Production of Dihomo-γ-linolenic Acid by *Mortierella alpina* 1S-4

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The mycelial dihomo-γ-linolenic acid content of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, was found to increase, with an accompanying marked decrease in its arachidonic acid content, on cultivation with sesame oil. The resultant mycelia were found to be a rich source of dihomo-γ-linolenic acid. This unique phenomenon was suggested to be due to specific repression of the conversion of dihomo-γ-linolenic acid to arachidonic acid by the oil. After fractionation of the oil with acetone into oil and non-oil fractions, it was found that the effective factor(s) was present in the non-oil fraction. In a study on optimization of the culture conditions for the production of dihomo-γ-linolenic acid by *M. alpina* 1S-4, a medium containing glucose, yeast extract and the non-oil fraction was found to be suitable for the production. Under the optimal conditions in a 50-l fermentor, the fungus produced 107 mg of dihomo-γ-linolenic acid/g dry mycelia (2.17 g/l of culture broth). This value accounted for 23.1% of the total fatty acids in the lipids extracted from the mycelia. The mycelia were also rich in arachidonic acid (53.5 mg/g dry mycelia, 11.2%). Other major fatty acids in the lipids were palmitic acid (24.1%), stearic acid (7.0), oleic acid (20.1), linoleic acid (6.6) and γ-linolenic acid (4.1).

Dihomo-γ-linolenic acid (8,11,14-*cis*-eicosatrienoic acid, DHGLnA), a C-20 polyunsaturated fatty acid (PUFA) with three double bonds, has attracted great interest recently due to its several unique biological activities (1,2). In addition, it is a natural precursor of a large family of structurally related C-20 compounds, including the prostaglandin$_1$ and thromboxane$_1$ groups. DHGLnA has been detected, in a small amount, as a component of cellular lipids in fungi (3), algae (4), protozoa (5) and animals (6). However, there has been no report so far of the production of DHGLnA in a practical amount.

In recent studies, we found that several fungal strains belonging to the genus *Mortierella* accumulate other C-20 PUFAs, i.e., arachidonic acid (Ara) and eicosapentaenoic acid (EPA), in their mycelia when grown with glycose as a major carbon source, and that the resultant mycelia are rich in Ara and/or EPA (3,7-11). We have also reported that these fungi produce a small amount of DHGLnA as a by-product in the production of Ara (9). These findings prompted us to search for potent strains or suitable culture conditions for the accumulation of a large amount of DHGLnA. We report here that *Mortierella alpina* 1S-4, one of the potent producers of Ara reported in our previous papers (7,9), can accumulate a large amount of DHGLnA in its mycelia when grown in a medium containing sesame oil. The culture conditions under which maximum DHGLnA productivity can be obtained are also given.

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MATERIALS AND METHODS

Chemicals. Sesame oil (crude oil) was purchased from Wako Pure Chemicals, Osaka. The oil contained palmitic acid (11.9%, by weight), stearic acid (5.3), oleic acid (42.4), linoleic acid (40.1) and others (0.3). Other oils used in this study were obtained from Yamakei Sangyo, Osaka; Wako Pure Chemicals, Osaka; Nakarai Chemicals, Kyoto, and Sigma Chemical Co., St. Louis, Missouri. Methyl dihomo-γ-linolenate was from Funakoshi Chemicals, Tokyo. All other chemicals used in this work were obtained as described previously (10).

Microorganism, media and cultivation. Mortierella alpina 1S-4 (AKU 3998; Faculty of Agriculture, Kyoto University, Kyoto) (3,7), which was isolated from soil in Kyoto Prefecture, was used. Medium A (2% glucose and 1% yeast extract, pH 6.0) and medium B (4% glucose and 1% yeast extract, pH 6.0) were used as basal media for cultivation. The fungus was inoculated into a 50-ml shaking flask containing 10 ml of a basal medium supplemented with 0.5-3.0% oil followed by incubation at 28 C with reciprocal shaking (120 strokes/min), unless otherwise stated. Other conditions are given in the legends to the respective figures.

Extraction and determination of fatty acids. Fungal mycelia were harvested by suction filtration and washed with 50 ml of ether (acidified with 0.5 ml of 2 N HCl) and then 50 ml of water. The filtered mycelia were dried at 100 C overnight. Transmethylation of the fatty acids in the mycelia with methylene chloride-10% methanolic HCl and extraction of the fatty acid methyl esters with *n*-hexane were carried out with *n*-heptadecanoic acid as an internal standard as described previously (7, 10). The resultant fatty acid methyl esters were analyzed by gas liquid chromatography (GLC), unless otherwise stated. In some cases, fatty acid methyl esters were analyzed by high performance liquid chromatography (HPLC) according to the procedure described previously (7), except for the mobile phase used (acetonitrile/water, 85:15, v/v). The fatty acid concentrations determined on HPLC were essentially the same as those in the case of GLC. The mycelial fatty acid composition values are given in weight %.

Isolation of the DHGLnA methyl ester from fungal mycelia. A portion of mycelia of *M. alpina* 1S-4, which was grown under the conditions given elsewhere in the paper was collected by suction filtration and then washed with water. The procedures used for transmethylation and purification of DHGLnA by HPLC were essentially the same as described previously (7).

Preparation of sesame oil extracts. Sesame oil (22 g) was dissolved in acetone (150 ml) and then allowed to stand at −80 C overnight. After suction filtration, the precipitate was evaporated under reduced pressure

to give an oil fraction (about 22 g). The filtrate was concentrated under reduced pressure to give a non-oil fraction (0.45 g).

Other methods. The methods used for measurement of fungal growth and analyses of mass and ^1H NMR spectra were described previously (10). Glucose concentrations were measured with a commercially available kit (Blood Sugar-GOD-Pred-Test, Boehringer, Mannheim), essentially according to the method of Werner et al. (12).

RESULTS

Effects of various oils on the production of DHGLnA. The production of C-20 PUFAs, i.e., DHGLnA and Ara, by *M. alpina* 1S-4 was assayed in medium A containing various oils or waxes. Only sesame oil was found to effectively increase the accumulation of DHGLnA. The mycelial accumulation of DHGLnA reached 31.2 mg/g dry mycelia (0.47 mg/ml) on incubation with 0.5% sesame oil. This value was about 3-fold higher than that obtained with unsupplemented medium A. No significant increase in the production of DHGLnA was observed on incubation with the other 57 oils and the eight waxes tested. Some of the results are summarized in Figure 1. When the fungus was grown in a medium containing 0.5% glucose, 1% yeast extract and 2% sesame oil, pH 6.0, DHGLnA accumulation reached 40.9 mg/g dry mycelia (1.02 mg/ml). On incubation with the same medium without sesame oil, mycelial mass was poor (5.3 mg/ml) and mycelial D DHGLnA was undetectable.

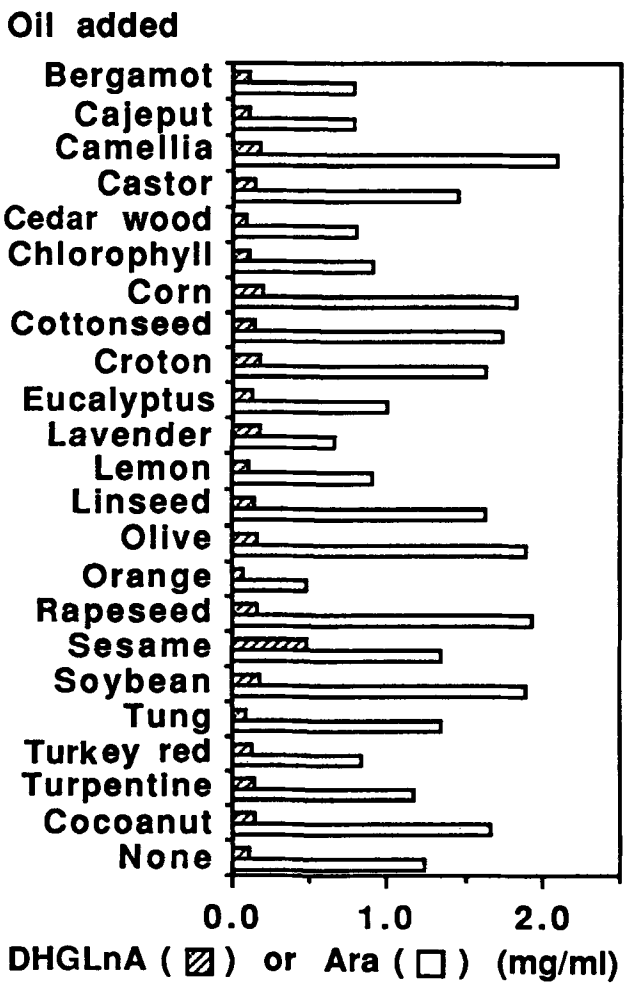

FIG. 1. Comparison of the DHGLnA productivities of *M. alpina* 1S-4 in the presence of various oils. *M. alpina* 1S-4 was grown for 7 days in medium A with each indicated oil (0.5%). Other conditions are given under Materials and Methods.

Effects of various factors affecting DHGLnA production in the presence of sesame oil. (i) Time course of DHGLnA accumulation. The changes in glucose and fatty acids of the sesame oil in medium B containing 2.0% sesame oil, and in those of DHGLnA and Ara during the growth of the fungus, were monitored. The glucose and the fatty acids of the oil in the medium were almost completely consumed during the first three days of cultivation, the mycelial mass reaching 27 mg/ml of culture broth. The mycelial DHGLnA/Ara ratio was about 1.4 at this point, and it did not change significantly throughout the cultivation. After eight days, 34 mg of mycelia (37.3 mg of DHGLnA and 24.6 mg of Ara/g dry mycelia) was obtained per ml of culture medium. On the other hand, the ratio drastically decreased with increasing cultivation time on incubation without sesame oil. The ratio after eight days was only 0.12, the amounts of DHGLnA and Ara being 23.6 and 191.6 mg/g dry mycelia (0.40 and 3.24 mg/ml), respectively.

(ii) Effect of the sesame oil concentration. As shown in Figure 2a, the amount of DHGLnA per ml of culture broth increased with increasing sesame oil concentration. With 3% sesame oil, the amount of DHGLnA accumulated reached 1.37 mg/ml of culture broth. This value was about three times higher than that obtained without addition of the oil. Conversely, the amount of Ara decreased markedly with increasing sesame oil concentration. The amount obtained with 3% sesame oil (0.68 mg/ml) was only about 30% of that obtained without addition of the oil. It should be noted that there was no significant change in the total amount of C-20 PUFAs (i.e., the sum of DHGLnA and Ara) with any concentration tested.

(iii) Effect of the glucose concentration. The data in Figure 2b indicate that the amount of DHGLnA increased in parallel with the concentration of glucose. The maximum production of DHGLnA (2.23 mg/ml) occurred at a concentration of 5%, the mycelial ratio of DHGLnA/Ara and the total C-20 PUFA value reaching 1.43 and 3.84 mg/ml of culture broth, respectively. Further increase in glucose concentration (7 and 10%) repressed production of mycelia, which resulted in a decrease in total C-20 PUFA value (1.83 and 0.95 mg/ml at 7 and 10%, respectively), although the DHGLnA/Ara ratio did not change significantly (data not shown).

(iv) Time of the addition of sesame oil. The mycelial DHGLnA content varied markedly, depending on the growth phase at which the oil was added. Figure 3 indicates that addition at an early phase of growth is more effective for high DHGLnA production than addition at a late phase. On addition 0 to 3 days after inoculation, the mycelial DHGLnA/Ara ratio ranged between 1.3 and 1.5, the DHGLnA production being about 1.5 mg/ml of culture broth. The amount of DHGLnA produced was only 0.61 mg/ml of culture broth, the DHGLnA/Ara ratio being 0.28, when the oil

was added on the sixth day of cultivation and analysis was performed after the mycelia had been cultivated for a further two days.

Extraction of effective fraction from sesame oil. In order to determine whether or not the fatty acid composition of the oil affects the DHGLnA production, the composition of the oil was modified by mixing it with other natural oils, such as olive oil, linseed oil, palm oil and so on, and then the DHGLnA productivities on incubation with the modified oil mixtures were compared. In every case, a significant increase in mycelial DHGLnA was observed. On the other hand, mixtures that did not include sesame oil were not effective. These results suggest that the fatty acid composition is not an essential factor for the enhancement of DHGLnA production. Therefore, we fractionated the sesame oil into two fractions, oil and non-oil fractions, according to the procedure described under Materials and Methods, and then assayed the DHGLnA productivity in the presence of each fraction, as shown in Figure 4. The addition of the oil fraction, at any concentration tested, did not change the DHGLnA/Ara ratio significantly (0.14–0.24) and did not result in significant stimulation of DHGLnA production. Reversal of the DHGLnA/Ara ratio was observed only when the fungus was incubated with the non-oil fraction. The mycelial DHGLnA content increased as did the amount of the non-oil fraction. The highest DHGLnA/Ara ratio (3.7) was obtained at a concentration of 0.025%, at which the maximum production (34.0 mg/g dry mycelia or 1.17 mg/ml) was also attained. At 0.05%, however, mycelial yield was about 65% of that at 0.025%, which resulted in a decrease in DHGLnA production (0.70 mg/ml). All the tested non-oil fractions, which were prepared separately from different lots of sesame oil, showed essentially the same stimulatory activity. Because the non-oil fraction comprises only about 2%, by weight, of the sesame oil, some minor component(s) in the oil may be effective for enhancement of the production of DHGLnA.

Bench-scale production of DHGLnA under optimal culture conditions. From the results of the experiments on individual and combined factors affecting DHGLnA production described above, the optimal culture conditions were determined to be as given in the legend to Figure 5. The intermittent addition of glucose, as shown in Figure 5a, was also necessary for bench-scale fermentor production. Under these conditions, the mycelial accumulation of DHGLnA was 107 mg/g dry mycelia (2.17 g/l). This value accounts for 23.1% of the total extractable fatty acids. The mycelia were also rich in Ara (53.5 mg/g dry mycelia; 11.2%). Other major fatty acids in the lipids were palmitic acid (24.1%), stearic acid (7.0), oleic acid (20.1), linoleic acid (6.6) and γ-linolenic acid (4.1).

Isolation of the DHGLnA methyl ester from fungal mycelia. The DHGLnA methyl ester (34 mg) was isolated from the lipids extracted from 50 g of wet mycelia of *M. alpina* 1S-4 grown under the conditions given in the legend to Figure 5. The isolated methyl ester gave single peaks at Rt = 11.7 and 10.6 min on GLC and HPLC analysis, respectively, which corresponded

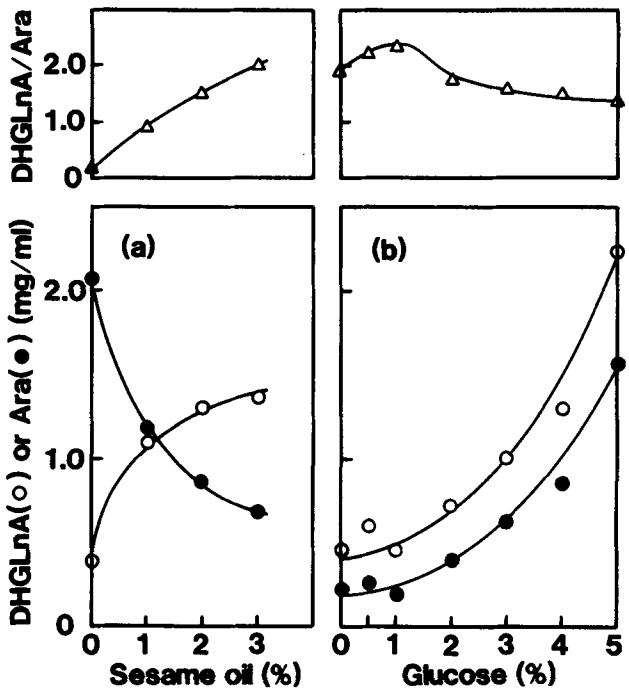

FIG. 2. Effects of sesame oil and the glucose concentration on the production of DHGLnA. In (a), *M. alpina* 1S-4 was grown for 7 days in medium B under the conditions given under Materials and Methods except for the addition of sesame oil as indicated. In (b), the organism was grown for 7 days in a medium containing 1% yeast extract, 2% sesame oil and glucose. The concentration of glucose in the medium was varied, as indicated.

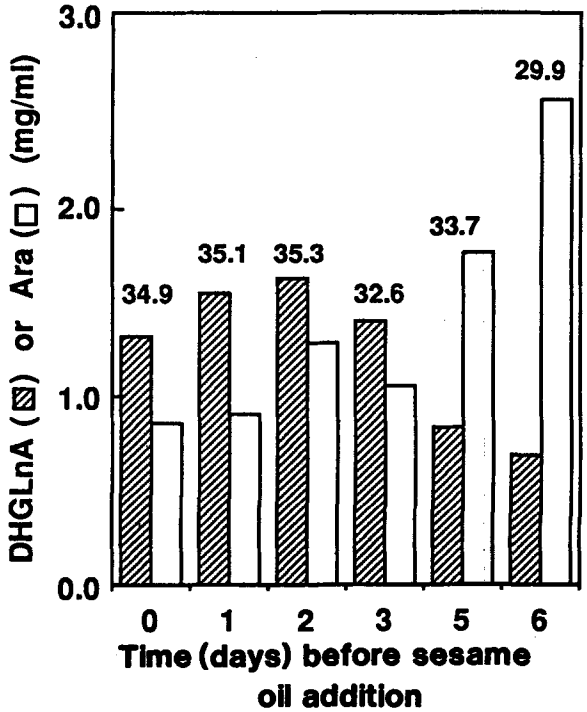

FIG. 3. Effect of the time of sesame oil addition on the production of DHGLnA. *M. alpina* 1S-4 was grown in medium B for 8 days under the conditions given under Materials and Methods except for the time of addition of the oil (2%), as indicated. The value of the top of each bar is the mycelial mass (mg/ml) after cultivation.

exactly to those in the case of authentic methyl dihomo-γ-linolenate. The mass spectrum of the isolated methyl ester showed a molecular ion peak at *m/z* 320 (relative intensity, 24%) and intense fragment ion peaks at *m/z* 222, 150, 135, 121, 93, 79, 67, 55, 41 and 28 (relative intensities, 23, 43, 22, 25, 60, 95, 100, 55, 54 and 41%, respectively). The ^1H NMR spectrum in $CDCl_3$ with tetramethylsilane as an internal standard showed signals at 0.90 (t, 3H, CH_3), 1.33 (m, 14H, CH_2), 2.06 (m, 4H, CH_2), 2.31 (t, 2H, CH_2), 2.81 (m, 4H, CH_2), 3.67 (s, 3H, CH_3) and 5.36 ppm (m, 6H, C=C). These data corresponded well to those for authentic methyl dihomo-γ-linolenate.

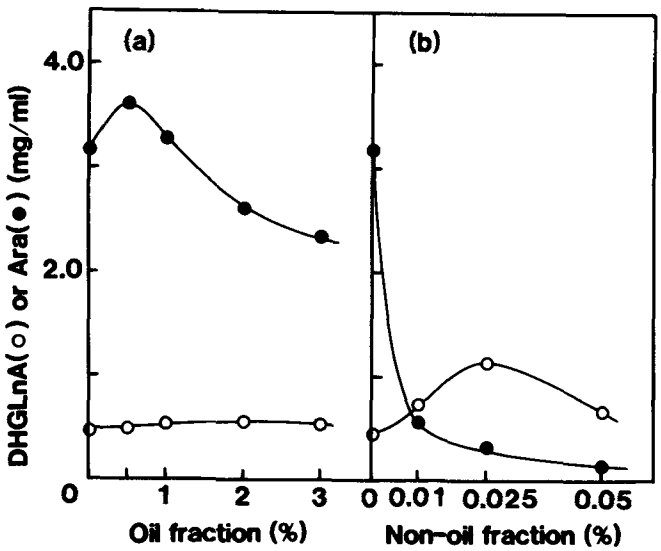

FIG. 4. Effects of the oil and non-oil fractions of sesame oil on the production of DHGLnA and Ara. *M. alpina* 1S-4 was grown in medium B containing each fraction for 8 days under the conditions given under Materials and Methods. The concentrations of the oil and non-oil fractions in (a) and (b) respectively, were varied as indicated.

DISCUSSION

We previously reported that *M. alpina* 1S-4, which was used in the present study, is a potent producer of Ara (7, 9). The amount of Ara produced by the fungus reached 3 to 4 g/l of culture broth under the optimal culture conditions (9). The fungus also produced a small amount of DHGLnA under the same conditions, but the mycelial DHGLnA/Ara ratio was only about 0.2 (9). The biosynthetic route responsible for the production of Ara from common C-18 fatty acids, such as oleic acid and linoleic acid, in this fungus has been suggested to be the n-6 route, which involves three successive reactions, Δ6-desaturation of linoleic acid to γ-linolenic acid, elongation of the γ-linolenic acid to DHGLnA and then Δ5-desaturation of the DHGLnA to Ara, based on the observation that all the intermediates in this route were detected in the mycelia (3, 7, 9). Because DHGLnA is a precursor of Ara in the n-6 route, the Ara found in the mycelia was produced via DHGLnA, suggesting that the fungus potentially has the ability to produce a large amount of DHGLnA.

The interesting finding reported here, that the addition of sesame oil caused an increase in DHGLnA accumulation accompanied by a decrease in the mycelial Ara content, may be useful for controlling the DHGLnA/Ara ratio in mycelia. Probably, the sesame

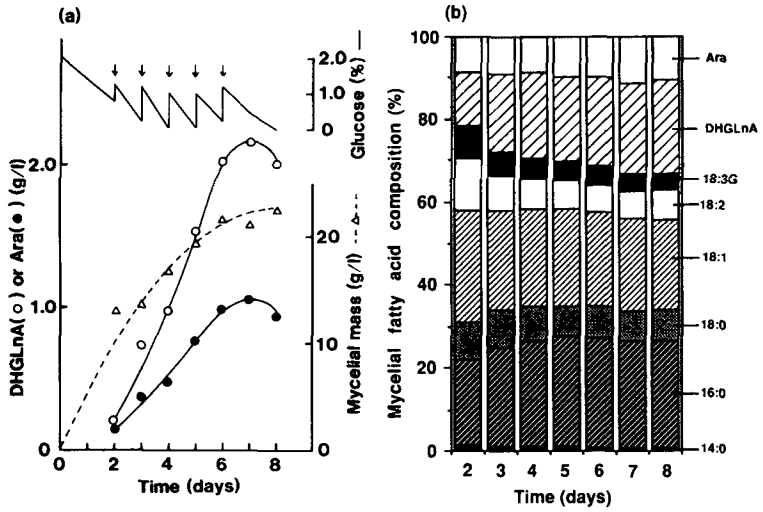

FIG. 5. Production of DHGLnA by *M. alpina* 1S-4 under optimal culture conditions. *M. alpina* 1S-4 was cultivated in a 50-l bench-scale fermentor (Mituwa Rikagaku Kogyo KMJ-50MGU-FP, Osaka) containing 30 l of medium A supplemented with 6.7 g of the non-oil fraction of sesame oil. Cultivation was carried out at 28 C with aeration at one volume/volume/min and agitation at 200 rpm. On the first and second day, the non-oil fraction (3.4 g each) was further added. Glucose was added to the medium at the time indicated by the arrow in Fig. 5a. Changes in the mycelial fatty acid composition during growth are shown in Fig. 5b. The following abbreviations are used for fatty acids: 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3G, γ-linolenic acid.

oil added to the medium specifically repressed the conversion of DHGLnA to Ara, i.e., Δ5-desaturation, which caused the reversal of the mycelial DHGLnA/Ara ratio. As a result, *M. alpina* 1S-4 accumulated a large amount of DHGLnA in its mycelia, as reported here.

The data in Figure 5 indicate that the effective component(s) responsible for this unique phenomenon was separated from sesame oil as a minor non-oil fraction. Because this non-oil fraction can be prepared easily and effectively causes a specific change in the DHGLnA/Ara ratio, it is more advantageous for practical purposes than the oil itself. The isolation and identification of the effective factor(s) are now in progress in our laboratory.

In conclusion, the results reported here indicate that the mycelia of *M. alpina* 1S-4 are very promising not only as a practical source for Ara but also for DHGLnA.

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