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ORIGINAL PAPER

Effect of Lipase Activity and Specificity on the DAG Content of Olive Oil from the Shodoshima-Produced Olive Fruits

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Abstract Olive oils have a higher relative diacylglycerol (DAG) content than other plant oils. The lipase in olive fruits is involved in DAG production and is directly related to the acidity of the olive oil. However, the lipase activity and positional selectivity have not been clarified. To investigate the properties of olive fruit lipase, olive fruits of the Mission variety harvested during mid-December of 2005 on Shodoshima Island (Japan) were stored at 20, 30 or 40 °C for 4 weeks. Changes in the acidity and acylglycerol content of the oils extracted from the stored fruits were analyzed. The acidity and DAG content of the olive oils increased due to triacylglycerol (TAG) hydrolysis during storage. *sn*-1,2-DAGs preferentially increased during the early stages of storage, indicating that the olive fruit lipase is enantioselective for the *sn*-3 position, while non-enzymatic isomerization of *sn*-1,2-DAGs was observed throughout the entire duration of storage. Kinetic analysis revealed that the enantioselectivity of olive fruit lipase for the *sn*-3 position was approximately four times greater than for the *sn*-1 position. The lipase was gradually inactivated at temperatures of 30 °C or higher, and the ratios of the rate constant for inactivation to TAG hydrolysis at the *sn*-3 position was 0.2, 13, and 23 at 20, 30, and 40 °C, respectively.

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Abbreviations

TAG	Triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
FFA	Free fatty acid

Introduction

Olive oil has a higher relative diacylglycerol (DAG) content than other plant oils [1]. Olive oil is reported to contain approximately 20 mol% DAG (predicted to be approximately 20 wt%) in Mallorca Island (Spain) [2]. DAGs in olive oil are generated during the biosynthesis or hydrolysis of triacylglycerol (TAG). The DAG content is relatively high in acidic oils, suggesting that hydrolysis is the primary cause of DAG accumulation [3, 4]. Hydrolysis occurs during fruit storage prior to oil extraction, and may also occur at slow rates even after the oil has been extracted [5, 6]. In addition, the ratio of 1,2-DAG to total DAG is high in fresh oil, while the ratio of 1,3-DAG increases when fruits are damaged by storage, freezing, fungi, or harmful insects [7]. Therefore, the DAG content and the isomer ratio have been proposed as an index of olive oil quality [8]. Lipase contained in olive fruit hydrolyzes TAG [9]. Therefore, DAG content and acidity, which is another important index of olive oil quality, are directly affected by lipase activity. However, no hydrolysis activity or positional selectivity of olive fruit lipase has been reported, except the optimal pH for activity [9]. In this study, olive oil was extracted from Mission variety olive

fruits that were harvested during mid-December of 2005 on Shodoshima Island (Japan). Olive fruits are extracted immediately after harvest in general. In this study, fruits were stored at 20, 30 or 40 °C for 4 weeks prior to extraction to indirectly investigate the properties of olive fruit lipase. The effect of fruit storage on the quality of olive oil and the possibility of DAG accumulation based on the activity and/or positional specificity of olive fruit lipase are discussed.

Experimental Procedures

Sample Oil Preparation

Olive fruits of the Mission variety harvested on Shodoshima Island (Kagawa, Japan) during mid-December of 2005 were stored at 5 °C. The harvested fruits had an entirely black skin and deep purple flesh, and were well ripened. One day after harvesting the fruits, their surfaces were washed well with distilled water. Afterwards, the fruits were stored in clean and sealed polyethylene bags (approximately 100 g/bag) at 20, 30, or 40 °C. After various storage times, the bags were removed from storage and the olives were frozen in liquid nitrogen and ground using a mill. To minimize case error due to individual fruits, all of the fruits in each bag (30–40 fruits) were ground and homogenized. The ground fruits were transferred to centrifuge tubes, defrosted at room temperature for 1 h, and centrifuged (20,000*g* for 30 min) to obtain the oil samples. Because damaged fruits are known to be highly acidic [7], the induction of enzyme activity in damaged fruits were also investigated. Olive fruits were damaged by dropping the polyethylene bags containing fresh fruit from a height of 4 m onto a linoleum-covered concrete floor. The partially damaged fruits were then stored at 20 °C. After storing the fruits for nearly 4 weeks, softening of the flesh and dripping occurred; thus, the longest storage period was set at 4 weeks.

Composition Analysis

An oil sample of approximately 40 mg was accurately weighed into a vial containing 10 mg of internal standard (trioctanoate), derivatized with a silylation agent consisting of hexamethyldisilazane, trimethylchlorosilane, and pyridine, and analyzed using an Agilent 6890 gas–liquid chromatograph system with a flame ionization detector (Agilent Technologies Inc., Santa Clara, CA, USA), as previously reported [10]. Calibration curves were prepared using analytical grade oleic acid, glycerolmonooleate, glycerol dioleate, and glycerol trioleate, and the fatty acid and acylglycerol contents of the samples were calculated from the curves, and presented as the molar concentration in the extracted oil. The coefficient of variation for this procedure was 0.5% for both 10 mmol/kg glycerolmonooleate and 30 mmol/kg glycerol dioleate.

Enantiomer Composition

The enantiomer ratio analysis was performed according to the method reported by Itabashi et al. [11]. Briefly, DAG-dinitrophenyl urethane (DNPU) derivatives were prepared by combining a 20-mg oil sample with 0.5 ml dinitrophenyl isocyanate (DNPI) reagent (toluene solution containing 10 mg of DNPI and 0.05 ml of pyridine), followed by mixing at room temperature for 3 h. The reaction was stopped by the addition of 0.5 ml methanol. The reaction products were separated using TLC (silica-gel, hexane:dichloroethane:ethanol = 40:10:3) to obtain the DAG-DNPU fraction (Rf = 0.5–0.6). The DAG–DNPU fraction underwent additional separation using TLC (silica-gel, chloroform:acetone = 99:1). The *sn*-1,2(2,3)-DAG–DNPU fraction (Rf = 0.25) was applied to a chiral column (AK-03, YMC Co. Ltd., Kyoto, Japan)-equipped high performance liquid chromatograph system (Elite, Hitachi High-Technologies Corp., Tokyo, Japan), using a detection wavelength of 254 nm.

Results and Discussion

Changes in the free fatty acid and acylglycerol contents during storage are shown in Fig 1. The free fatty acid and DAG contents increased, while TAG content decreased during storage. The decrease in the fatty acid groups contained in TAG from the initial level was consistent with the increases in the sum of the fatty acid groups contained in MAG, DAG and FFA (Fig. 2), confirming that changes in the composition of the olive oil were primarily due to TAG hydrolysis. During the first week of storage, the rate of TAG hydrolysis increased with temperature. When the fruits were stored at 20 °C, TAGs were hydrolyzed at a constant rate throughout the storage period, but storage at 30 or 40 °C resulted in a gradual decline in the rate of TAG hydrolysis. In fact, after storing fruits at 40 °C for 1 week, TAG hydrolysis was negligible. TAG was the main lipid of the fruit throughout the storage period, and the changes in the TAG concentration were small, ranging from 1,101 to 1,007 mmol/kg. Therefore, the decrease in the TAG hydrolysis rate suggested that the lipase was inactivated at 30 and 40 °C. In fruits stored at 30 °C, the DAG content increased to 110 mmol/kg (6.8 wt%), but the rate of increase in DAG content during the late storage period was grater in fruits stored at 20 °C than in those stored at 30 °C. This result indicates that maximal DAG content may be

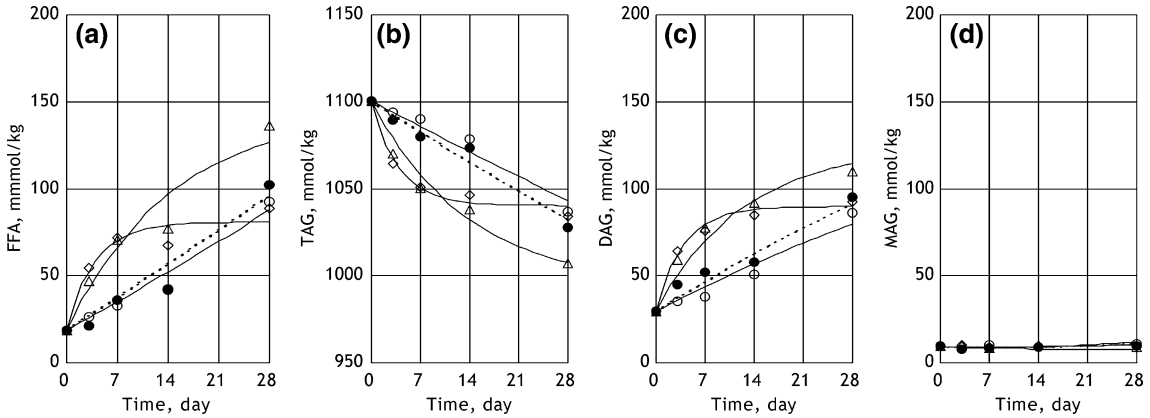

Fig. 1 Changes in **a** free fatty acid, FFA, **b** triacylglycerol, TAG; **c** diacylglycerol, DAG; **d** monoacylglycerol, MAG content in fruit stored at 20 °C (*open circles*), 30 °C (*open triangles*), 40 °C (*open diamonds*), and in damaged fruit stored at 20 °C (*filled circles*). Calculated curves by the kinetic model shown in Fig. 4 are displayed by *solid lines* for 20, 30 and 40 °C, and by *dotted lines* for damaged fruit stored at 20 °C. The unit of (mmol/kg) is converted into (wt%) by multiplying by 28.2 for FFA, 88.4 for TAG, 62.0 for DAG and 35.6 for MAG, using oleic acid to approximate all other fatty acids

obtained in fruits stored at 20 °C for longer than one month. The MAG content did not change throughout the storage period. Because the production of FFAs was only slightly greater than that of DAGs, it appears that hydrolysis of DAGs to MAGs and FFAs was minimal, and only a small amount of the resulting MAGs were subsequently hydrolyzed to FFAs and glycerol. Damage to the fruit did not affect TAG hydrolysis or lipase activity, as the composition changes in damaged fruits were not markedly different from those in the undamaged fruits. The reported high acidity of the oils from damaged fruit [7] seems to be related not only to fruit damage, but also to invasion by microorganisms and/or insects.

Changes in the DAG isomer contents are shown in Fig. 3. Prior to storage, *sn*-1,2-DAGs accounted for the majority of DAGs, and during early storage period, *sn*-1,2-DAGs preferentially increased, suggesting that the olive fruit lipase has high enantioselectivity for the *sn*-3 position. During the early storage period, *sn*-1,2-DAGs increased more rapidly at higher storage temperatures, but this rate gradually decreased in fruits stored at 30 or 40 °C; the rate of TAG hydrolysis also decreased. The *sn*-1,2-DAG content started to decrease after 2 weeks at 30 °C and after 1 week at 40 °C. In damaged fruits stored at 20 °C, the maximal *sn*-1,2-DAG content observed during the study period was 74 mmol/kg (4.6wt%). In contrast, the 1,3-DAG content continued to increase throughout the storage period, and the rate of increase was higher in fruits stored at 30 and 40 °C. 1,3-DAG production was not affected by the enzyme inactivation predicted from the TAG hydrolysis pattern, and accompanied the decrease in *sn*-1,2-DAG content in fruits stored at 30 or 40 °C, suggesting that 1,3-DAGs were produced by non-enzymatic isomerization of *sn*-1,2-DAGs. Although the amount of *sn*-2,3-DAG produced was small, the trends for the changes in *sn*-2,3-DAG content were analogous to those for *sn*-1,2-DAG, suggesting that hydrolysis of TAG at the *sn*-1 position occurred because of incomplete lipase enantioselectivity.

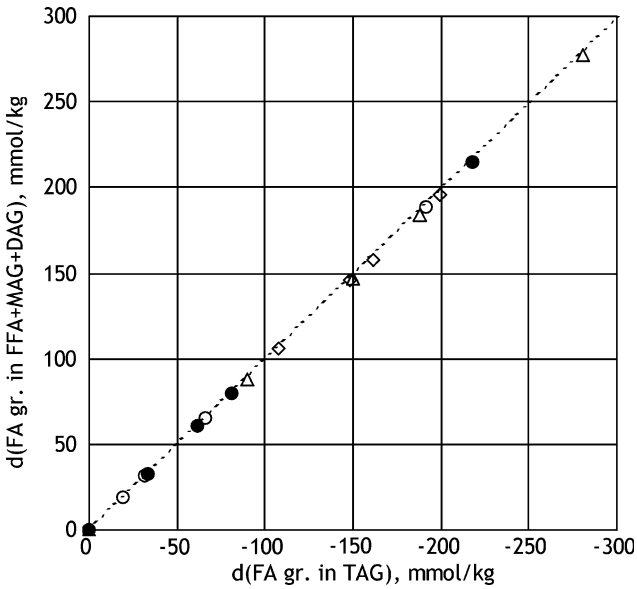

Fig. 2 Mass balance of fatty acid groups in samples during storage at 20 °C (*open circles*), 30 °C (*open triangles*), 40 °C (*open diamonds*), and in damaged fruit stored at 20 °C (*filled circles*)

As mentioned above, it was assumed that olive oil was hydrolyzed by an enantioselective lipase present in the olive fruit, according to the pathway shown in Fig. 4. To evaluate the enzyme characteristics, a hydrolysis model (Eqs. (1)–(6)) based on the scheme presented in Fig. 4 was constructed, and the reaction rate analyzed. The reaction rate was assumed to be proportional to the linear substrate

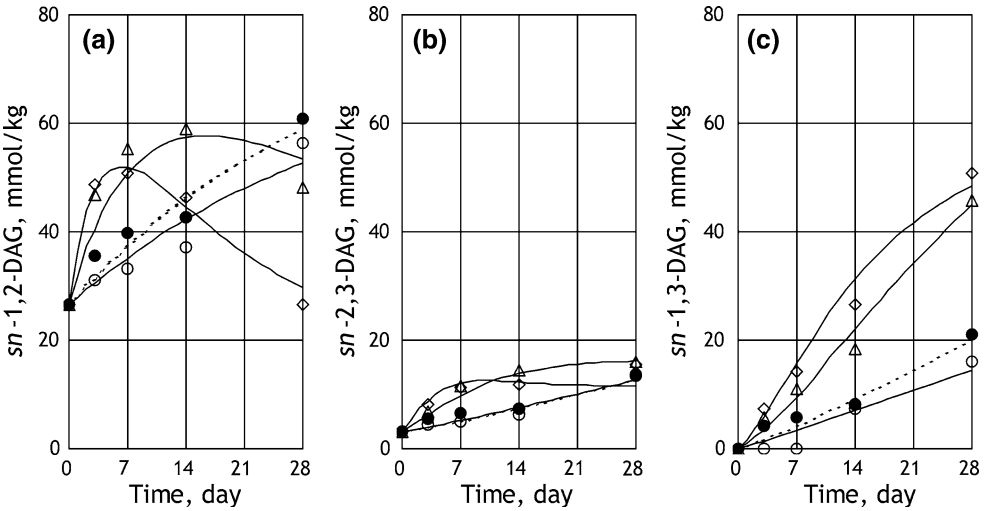

Fig. 3 Changes in the content of DAG isomers in olive fruit stored at 20 °C (*open circles*), 30 °C (*open triangles*), 40 °C (*open diamonds*), and in damaged fruit stored at 20 °C (*filled circles*): **a** *sn*-1,2-diacylglycerol, *sn*-1,2-DAG; **b** *sn*-2,3-diacylglycerol, *sn*-2,3-DAG; **c** 1,3-diacylglycerol, 1,3-DAG. Calculated *curves* by the kinetic model shown in Fig. 4 are displayed by *solid lines* for 20, 30 and 40 °C, and by *dotted lines* for damaged fruit stored at 20 °C

concentration [12]. Because the water content of the olive fruit is far too excessive for hydrolysis, the reverse reaction, esterification, and the water term were omitted. The hydrolysis rate constants representing the hydrolytic activity, k1–k6 (1/day), were assumed to decrease at the inactivation rate constant, ki (1/day); whereas, it was assumed that the non-enzymatic isomerization reaction did not decrease, but progressed at reaction rate constants of k7 and k8 (1/day).

$$\mathrm{d[TAG]}/\mathrm{d}t = -\mathrm{k1[TAG]} - \mathrm{k2[TAG]} \tag{1}$$

$$\begin{aligned}\mathrm{d}[sn-1,2-\mathrm{DAG}]/\mathrm{d}t = {} & \mathrm{k1[TAG]} - \mathrm{k3}[sn-1,2-\mathrm{DAG}] \\ & + \mathrm{k8}[1,3-\mathrm{DAG}] \\ & - \mathrm{k7}[sn-1,2-\mathrm{DAG}]\end{aligned} \tag{2}$$

$$\begin{aligned}\mathrm{d}[1,3-\mathrm{DAG}]/\mathrm{d}t = {} & -\mathrm{k4}[1,3-\mathrm{DAG}] - 2\mathrm{k8}[1,3-\mathrm{DAG}] \\ & + \mathrm{k7}[sn-1,2-\mathrm{DAG}] \\ & + \mathrm{k7}[sn-2,3-\mathrm{DAG}]\end{aligned} \tag{3}$$

$$\begin{aligned}\mathrm{d}[sn-2,3-\mathrm{DAG}]/\mathrm{d}t = {} & \mathrm{k2[TAG]} - \mathrm{k5}[sn-1,2-\mathrm{DAG}] \\ & + \mathrm{k8}[1,3-\mathrm{DAG}] \\ & - \mathrm{k7}[sn-2,3-\mathrm{DAG}]\end{aligned} \tag{4}$$

$$\begin{aligned}\mathrm{d[MAG]}/\mathrm{d}t = {} & \mathrm{k3}[sn-1,2-\mathrm{DAG}] + \mathrm{k4}[1,3-\mathrm{DAG}] \\ & + \mathrm{k5}[sn-2,3-\mathrm{DAG}] - \mathrm{k6[MAG]}\end{aligned} \tag{5}$$

$$\mathrm{dk}(i)/\mathrm{d}t = -\mathrm{ki} \times \mathrm{k}(i), \quad i = 1,2,3,4,5,6 \tag{6}$$

d[*X*]/d*t* represents the rate of change in the concentration of component *X*. Based on the thermodynamic equilibrium between *sn*-1,2(2,3)-DAG and 1,3-DAG (*sn*-1,2(2,3)-DAG: 1,3-DAG = 1:2), k7 was set to 4-times k8. The Runge–Kutta method was used for integration, and the Newton method was used for fitting. The results of fitting for the

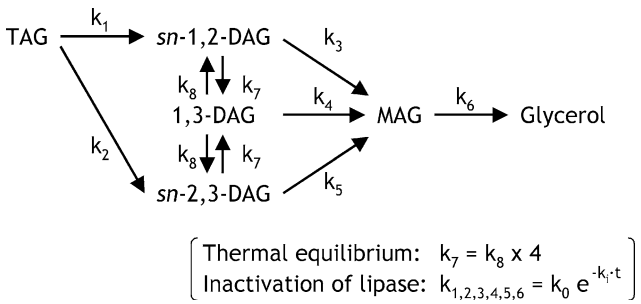

Fig. 4 Hydrolysis scheme of olive oil

fruit stored at 20, 30 and 40 °C (solid lines) and for the damaged fruit stored at 20 °C (dotted line) are shown in Figs. 1 and 3. The multiple correlation coefficients between analytical data and calculated values from the hydrolysis model in each of the storage conditions were not less than 0.97, suggesting that this model fits the experimental results well. The reaction rate constant (kx) and the ratio (kx/k1) of each reaction rate to the rate constant for TAG hydrolysis (k1), which is a primary hydrolysis reaction, are shown in Table 1. Lipase activity (μmol-FFA release/g-oil/day) of the fruits stored at 20, 30 and 40 °C calculated by Eq. (7) was 2.2, 8.2 and 16, respectively.

$$\text{Lipase activity} = 1130 \cdot (\mathrm{k1} + \mathrm{k2}) \tag{7}$$

where the value of 1,130 represents millimolar concentration of TAG in pure triolein substrate. Although most of the rate constants increased with temperature, the value of kx/k1 revealed that the isomerization reaction (k7) was not particularly accelerated in fruits stored at high temperatures, but inactivation (ki) of hydrolysis was markedly increased. Because of the inactivation of the hydrolysis activity, isomerization reaction is prominent at high temperature. In addition, k1/(k1 + k2), representing

Table 1 Rate constant (1,000/day) and selectivity

Temperature	20 °C		30 °C		40 °C		20 °C/Damaged	
	kx[a]	(kx/k1)[b]	kx[a]	(kx/k1)[b]	kx[a]	(kx/k1)[b]	kx[a]	(kx/k1)[b]
k1	1.4	(1.0)	5.7	(1.0)	11	(1.0)	1.8	(1.0)
k2	0.50	(0.4)	1.5	(0.3)	3.1	(0.3)	0.43	(0.2)
k3	0.97	(0.7)	3.0	(0.5)	4.4	(0.4)	0.49	(0.3)
k4	13	(9.4)	32	(5.6)	33	(3.0)	17	(9.5)
k5	13	(9.2)	16	(2.8)	24	(2.1)	3.6	(2.0)
k6	16	(11.5)	65	(11.3)	44	(3.9)	14	(7.6)
k7	3.3	(2.4)	8.6	(1.5)	11	(1.0)	4.5	(2.5)
ki	0.28	(0.2)	72	(12.6)	256	(23.0)	0.23	(0.1)
Selectivity[c]	0.74		0.79		0.79		0.81	

[a] *kx* rate constant, [b] *(kx/k1)* ratio of rate constant to that for TAG hydrolysis at *sn*-3 position, [c] selectivity k1/(k1 + k2)

enantioselectivity for hydrolysis of fatty acid from the *sn*-3 position, was approximately 0.8 regardless of the temperature. In addition, the enantiomeric excess of the hydrolysis product, *sn*-1,2-DAG, was calculated to be approximately 60%. Although information about the enantioselectivity of plant lipases is limited, the olive fruit lipase showed similarly high enantioselectivity as the *Carica papaya* latex lipase [13] and gastric lipase [14]. Because extraction and purification of the enzyme was not carried out in this study, the specific activity and the amount of olive fruit lipase were not determined. Further investigation is needed to detail the properties of olive fruit lipase.

Because the lipase activity contained in olive fruits is enantioselective for the *sn*-3 position, when lipase activities are present, and the isomerization is not so active, as was evident in the fruits stored at 20 °C, TAG hydrolysis is likely to stop at *sn*-1,2-DAG, allowing this isomer to accumulate in large amounts. When the storage temperature is 30 °C or higher, accumulation of DAGs may be suppressed, not only by the increase in DAG hydrolysis via 1,3-DAG generated through the accelerated isomerization, but also by the decreased rate of DAG production due to lipase inactivation. As mentioned above, olive oil produced on Mallorca containing about 20% DAG has been reported [2]. Based on the results of the present study, long-term storage at low temperatures may allow large amounts of DAGs to accumulate in olive oil, as occurred in oils produced on Mallorca a long time ago.

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