Partial Purification of *Nigella sativa* L. Seed Lipase and Its Application in Hydrolytic Reactions. Enrichment of γ-Linolenic Acid from Borage Oil

Melek Tuter[a], H. Ayşe Aksoy[a,*], Guldem Ustun[a], Sergio Riva[b], Francesco Secundo[b], and Serhat İpekler[a]

[a]Istanbul Technical University, Chemical Engineering Department, 80626 Maslak, Istanbul, Turkey, and [b]Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, 20131, Milano, Italy

ABSTRACT: Selective hydrolysis of borage (*Borago officinalis* L.) oil was catalyzed by two lipase preparations of *Nigella sativa* L. seeds at 40°C in a mixture of borage oil, water, and hexane. Ammonium sulfate-precipitated lipase *(Nigella PL)* and lipase partially purified by DEAE-ion exchange chromatography *(Nigella CPL)* exhibited a negative specificity toward γ-linolenic acid (GLA). Best results were obtained in the experiments conducted with 330 U/g oil of *Nigella PL* and 200 U/g oil of *Nigella CPL*. When 330 U/g oil of *Nigella PL* was used, after 8 h the GLA level rose from 21.9% in the starting oil to 29.6 and 41.8% in TAG and DAG fractions of the product mixtures, respectively (1.5-fold enrichment of GLA in the total unhydrolyzed acylglycerol fraction). At 200 U/g oil enzyme concentration of *Nigella CPL,* after 77 h maximum GLA enrichment was observed in the DAG fraction. The GLA content of the DAG increased to 34.6%, corresponding to almost 1.6-fold enrichment. The relative inability of *Nigella sativa* lipase(s) to hydrolyze γ-linolenoyl moieties of TAG can be used for the enrichment of this acid in the unhydrolyzed acylglycerol fractions of GLA-containing oils.

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γ-Linolenic acid (all-*cis*-6,9,12-octadecatrienoic acid; 18:3n-6; GLA) is a pre-essential FA in humans and other mammals and is valuable as a medicine (1–3). In recent years, GLA-rich acylglycerols have been reported to have beneficial effects in treating certain diseases, such as atopic eczema, multiple sclerosis, and rheumatic arthritis (2). Therefore, several studies have been undertaken on the preparation of GLA-enriched products from GLA-containing oils, particularly by means of lipase-catalyzed hydrolysis and esterification reactions (1–4).

Borage (*Borago officinalis* L.) oil (GLA content, 22%) is one of the most important and commercially available sources of GLA. Lipase-catalyzed selective hydrolysis of borage oil performed with Pancreatin or with lipase from *Candida cylindracea* led to enrichment in GLA from 20.4% in the starting oil to 33.5 and 47.8% in the unhydrolyzed acylglycerol fractions, respectively (1). Additionally, Huang *et al.* (2) reported that by using *C. rugosa* lipase immobilized on microporous polypropylene for the selective hydrolysis of borage oil in isooctane, the GLA content could be raised from 23.6 mol% in borage oil to 51.7% in the unhydrolyzed acylglycerols. Shimada *et al*. (4) purified GLA from borage oil using a two-step enzymatic method. In the first step, hydrolysis of borage oil catalyzed by a lipase from *Pseudomonas* sp. (LIPOSAM) resulted in 91.5% conversion after 24 h. The resulting FFA were extracted from the reaction mixture with *n*-hexane (GLA content, 22.5%; recovery of GLA, 92%). In the second step, the FFA obtained from borage oil were esterified with lauryl alcohol by using *Rhizopus delemar* lipase. It was found that 74.4% of borage-FFA were esterified, and the GLA content in the FFA fraction was enriched from 22.5 to 70.2% with a recovery of 75.1% of the initial content. Shimada *et al*. (5) investigated the kinetics of the selective hydrolysis of borage oil (GLA content, 22%; GLA-22) by *C. rugosa* lipase (Lipase-OF) in different amounts with stirring at 500 rpm for 15 h at 35°C. GLA was enriched from 22 to 35% with 94% recovery after 2 h of reaction, and to 46% with 76% recovery after 15 h of reaction. The acylglycerols obtained from 2- and 15-h reaction mixtures were coded as GLA-35 and GLA-46, respectively, and they were then hydrolyzed separately under the same conditions. The hydrolysis rate decreased with increasing GLA content of acylglycerols, but the release rate of GLA did not depend on the GLA contents in the oil (5).

Nigella sativa L., also known as black cumin or fennel flower, is a member of the *Ranunculaceae* family and is native to some parts of the Mediterranean region (6). Üstün *et al. (7)* reported on the use of *N. sativa* L. lipase to catalyze hydrolytic reactions at ambient temperatures during harvesting, handling, and oil processing (7). Specifically, the ground *N. sativa* L. seeds, the pressed seeds, an acetonic powder form of the crude lipase, and a lipase sample immobilized by adsorption on Celite 535 from phosphate buffer solutions are effective biocatalysts for the hydrolysis and glycerolysis of TAG and for the esterification of FA (8–11).

In this study, we report on the selective hydrolysis of borage oil catalyzed by two lipase preparations of *N. sativa* L. seeds in order to enrich the GLA content of the acylglycerol fractions. To our knowledge, this is the first report on the selective hydrolysis of borage oil by a plant lipase.

*To whom correspondence should be addressed.
E-mail: aksoyha@itu.edu.tr

EXPERIMENTAL PROCEDURES

Materials. Borage oil was obtained from Sigma Chemical Co. (Deisenhofen, Germany). *Nigella sativa* L. seeds of Denizli region of Turkey were purchased locally. All other chemicals used in the studies were purchased from Merck Chemical Co. (Darmstadt, Germany).

Analysis of borage oil. For the determination of FA composition, borage oil was converted to its corresponding methyl esters by BF_3/methanol esterification (12), which was analyzed by a GLC method using a capillary column, Ultra 2 (25 m × 0.32 mm × 0.52 μm film thickness of 5% diphenyl and 95% dimethyl polysiloxane) and FID in an HP-5890 series II gas chromatograph (Hewlett-Packard, Waldron, Germany). Nitrogen was used as the carrier gas at a flow rate of 1.6 mL/min. Air and hydrogen flow rates were 460 and 33 mL/min, respectively. The detector and injector temperatures were 280 and 250°C, respectively. The oven temperature was set to 150°C for 5 min and heated to 225°C at a rate of 5°C/min and maintained at this temperature for 30 min.

The FA composition of borage oil is given in Tables 1 and 2.

Extraction of lipase from seeds. Nigella sativa seeds were initially defatted using hexane in a special Soxhlet extraction apparatus equipped with a cooling system (6-h extraction). The defatted seeds (250 g) were then extracted with 1,100 mL distilled water containing 1% (vol/vol) Triton X-100 (triton) for 6 h at room temperature. After placing the suspension in the refrigerator overnight, this homogenate was filtered through cheesecloth, and the turbid solution was then centrifuged for 20 min at 23,000 × *g*. A slightly turbid supernatant was obtained and designated as "crude lipase extract."

Preparation of ammonium sulfate precipitated enzyme (Nigella PL). Solid ammonium sulfate was added to the crude extract up to 35% saturation under constant stirring. The resulting precipitate was separated by centrifugation and dried and designated as *Nigella PL.*

Preparation of partially purified enzyme (Nigella CPL) *by diethyl amino ethyl cellulose (DEAE)–ion exchange chromatography*. The above described precipitates were dissolved in potassium phosphate buffer (5 mM, pH 7.5) and dialyzed against the same buffer overnight. The solution was loaded on a DEAE column (15 × 2.5 cm) previously equilibrated with the same buffer, and the enzymes were then eluted over 2.5 h using a linear gradient from potassium phosphate buffer (5 mM, pH 7.5) to the same buffer containing 1 M NaCl and 0.5% triton. The eluate containing lipase activity was lyophilized and designed as *Nigella CPL.*

Determination of lipase activity. The activities of the two enzymatic preparations (*Nigella PL* and *Nigella CPL*) were determined using the olive oil emulsion method without addition of surfactant. Approximately 20 mg of lipase was added to 1 mL of olive oil previously incubated at 37°C for 10 min in a shaking bath with 0.5 mL 0.1 M $CaCl_2$, 3 mL phosphate buffer (0.06 M, pH 7), and 5 mL water. After 20 min, the reaction was stopped by adding 20 mL of an acetone/ethanol solution (1:1 vol/vol). The amount of FFA was titrated with a 0.02 M NaOH solution in the presence of phenolphthalein until the equivalence point of phenolphthalein was reached (solution changes from colorless to red at about pH 9). Blank samples were treated similarly. One unit of lipase activity (U) was defined as the amount of enzyme that liberated 1 μmol of free acid in 1 min at 37°C. It is assumed

TABLE 1
Effect of Amount of *Nigella PL* on the Composition of Borage Oil Hydrolysis Products and on Their FA Compositions

Sample	FA composition (wt%)								Enrichment of GLA (-fold)
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	24:1	
Borage oil	10.7	3.8	12.4	40.6	21.9	4.5	3.0	1.9	1.0
Component of reaction mixture[a] (165 U/g oil)									
TAG (68.0%)	10.7	3.8	13.8	37.4	24.6	4.2	2.8	1.8	1.1
DAG (6.2%)	11.7	3.6	13.2	34.9	29.3	3.5	1.9	1.2	1.3
MAG (4.5%)	17.8	6.1	30.3	17.2	17.6	5.1	3.5	2.4	—
FFA (21.3%)	11.2	5.3	22.4	37.3	11.0	6.3	3.9	1.2	—
Component of reaction mixture[a] (250 U/g oil)									
TAG (58.0%)	10.6	3.6	14.3	36.3	26.9	3.6	2.4	1.4	1.2
DAG (5.2%)	12.4	3.5	14.8	28.8	31.3	3.8	2.6	1.8	1.4
MAG (6.5%)	15.0	4.6	23.0	27.4	18.5	5.3	3.8	2.3	—
FFA (30.3%)	12.5	4.0	20.9	40.4	10.6	5.2	3.2	1.6	—
Component of reaction mixture[a] (330 U/g oil)									
TAG (48.1%)	9.2	2.8	11.6	39.0	29.6	3.8	2.5	0.7	1.4
DAG (6.2%)	7.9	3.0	11.1	29.5	41.8	3.2	1.7	1.1	1.9
MAG (8.2%)	14.8	4.2	17.4	31.5	16.4	6.0	5.4	3.8	—
FFA (37.6%)	12.4	5.6	19.6	37.6	10.4	6.7	3.8	2.2	—
Component of reaction mixture[a] (500 U/g oil)									
TAG (28.3%)	11.0	4.2	15.2	36.8	21.3	4.9	3.2	2.3	~1.0
DAG (4.3%)	12.5	3.8	14.1	35.2	25.5	4.3	2.3	1.5	1.1
MAG (14.8%)	20.0	4.8	19.5	27.3	19.7	4.2	2.6	1.6	—
FFA (66.3%)	8.9	4.3	14.5	40.3	21.7	5.0	2.0	1.0	~1.0

[a]Weight percentage of total reaction mixture. Experimental conditions: 40°C; oil/water/hexane, 2 g/5 mL/50 mL; reaction time, 8 h.

TABLE 2
Effect of Amount of *Nigella CPL* on the Composition of Borage Oil Hydrolysis Products and on Their FA Compositions

Sample	FA composition (wt%)								Enrichment of GLA (-fold)
	16:0	18:0	18:1	18:2	18:3	20:1	22:1	24:1	
Borage oil	10.7	3.8	12.4	40.6	21.9	4.5	3.0	1.9	1.0
Component of reaction mixture[a] (200 U/g oil)									
TAG (74.6%)	10.3	2.9	5.0	48.7	22.1	4.4	3.0	1.9	1.0
DAG (3.9%)	10.8	3.7	10.9	32.0	34.6	4.3	2.1	0.1	1.6
MAG (‹1%)	18.4	5.8	12.8	32.0	23.9	3.3	0.1	0.1	1.1
FFA (19.7%)	11.8	7.6	43.0	8.8	18.0	5.1	3.3	2.3	—
Component of reaction mixture[a] (1,350 U/g oil)									
TAG (42.7%)	14.9	6.7	23.3	20.9	11.0	7.0	4.8	3.5	—
DAG (3.6%)	11.5	3.9	22.1	18.3	16.0	8.5	6.8	5.1	—
MAG (3.1%)	22.0	5.2	15.6	27.5	10.3	4.3	2.2	1.1	—
FFA (50.5%)	6.3	1.2	2.4	56.1	30.3	2.1	1.2	0.4	1.4

[a]Weight percentage of total reaction mixture. Conditions: 40°C; oil/water/hexane, 2 g/5 mL/50 mL; reaction time: 77 h.

here that the activity at 37°C will be the same as that at 40°C, where a group of the assays in this work were conducted.

Hydrolysis of borage oil. The hydrolytic reactions were conducted using 2 g of borage oil, 5 mL of water, and 50 mL of hexane in a glass reactor flask (100 mL) that was placed in a water bath. Heating of the water bath and stirring of the reaction mixture were performed with a magnetic stirrer equipped with a heating unit (Framo-Geraetetechnik M22/1 5655, Franz Morat KG, Eisenbach, Germany). The stirring rate was adjusted to 500 rpm, and the reaction temperature was kept constant at 40°C with an accuracy of ±1°C by a temperature controller. The hydrolysis reaction was started by adding the proper amount of enzyme to the reaction mixture. Samples were taken at selected time intervals and heated in a water bath at 90°C for 15 min to inactivate the enzyme and then centrifuged to separate the oil and the water phase.

Analysis of the hydrolyzed samples. The hydrolyzed mixtures were composed of TAG, DAG, MAG, and FFA. They were analyzed by TLC–FID using an Iatroscan TH-10 analyzer with SIII rods (Iatron Lab, Inc., Tokyo, Japan) under the conditions described in a previous report (10). Separation and recovery of the TAG, DAG, and MAG fractions from the reaction mixtures were carried out by column chromatography. The crude reaction mixtures were passed through a glass column (18 mm i.d.) filled with Florisil (25 g). TAG was eluted with 200 mL hexane/diethyl ether (85:15, vol/vol), whereas DAG and MAG were eluted with 250 mL hexane/diethyl ether (50:50, vol/vol) and 200 mL diethyl ether, respectively. Solvent flow was adjusted to 2 mL/min, and the eluates were collected in 10-mL fractions. Samples of 1 µL were taken from each fraction and analyzed by TLC, using silica gel G (Merck) plates, development with hexane/diethyl ether/acetic acid (70:30:1, by vol), and visualization with iodine vapor. The pure acylglyceride fractions (TAG, DAG, and MAG) were obtained after pooling of pure fractions and evaporation of solvent.

For the removal of FFA from the reaction mixture, another sample of reaction mixture (2 g) was dissolved in hexane (50 mL) and titrated with aqueous 0.5 *N* NaOH. The aqueous phase was then neutralized with 0.5 N HCl. FFA were extracted with diethyl ether and recovered by evaporation of solvent.

The TAG, DAG, MAG, and FFA fractions were separately converted into their methyl esters by reaction with the BF_3-MeOH esterification method, and the FA compositions were determined by capillary GC, as described above.

RESULTS AND DISCUSSION

Characterization of N. sativa *seeds.* The proximate compositions of the seeds determined according to standard AOCS methods (12) showed that the seeds contained (in %) moisture 9.4, ash 4, oil 35.4, protein 23.3, crude fiber 6.7, and carbohydrates 21.2.

Enzymatic hydrolysis of borage oil by enzyme preparation of Nigella PL. To study the influence of enzyme purity on the selectivity toward different FA, especially GLA, two enzymatic preparations, *Nigella PL* and *Nigella CPL*, were used in the hydrolytic reactions. At first, to evaluate the effect of *Nigella PL* on the course of hydrolysis of borage oil, a set of experiments was conducted at large intervals of enzyme concentration. The reactions were carried out for 24 h by varying the enzyme concentration in the range of 165–2,000 U/g oil. The extent of hydrolysis was followed by the change of TAG in the reaction mixture.

As observed in Figure 1, the TAG content in the reaction mixture decreased sharply with an increase in the enzyme concentration in the range of 165–500 units, approaching almost the same value after 8 and 24 h of reaction. Therefore, 500 units of enzyme was sufficient to saturate the reaction system in terms of the enzyme load.

The changes in the concentration of TAG, DAG, MAG, and FFA in the reaction mixtures during the hydrolysis of borage oil for 8 h by action of *Nigella PL* in the concentration range of 165–500 U per g oil are given in Table 1. After 8 h, increasing the enzyme concentration from 165 to 500 units resulted in a decrease of the concentration of the unhydrolyzed TAG from 68.0 to 28.3% and an increase of the FFA

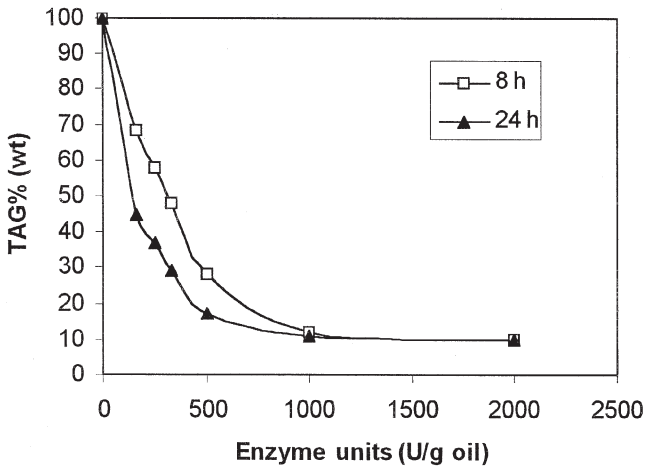

FIG. 1. The effect of enzyme concentration on the extent of borage oil hydrolysis catalyzed by *Nigella PL*. Conditions: 40°C; oil/water/hexane, 2 g/5 mL/50 mL).

fraction from 21.3 to 66.3%. All reaction mixtures contained practically the same amount of DAG (about 4–6%) and of MAG (5–15%) fractions.

To evaluate the effect of enzyme concentration on the FA composition of each reaction product, the FA data for the TAG, DAG, MAG, and FFA fractions obtained at different enzyme concentrations, after 8 h, were also incorporated in Table 1.

When 165 U of *Nigella PL* per gram oil was used in the experiments, the FA composition of the hydrolysis products indicated that the level of GLA was raised from 21.9% in the starting material to 24.6 and 29.3% in the TAG and DAG fractions, respectively (Table 1). No enrichment of GLA was observed in the MAG fraction, and the FFA fraction contained only 11.0% of GLA.

Increasing the amount of enzyme in the mixture, from 165 to 250 U/g oil, increased the extent of enrichment of GLA in the TAG and DAG fractions. In fact, the GLA content was 26.9% in the TAG and 31.3% in the DAG fraction, corresponding to 1.2- and 1.4-fold enrichments of GLA compared to borage oil, respectively.

The level of GLA in the TAG and DAG fractions reached maximal values of 29.6 and 41.8%, respectively, corresponding to almost twofold enrichment of GLA content in the DAG fraction, when the enzyme was present at 330 U. Again, no enrichment of GLA was observed in the MAG fraction. The FFA fraction contained only 11.0% GLA, corresponding to 17.8% of total GLA in the original borage oil.

Further increase of the enzyme from 330 to 500 U led to a decrease in the extent of enrichment of GLA in both TAG and DAG fractions. Concomitantly, it caused a loss of large amounts of GLA (about 65% of total GLA) to the FFA fraction.

When the amount of enzyme was increased further to 1,000 and 2,000 U, the extent of hydrolysis reached about 90% for both reactions. However, the FA data for the reaction products indicated that GLA was not concentrated in any of the TAG, DAG, and MAG fractions.

The effect of reaction time on the course of hydrolysis and on the FA composition of the resulting reaction products was also investigated by conducting another set of hydrolysis experiments, under the same conditions, for 24 h. As observed in Figure 1, prolonging the reaction time from 8 to 24 h resulted in further decreases in the TAG content of the reaction mixtures at enzyme concentrations up to 500 U, above which the level of TAG was essentially the same as at 8 h of reaction time. On the other hand, extending the reaction time to 24 h only slightly, if at all, increased the level of GLA in the TAG, DAG, and also in the FFA fractions when 165–500 U of enzyme were employed, i.e., at 330 units, the GLA content was found to be 31.4% in the TAG, 42.4% in the DAG, and 14.3% in the FFA fractions. To increase the content of GLA in TAG and DAG fractions in a reasonable time, and at the same time cause minimal GLA loss to the FFA fraction, the appropriate reaction time was concluded to be 8 h.

The above experimental results indicated that GLA is apparently a poor substrate for *Nigella PL,* and therefore it is possible to accumulate this FA in the TAG and DAG fractions, particularly in the DAG, by a careful selection of the hydrolytic conditions. Best results were obtained in the experiments conducted with 330 U *Nigella PL* for 8 h.

Enzymatic hydrolysis of borage oil by Nigella CPL. To investigate the effect of the partial purification of the enzyme on the hydrolysis of borage oil, reactions were carried out using different amounts of *Nigella CPL* (200 and 1350 U per g oil).

In preliminary experiments, hydrolysis by *Nigella CPL* proceeded very slowly, resulting in small changes in the composition of the reaction mixtures after 8 h of reaction. As a consequence, reaction time was increased to 77 h. The compositions of the obtained hydrolysis products are reported in Table 2.

Results indicated that approximately 76% of the TAG remained unhydrolyzed in the reaction mixture containing 200 U/g oil, whereas by increasing the enzyme amount to 1350 U/g oil, the residual TAG content declined to 42.7%. Both reaction mixtures contained about 4% DAG, with a minimal presence of MAG.

The FA data for the components of the reaction mixture carried out at 200 U enzyme concentration revealed that GLA was considerably concentrated in the DAG fraction and its content increased to 34.6%, corresponding to a 1.6-fold enrichment (see Table 2). The TAG and MAG fractions were also slightly enriched in GLA, namely, 1.0 and 9.0%, respectively.

At a higher concentration of *Nigella CPL* (1350 U), GLA was released extensively from all acylglycerol fractions, leading to an increase of GLA in the FFA. As a consequence, the GLA content in each acylglycerol fraction (TAG, DAG, and MAG) was lower than in the original borage oil, similarly to what was previously observed with *Nigella PL* above 500 U/g oil enzyme concentrations.

The data presented here show that GLA is apparently a poor substrate for partially purified preparations of *N. sativa* lipase (*Nigella PL* or *Nigella CPL*). GLA-containing acylglycerols were barely hydrolyzed by these enzymes, leading to the concentration of GLA in the TAG and DAG fractions, particularly in the DAG during the process of hydrolyzing borage oil. Ammonium sulfate precipitation gave a form of *N. sativa* lipase (*Nigella PL*) that was a more effective biocatalyst for the hydrolysis of borage oil than the enzyme partially purified by DEAE-ion exchange chromatography (*Nigella CPL*). In using the same units of *Nigella CPL* and *Nigella PL*, it was experimentally observed that almost 10 times longer reaction times were needed to achieve the same value of GLA enrichment when the transformations were conducted with *Nigella CPL.* Apparently, an increase in enzyme purity (moving from *Nigella PL* to *Nigella CPL*) was accompanied by a decrease in its hydrolytic ability toward borage oil. It might be possible that the purification protocol used, described in detail in Reference 13, caused the loss of hydrophobic membrane enzyme(s), extracted from the seeds due to the presence of triton, that were not loaded on the DEAE-column.

Best results were obtained in the experiments conducted with *Nigella PL*, where GLA accumulated, particularly in the DAG fraction where its content reached 42%. The relative inability of *N. sativa* lipase preparations to hydrolyze γ-linolenoyl moieties of TAG could be used for the enrichment of GLA in the unhydrolyzed acylglycerol fractions of GLA-containing oils, such as borage and evening primrose oils.

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