura, 1984; Antonian, 1988). This suggests that one could probably find a suitable lipase from nature that would be suitable for a desired application. In contrast to the wealth of information pertaining to lipases from other biological sources, none has been reported on lipase from neon flying squid (*Ommastrephes bartramii*).

The objective of this study was to investigate the possibility of using the hepatopancreas of neon flying squid, commonly discarded during processing, as a potential source of industrially valuable lipase with special characteristics. In addition, by using an organ that is usually discarded as a source of enzyme, it is expected to reduce environmental pollution and increase the economic value of the waste. Our results for the purification and some properties of this lipase are described.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Procedures for screening the lipase and investigating its activity

Copper reagent was prepared according to the method of Lowry & Tinsley (1976). Oleic acid as fatty acid standard and *p*-nitrophenyl ester laurate were purchased from Tokyo Kasei Chemicals (Tokyo, Japan). Bis(2-ethylhexyl) sodium sulfosuccinate (abbreviated as AOT), iso-octane and other solvents and chemicals were obtained from the same source.

For lipase screening, the hydrolytic assay and activity test for lipase were carried out according to the methods of Han *et al.* (1987) and Kwon & Rhee (1986). Lipase and esterase assays were carried out by the methods of Stuer *et al.* (1986) and Isobe *et al.* (1988) using *p*-nitrophenyl laurate as a standard substrate, and also by the modified method of Sarda & Desnuelle (1958) using Tween 20 (polyoxyethylene sorbitan monolaurate) as a water-soluble substrate and triolein as an emulsified triglyceride (TG).

Preparation of the crude enzyme

The neon flying squids (*Ommastrephes bartramii*) were caught near the Midway Islands in August 1991. From ten squids collected, the means of the body length, body weight and hepatopancreas weight were 60.1 cm,

486.7 g and 29.3 g, respectively. Crude enzyme was prepared by extraction (defatting) of squid hepatopancreas using organic solvents. During the extraction, phenylmethylsulfonyl fluoride (PMSF) was also used as an inhibitor for serine proteinase and esterase. Fifty grams of squid hepatopancreas were mixed in 10 volumes of cold acetone for 30 min, homogenized for 5 min, then centrifuged at 10000*g* for 30 min. The precipitate formed was collected, treated again with cold acetone and then recentrifuged. The same procedure was carried out with ethyl acetate and diethyl ether. The final precipitate was vacuum-dried. The powder obtained by this procedure was used for further studies.

Preparation of colipase

A partially purified colipase was obtained using a modified method (Canioni *et al.*, 1977). A 2 g aliquot of crude enzyme was stirred in 20 ml of 0.1 M H_2SO_4 for 1 h at room temperature, then centrifuged for 25 min at 10000*g*, 4 °C. The supernatant was adjusted to pH 7.0 with 5 M NaOH, and then recentrifuged. Ammonium sulfate (2 g ml^{-1}) was added slowly to the supernatant and the solution was stirred for 30 min at 4 °C. The precipitate was dissolved in 2 mM Tris–HCl buffer pH 7.0 to give a volume of 10 ml. The solution was then mixed slowly with ethanol (10 ml) and kept at 4 °C for 1 h under stirring. Insoluble material was removed by centrifugation and the supernatant was mixed with ethanol (80 ml); again the mixture was stirred for 1 h at 4 °C and centrifuged to obtain a colipase pellet. The pellet was vacuum-dried and then stored at −20 °C.

Chromatographic purification of lipase

The crude enzyme obtained from organic solvent extraction was dialyzed against 0.01 M Tris–HCl buffer, pH 7.5, for 24 h at 4 °C, and then loaded onto a DEAE-cellulose column (2.5 cm×40 cm). Elution was carried out with 0.05 M Tris–HCl buffer, pH 7.5, containing 0.3 M NaCl and 0.02% benzalkonium salt, at 4 °C. Fractions of 5 ml were collected by a fraction collector at a flow rate of 8 ml cm^{-2} h^{-1}. The effluent was monitored at 280 nm using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

The active fraction was applied to a 2.0 cm×85 cm glass column packed with Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was carried out with 0.05 M phosphate buffer, pH 7.5, containing 0.02% sodium azide (NaN_3) at 4 °C; the flow rate was 2 ml cm^{-2} h^{-1}. The effluent was monitored at 280 nm using a Hitachi U-2000 spectrophotometer. The protein content of each fraction was determined by the method of Lowry *et al.* (1951). Vacuum-drying was carried out using a Tokyo Rikakikai FD-5 freeze-drier. The active fraction was then used for further studies.

Molecular weight determination

The enzyme purity was confirmed by disc gel electrophoresis (slab gel) according to Davis (1964). The molecular weight of the enzyme was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a modified Laemmli discontinuous buffer system (Anonymous, 1986).

Measurement of optimum pH and pH stability

Partially purified lipase and colipase were dissolved in deionized water containing 0.25% sodium taurocholate, 0.15 M NaCl, 1 mM $CaCl_2$ and 0.3 mM NaN_3. The enzyme reaction was determined at various pH values between 4.0 and 9.0 at 25 °C for 30 min. The hydrolytic reaction was terminated by the addition of 10 ml of acetone/ethanol mixture (1:1, v/v). The liberated free fatty acid was titrated with 0.1 M NaOH solution using phenolphthalein as an indicator. One unit of lipase activity was defined as the amount which liberated 1 μmole of acid per minute at 25 °C. The relative rate was based on the highest activity of the enzyme at a certain pH with olive oil as substrate (as 100%). For measuring pH stability of the enzyme, the pH of lipase and colipase solutions was adjusted with the buffer solutions and kept at 4 °C for 6 h. After the pH treatment, the enzyme solution was diluted with 0.1 M phosphate buffer, pH 7.0. Activity then was determined titrimetrically as described above. Residual activity was expressed as a percentage of non-treated lipase activity.

Measurement of optimum temperature and thermal stability

The optimum temperature of the enzyme was determined (at pH 7.0 for 30 min) at various temperatures between 4 °C and 42 °C. The thermal stability was investigated by determining the residual activity (at 25 °C, 30 min) after leaving the enzyme and colipase solutions (pH 7.0) at various temperatures, between 25 °C and 50 °C, for 10 min. The activity was assayed titrimetrically as described above.

Gas–liquid chromatography

The fatty acid composition of lipid hydrolyzed with the lipase examined was determined by gas–liquid chromatography (GLC). Methyl esters of fatty acids were prepared according to the method of Metcalfe & Wang (1981) and analyzed using a Hitachi gas chromatograph equipped with a 3 mm × 300 cm glass column packed with Unisole 3000 (GL Sciences, Tokyo, Japan) on Uniport C (80–100 mesh) and with flame ionization detection. Samples were run under the following conditions: column temperature, 210 °C; detector temperature, 250 °C, injection temperature, 240 °C; nitrogen flow rate, 25 ml min^{-1}.

Determination of lipid classes

The composition of the hydrolyzed TG was analyzed by thin-layer chromatography (TLC). The developing solvent system used was *n*-hexane/diethyl ether (7:3, v/v). The composition of the lipid after hydrolysis was quantified on a densitometer after spraying the TLC plate with either 3% cupric sulfate or 3% cupric acetate in 8% phosphoric acid and heating at 160–180 °C for 10–15 min.

Preparation of TG molecular species as substrate for lipase specificity analysis

Fish oil was obtained from Nippon Oil (Tokyo, Japan). Olive oil and linseed oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cocoa butter was a generous gift from Yunokawa Seiyaku (Hakodate, Japan). The TGs from these oils were purified by preparative TLC using *n*-hexane/diethyl ether (7:3, v/v) as developing solvent.

The molecular species of some TGs from cocoa butter and linseed oil were prepared by high-performance liquid chromatography (HPLC). Before applying the sample, the TGs prepared by TLC were filtered through a 0.45 μm Ekicrodisc 3 CR filter (Gelman Sciences, Tokyo, Japan). Separation of TGs with HPLC was carried out on a Wakosil-5C18N (Wako) 4 mm× 300 mm column. The instrument used consisted of a Hitachi 665A-11 liquid chromatograph equipped with a Hitachi 655A-30 refractive index detector and Hitachi D-2500 Chromato-Integrator (Hitachi). The eluting solvent used for TGs of cocoa butter and linseed oil was acetone/acetonitrile with ratios of 4:1 and 4.3:1 (v/v), respectively. The TGs were applied to the column at room temperature (about 25 °C). The flow rate was 0.5 ml min^{-1}. Peaks on HPLC chromatograms were numbered in sequence of elution. The fatty acid composition of each collected peak was analyzed by GLC.

Determination of specificity

For lipase specificity determination, the substrates used were TGs of fish oil, squid oil, olive oil, linseed oil, cocoa butter, and an equimolar mixture of tripalmitin, triolein and tristearin. The hydrolysis of substrate was carried out in a 50 ml glass-stoppered flask containing 7.5 ml of 100 mM AOT–iso-octane solution. To the flask, 2.5 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.25% sodium taurocholate, 0.15 M NaCl, 1 mM $CaCl_2$, 0.3 mM NaN_3, and an aliquot of lipase and colipase were added. Reaction was carried out for certain times at 37 °C. The fatty acid composition of each lipid class through squid hepatopancreatic lipase mediated hydrolysis was determined by GLC. For hydrolyzed TG of cocoa butter, treatments with and without undecenoic methyl ester, as an internal standard, were also conducted.

RESULTS AND DISCUSSION

Molecular weight

The molecular weight of the enzyme was estimated by SDS–PAGE and was calculated to be approximately 33 000.

Discrimination of lipase from esterase and cutinase

Besides lipase, there are other lipolytic enzymes such as esterase and cutinase. Assays for discrimination of lipase from other lipolytic enzymes were conducted using *p*-nitrophenyl laurate, an ester of medium-chain fatty acids, as a substrate. The results, as shown in Fig. 1, indicated that the enzyme examined could hydrolyze the substrate and release *p*-nitrophenol. Assays were also conducted in both emulsified and aqueous systems using triolein and Tween 20 as substrates, respectively. Figure 2 shows that the activity was observed in the emulsified system, but none in the aqueous system. These results indicated that the enzyme is not any lipolytic enzyme other than lipase. They are also in agreement with the reports of Hoshino *et al.* (1992), Kolattukudy (1984) and Desnuelle (1961). Hoshino *et al.* (1992) and Kolattukudy (1984) reported that lipases hydrolyze ester bonds of medium- and long-chain fatty acids, but esterases and cutinases do not. Kolattukudy (1984) also mentioned that cutinases catalyze hydrolysis of ester bonds in the polymer and in certain model substrates; purified cutinases from fungal and pollen sources catalyze hydrolysis of *p*-nitrophenyl esters of short-chain fatty acids. Meanwhile, Desnuelle (1961) suggested that the term lipases should be restricted to hydrolytic enzymes that have little or no action on esters present in true aqueous solution but are dependent on the presence of an interface, i.e. on an

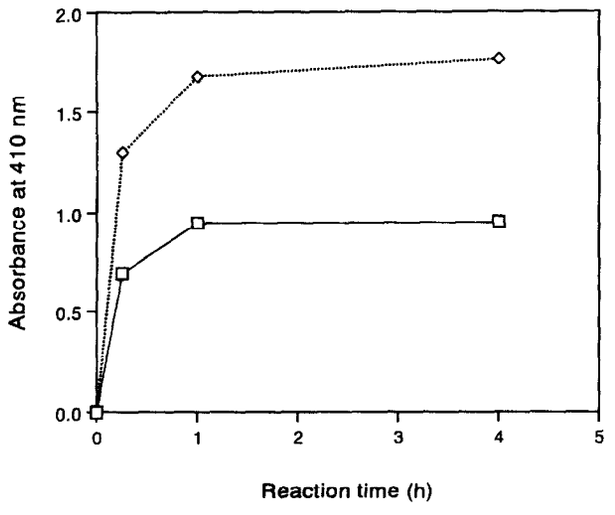

Fig. 1. Activity of the lipase examined towards *p*-nitrophenyl laurate. The liberated *p*-nitrophenol is expressed as absorbance at 410 nm. □, control; ◇, enzyme.

emulsified system, for activity; the term esterases should be applied to enzymes whose activities are confined to the hydrolytic cleavage of the ester bonds of water-soluble substrates. Moreover, the Tween-hydrolyzing enzyme (esterase) extracted from rat adipose tissue as reported by Wallach *et al.* (1962) attacks Tween 20 but is inactive against the other Tweens and long-chain TGs.

A number of esters have been employed for lipase and esterase assays. Tween 20, a commercial water-soluble ester, has been recommended and has often been used as a substrate for testing esterase activity (Renold & Marble, 1950; Sarda & Desnuelle, 1958; Wallach *et al.*, 1962; Desnuelle & Savary, 1963; Laws & Moore, 1963; Nagaoka & Yamada, 1973; Von Tigerstrom & Stelmaschuk, 1989). On the other hand, phenolic esters such as *p*-nitrophenyl have also been recommended and often used for testing lipase activity in various tissues or biological fluids (Hoshino *et al.*, 1992; Desnuelle & Savary, 1963).

The presence or absence of protease was also checked by using inhibitors such as antipain, pepstatin A, and trypsin inhibitor. It was assumed that adding these inhibitors would inhibit the protease and, therefore, the lipase would be free from the action of protease. Consequently, the activity of lipase would increase. However, treatments with and without these inhibitors in the hydrolytic system gave no significant differences in the activity of lipase. This result indicated that the enzyme examined was free from protease. The absence of protease, esterase and other lipolytic enzymes was due to the treatment with PMSF during the purification process. In most cases, PMSF acts as an inhibitor for serine proteases and serine esterases (Brockman, 1981).

Effect of colipase on hydrolysis

Treatment with and without colipase gave a significant difference in the activity of the enzyme. The presence of colipase in the hydrolytic system increased the initial activity of the enzyme (approximately ten times). This is in agreement with Brockman (1981) and Borgstrom & Charlotte-Erlanson (1978) who reported that the presence of colipase was desirable in most of the pancreatic lipases. Colipase can help to stabilize the enzyme and allow it to function in the presence of detergents and hydrophobic proteins. There was no evidence that our colipase contained lipase.

Lipase, by definition, acts at lipid/water interfaces. From numerous studies on lipases and related enzymes, it has been confirmed that catalysis proceeds in two steps: interaction of the substrate with the interface, followed by catalysis within the interfacial plane. It follows, therefore, that the regulation of catalysis is a function not only of the chemical structure of the substrate, but also of the physical properties of the interface at which catalysis occurs (Brockman, 1981). In the presence of bile salts such as taurodeoxycholate, pancreatic lipase loses its affinity for the substrate and bile salt/water interface (Momsen & Brockman, 1976). Similarly, hydrophobic proteins can interfere with lipase adsorption (Borgstrom & Charlotte-Erlanson, 1978). In each case, lack of adsorption implies lack of catalysis, because the substrate is located at the interface. The binding of colipase is unaffected by these agents, and at interfaces it has a high affinity for lipase. Colipase, therefore, allows catalysis to proceed in the presence of bile salts and other proteins (Brockman, 1981).

Effect of pH and temperature on enzyme activity

The optimum pH of the enzyme reaction was determined (25 °C, 30 min) at various pH values from 4.0 to 9.0. The enzyme showed the highest activity at around pH 7.0. The enzyme was relatively stable over the pH range 6.0–9.0 for 6 h at 25 °C (Fig. 3).

The enzyme activity was also investigated at various temperatures (i.e. 4, 15, 25, 37 and 42 °C). The optimum

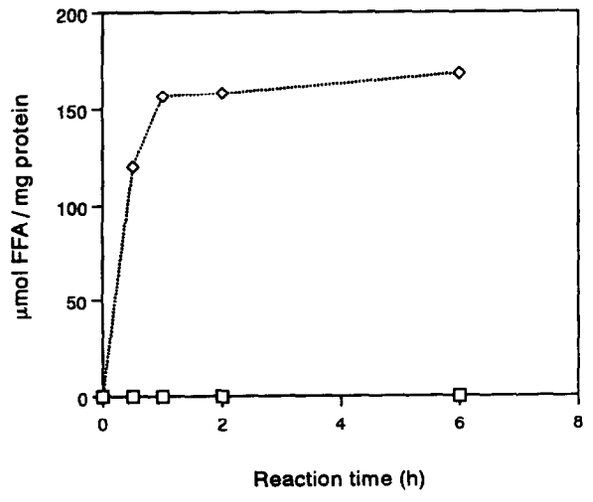

Fig. 2. Activity of the lipase and esterase in emulsified and aqueous systems, respectively. Enzymes used were from the same source. □, esterase; ◇, lipase.

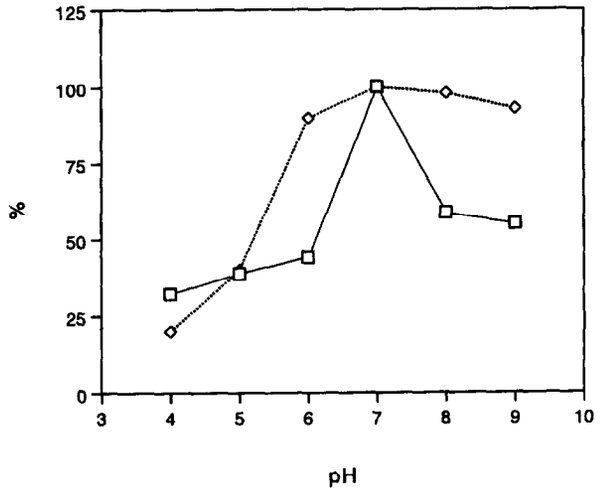

Fig. 3. Optimum pH and pH stability of the lipase examined. □, relative activity (%), optimum pH; ◇, residual activity (%), pH stability.

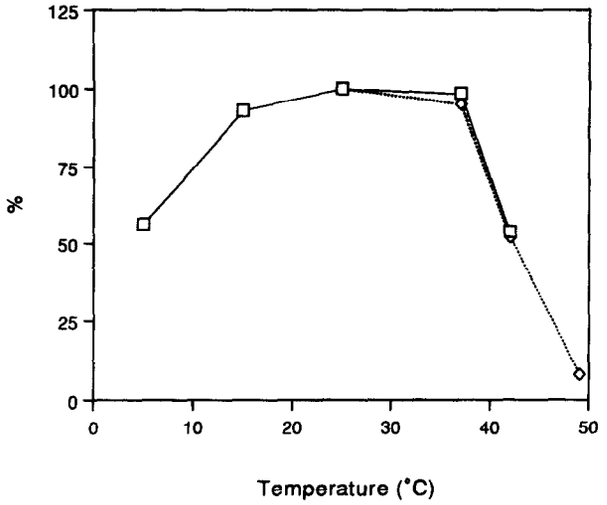

Fig. 4. Optimum temperature and thermal stability of the lipase examined. □, relative activity (%), optimum temperature; ◇, residual activity (%), thermal stability.

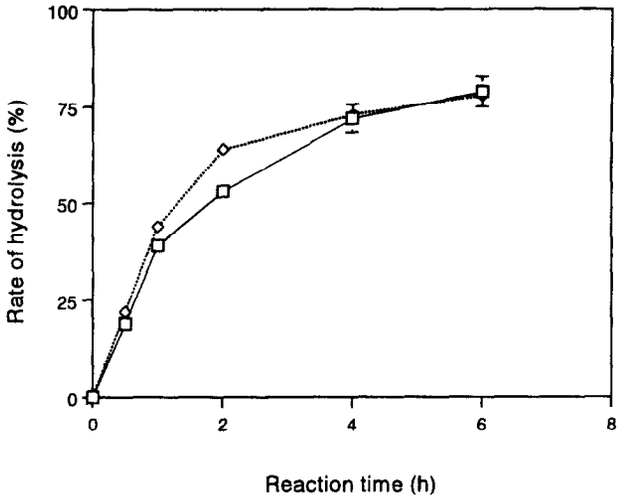

Fig. 5. Time course rate of lipase mediated hydrolysis using fish oil and olive oil triglycerides as substrates. □, fish oil; ◇, olive oil.

temperature of the enzyme reaction (pH 7.0, 30 min) was around 25 °C. The thermal stability was investigated by determining the residual activity, at 25 °C for 30 min, after leaving the enzyme solution (pH 7.0) at various temperatures, between 25 °C and 50 °C, for 10 min. The enzyme lost 50% of its activity at about 42 °C (Fig. 4).

Positional and fatty acid specificities

Triglycerides from cocoa butter, olive oil, linseed oil, fish oil and squid oil were used to investigate the substrate specificity of the enzyme. After hydrolytic reaction, the fatty acids, monoglycerides and diglyceride fractions were subjected to GLC for their fatty acid analysis. As shown in Fig. 5 and Tables 1–3, the enzyme was capable of hydrolyzing a broad range of substrates, including most solid fats (such as cocoa butter) and some other oils (e.g. fish oil, squid oil, olive oil and linseed oil). In the hydrolytic system, it was found that saturated and monounsaturated fatty acids, especially palmitic, stearic and oleic acids, were rapidly released by the lipase examined, while highly unsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were poorly released by this enzyme. Among these fatty acids, oleic acid was found to be the most susceptible.

To know whether the enzyme has positional or fatty acid specificities, several TG molecular species were used as substrates. With respect to the hydrolyzate of TGs from fish oil, obtained through the action of the enzyme examined, the saturated and the monounsaturated fatty acids like palmitic, stearic and oleic acid were detected during the earlier stages of hydrolysis, but

Table 1. Fatty acid composition of each lipid class through squid hepatopancreatic lipase mediated hydrolysis of fish oil and squid oil triglycerides

Fatty acid	Substrate 0 h	Hydrolysis 4 h				Hydrolysis 20 h				Hydrolysis 44 h			
	TG	MG	DG	FFA	TG	MG	DG	FFA	TG	MG	DG	FFA	TG
16:0	15.7[a]	8.1	17.2	6.3	11.7	19.8	19.2	19.8	18.4	21.5	18.5	33.2	17.9
	16.2[b]	22.1	11.3	12.3	10.5	17.7	10.8	19.6	10.6	16.4	16.6	17.1	10.8
18:0	3.5	0.1	3.8	0.9	24.5	4.3	5.6	4.6	3.8	5.0	3.5	9.4	3.7
	4.4	7.9	3.7	3.8	4.3	7.5	3.6	5.3	3.4	7.3	7.1	2.8	4.5
18:1	13.8	16.7	13.6	33.2	10.8	15.6	31.5	33.0	14.8	13.7	14.4	25.6	15.0
	12.9	19.8	12.1	29.7	13.8	21.4	12.8	29.1	14.1	18.0	22.7	28.9	13.7
18:2	1.2	7.5	2.1	8.7	1.4	2.5	8.1	16.9	1.7	1.5	2.5	3.4	1.5
	1.3	1.3	1.7	3.5	1.1	0.8	2.2	5.0	0.2	0.3	2.2	0.0	0.8
20:5	20.3	16.0	16.7	3.8	9.4	15.9	8.3	2.4	17.9	15.5	15.1	2.0	18.6
	11.4	7.2	7.7	5.6	15.3	16.7	5.6	9.0	15.6	14.9	12.4	9.5	15.7
22:6	10.7	12.6	12.2	2.8	6.4	9.5	3.5	2.1	7.9	9.8	11.1	1.2	9.3
	18.9	14.4	34.8	9.5	21.1	3.0	36.3	9.3	21.8	12.1	12.0	13.6	20.9

[a]Values in first row refer to fish oil triglycerides.
[b]Values in second row refer to squid oil triglycerides.
MG, monoglyceride; DG, diglyceride; FFA, free fatty acid; TG, triglyceride.

Table 2. Fatty acid composition of each lipid class through squid hepatopancreatic lipase mediated hydrolysis of cocoa butter triglycerides (TGs)

Cocoa butter TGs	Fatty acid	Substrate 0 h	Hydrolysis 4 h				Hydrolysis 20 h				Hydrolysis 44 h			
		TG	MG	DG	FFA	TG	MG	DG	FFA	TG	MG	DG	FFA	TG
POP	16:0	63.7	11.4	21.8	21.1	55.6	16.9	42.2	30.3	61.7	9.1	32.6	35.4	48.3
	18:0	–	–	–	–	–	–	–	–	–	–	–	–	–
	18:1	31.5	14.3	28.4	46.5	39.9	21.1	27.2	52.1	34.7	26.9	35.5	54.3	45.4
POS	16:0	32.5	9.7	13.9	16.3	24.8	13.2	11.1	25.1	20.7	17.0	15.7	21.0	26.3
	18:0	31.9	6.4	3.7	8.9	42.7	6.2	8.9	6.0	53.3	7.3	8.2	7.4	53.6
	18:1	31.4	17.7	33.2	27.0	27.8	13.0	15.6	40.5	20.3	22.4	36.6	33.9	17.9
SOS	16:0	–	–	–	–	–	–	–	–	–	–	–	–	–
	18:0	64.2	9.9	13.6	5.3	72.7	10.7	16.0	18.3	79.6	10.5	15.6	22.2	87.3
	18:1	30.7	22.5	20.3	36.7	24.3	22.2	36.8	46.6	10.0	36.8	32.4	45.6	10.6

MG, monoglyceride; DG, diglyceride; FFA, free fatty acid; TG, triglyceride; POP, 1,3-dipalmitoyl-2-oleoyl glycerol; POS, 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl glycerol; SOS, 1,3-distearoyl-2-oleoyl glycerol.

Table 3. Fatty acid composition of each lipid class through squid hepatopancreatic lipase mediated hydrolysis of substrate containing equimolar tripalmitin, triolein and tristearin

Fatty acid	Hydrolysis 4 h				Hydrolysis 20 h				Hydrolysis 44 h			
	MG	DG	FFA	TG	MG	DG	FFA	TG	MG	DG	FFA	TG
16:0	11.2	32.6	28.9	15.6	13.1	40.1	33.2	15.3	10.9	37.2	35.4	20.1
18:0	50.9	12.9	7.8	69.7	49.8	15.6	8.2	78.5	57.3	16.9	11.0	65.3
18:1	37.8	54.5	54.5	13.5	35.5	43.9	53.0	6.1	30.0	45.1	46.8	13.8

MG, monoglyceride; DG, diglyceride; FFA, free fatty acid; TG, triglyceride.

the polyunsaturated fatty acids such as EPA and DHA were not (Table 1). In TGs of fish oil, EPA and DHA are dominantly bound at position *sn*-2 (Brockerhoff *et al.*, 1968; Litchfield, 1968; Menzel & Olcott, 1964; Wada, 1993). From these results, therefore, two possibilities might be considered: (1) *sn*-1,3 positionally specific, or (2) easy to release other fatty acids except EPA and DHA. Using TGs of cocoa butter in which oleic acid is predominantly at position *sn*-2 (Jensen *et al.*, 1983; Jurriens & Kroesen, 1965)—e.g. 1,3-dipalmitoyl-2-oleoyl glycerol (16:0–18:1–16:0), 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl glycerol (16:0–18:1–18:0) and 1,3-distearoyl-2-oleoyl glycerol (18:0–18:1–18:0)—as substrates, the GLC chromatograms of free fatty acid fractions of the hydrolyzates showed that the relative percentage of oleic acid was higher than palmitic or stearic acid, as shown in Table 2, indicating that the enzyme was not a *sn*-1,3 positionally specific lipase. To confirm the specificity of the enzyme, hydrolytic assay with monoacid TG mixtures was carried out. In this case, mixtures of equimolar tripalmitoyl glycerol, tristearoyl glycerol and trioleoyl glycerol were used as substrates. Table 3 shows that oleic acid was also detected more than the other two fatty acids. Substrates with TGs from linseed oil, which contains not only monounsaturated but also polyunsaturated fatty acids, gave the same result: i.e. oleic acid existed in higher amounts than the other fatty acids in the hydrolyzate free fatty acid fraction (data not shown).

These findings demonstrated that the enzyme examined was not an *sn*-1,3 or an *sn*-2 positionally specific lipase. The enzyme appeared to have specific activity on monounsaturated and saturated chain TGs, most likely oleic and/or palmitic acid.

ACKNOWLEDGEMENTS

The authors wish to thank Masahiro Nojima for his help throughout this work.

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