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Bioassay-Guided Purification and Identification of PPARα/γ Agonists from *Chlorella sorokiniana*

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This study isolated agonists of peroxisome proliferator activated receptors (PPARs) from the green algae *Chlorella sorokiniana*, using a bioassay-guided purification strategy. PPARs are widely recognized as the molecular drug targets for many diseases including hyperglycemia, diabetes, obesity and cancer. Two independent bioassays were developed. The first is the scintillation proximity assay, a ligand binding assay. The other is the cell-based transcriptional activation assay which uses the Dual-Luciferase® reporter system as the reporter gene under the control of the PPAR response element. Using these two assays, a PPARγ-active fraction, CE 3-3, was obtained from *C. sorokiniana* extracts, which was also able to activate PPARα-mediated gene expression.

To elucidate the active ingredients in the CE 3-3 fraction, GC-MS analysis was employed. The results showed that the CE 3-3 fraction consisted of at least ten fatty acids (FAs). The bioactivities of several of the individual FAs were evaluated for their PPARγ activity and the results showed that linolenic acid and linoleic acid were the most potent FAs tested. Our studies indicate that *Chlorella sorokiniana* could have potential health benefits through the dual activation of PPARα/γ via its unique FA constituents.

Keywords: peroxisome proliferator-activated receptors gamma (PPARγ); peroxisome proliferator-activated receptors alpha (PPARα); *C. sorokiniana*; scintillation proximity assay (SPA); transcriptional activation (TA) assay.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors belonging to the nuclear receptor (NR) superfamily. PPARs are involved in mediating lipid metabolism and glucose homeostasis (Mangelsdorf *et al.*, 1995). Three subtypes: PPARα, PPARδ and PPARγ have been identified and well characterized with respect to their biological functions (Lee *et al.*, 2003). PPARs form a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) to effect downstream gene expression. Upon activation by endogenous or synthetic ligands, PPARs regulate the expression of target genes involved in a variety of important physiological pathways such as lipid metabolism, insulin sensitivity, cell differentiation, inflammation, proliferation and apoptosis (Willson *et al.*, 2000; Moraes *et al.*, 2006; Zoete *et al.*, 2007). The insulin-sensitizing and lipid-lowering effects of glitazones and fibrates are mediated by PPARγ and PPARα, respectively. Several synthetic agonists of PPARα and PPARγ have been widely used for treatment of dyslipidemia and diabetes (Pershadsingh, 2004; Cheng and Mukherjee, 2005; Hogan *et al.*, 2003; Narayan *et al.*, 2003).

Many herbal or natural products are rich sources of PPAR agonists (Li *et al.*, 2005; Anandharajan, 2006; Li *et al.*, 2006; Huang *et al.*, 2006; Rau *et al.*, 2006). Similar to these medicinal plants, the health benefits of many algae have also been documented (Kittaka *et al.*, 2002; Singh *et al.*, 2005). Experiments with diabetic animals have illustrated that the extracts of certain algae could reduce blood glucose levels and minimize diabetic complications through various modes of action (Lamela *et al.*, 1989; Shibata *et al.*, 2003; Jin *et al.*, 2004). The green alga *Chlorella* has been endowed with a variety of medicinal properties (Noda *et al.*, 1996; Tanaka *et al.*, 1997; Guzman *et al.*, 2001). For example, a few species of *Chlorella* have well documented antidiabetic properties (Rodriguez *et al.*, 1971; Lee *et al.*, 1977). Recently, investigations have found that *Chlorella* increases insulin sensitivity in streptozotocin (STZ) induced diabetic mice by influencing glucose uptake in the liver and muscle (Jong and Mei, 2005; Cherng and Shih, 2006).

This study evaluated whether the antidiabetic properties of *Chlorella* could be mediated through PPAR activation, similar to the results obtained with many plant-derived products. To investigate this possibility, *Chlorella sorokiniana*, a single-cell, thermophilic green

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alga was chosen in the present study (Lin and Huang, 2002). Since PPARα/γ are the therapeutic targets for hyperglyceridemia and insulin resistance, a bioassay guided purification strategy was used to isolate, identify and characterize the activators for PPARα/γ.

MATERIALS AND METHODS

***C. sorokiniana* extract.** Crude *C. sorokiniana* W87-10 extract was provided by International Chlorella Co. Ltd, Chang-Hua County, Taiwan, R.O.C.

PPARγ ligand binding assay. To determine the binding capacity of *C. sorokiniana* fractions towards the PPAR ligand binding domain (LBD), a scintillation proximity assay (SPA) was used for PPARγ and a charcoal binding assay was used for PPARα as described previously (Mahindroo *et al.*, 2005; Lu *et al.*, 2006; Mahindroo *et al.*, 2006a, 2006b). The principle of SPA is schematically depicted in Fig. 1. Briefly, the ligand binding domains of two hPPARs (γ and α) were expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins. Recombinant proteins were then isolated by affinity purification using glutathione-sepharose following the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The SPA experiment was conducted using 96-well microtiter plates (Catalog number 6005290, Packard Instrument, Meriden, CT) with a 100 µL reaction volume. The assay buffer contained 10 mM Tris-Cl, pH 7.2, 1 mM EDTA, 10% (w/v) glycerol, 10 mM sodium molybdate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/mL benzamidine and 0.1% skim milk powder. Protein A-yttrium silicate SPA beads (catalog number RPN143, Amersham Biosciences, Piscataway, NJ) was suspended in 50 mL of the above assay buffer except that skim milk powder was replaced with 0.01% sodium azide.

The recombinant GST-hPPARγ^{LBD} preparations were used at a final concentration of approximately 5 nM. Goat anti-GST antibodies (Catalog number 27-4577-01, Amersham Biosciences, Piscataway, NJ) were used at a dilution of 1:2000. The test fractions were then dissolved in DMSO to obtain a final concentration of 5 µg/mL. The radiolabeled PPAR ligand [^{3}H] rosiglitazone (60 Ci/mmol) (American Radiolabeled Chemicals, St Louis, MO, USA) was diluted 425-fold in ethanol and used at a final concentration of 7.8 nM. Then, GST-PPARγ^{LBD}, goat anti-GST antibodies, well-suspended protein A-yttrium silicate SPA beads and the test fractions were sequentially added (20 µL each) to the microtiter plate. Finally, 20 µL of the diluted [^{3}H] rosiglitazone solution was added to each well. The plate was then incubated with gentle shaking at 4 °C for 24 h. Following which, the level of radioactivity was quantified using a Topcount® Microplate Scintillation and Luminescence Counter (Packard Instrument Co., Inc, USA).

PPARα charcoal binding assay. To study the binding activity of *C. sorokiniana* extracts to PPARα^{LBD}, a charcoal binding assay was performed in TEGM buffer (10 mM Tris, pH 7.2, 1 mM EDTA, 10% glycerol, 7 µL/100 mL of β-mercaptoethanol, 10 mM sodium molybdate, 1 mM dithiothreitol, 2 µg/mL benzamide and 0.5 mM phenylmethylsulfonyl fluoride) containing 2.5 nM [^{3}H] L-783 483 (79 µCi/mmol), with or without the test samples. Assay components were incubated at 4 °C for 24 h in a final volume of 300 µL. The unbound ligand was removed by incubation on ice with 200 µL of dextran/gelatin-coated charcoal for 10 min. After centrifugation at 3000 rpm for 10 min at 4 °C, 200 µL of the supernatant fraction was counted in a TRI-CARB 2100TR® liquid scintillation analyser. The [^{3}H] L-783 483 was synthesized in-house by the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan (Mahindroo *et al.*, 2005, 2006a, 2006b; Lu *et al.*, 2006).

Cell culture and PPAR transcriptional activation (TA) assay. Huh-7 cells were seeded (5×10^4 cells/well) in 24-well cell culture plates in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL penicillin G and 100 mg/mL streptomycin sulfate and 0.25 µg/mL amphotericin B at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h, transfections were performed using Fugene 6® transfection reagent (Roche, Penzberg, Germany) according to the instructions of the manufacturer. Specifically, a transfection mixture was prepared by adding 0.5 µL of Fugene 6, 0.05 µg of pGAL4-PPARγ^{LBD} plasmid, 0.14 µg of pG5-TK-Luc reporter and 0.25 ng of a pRL-SV40 renilla luciferase plasmid as the transfection internal control to each well. The cells were then incubated in the transfection mixture at 37 °C overnight in 5% CO_2. The cells were then incubated for 1 day in fresh high glucose Dulbecco's modified Eagle's medium with increasing concentrations of a test sample. Since the test samples were dissolved in DMSO, control cells were incubated in a DMSO solution of equivalent concentrations. Figure 2 schematically depicts the principle of TA.

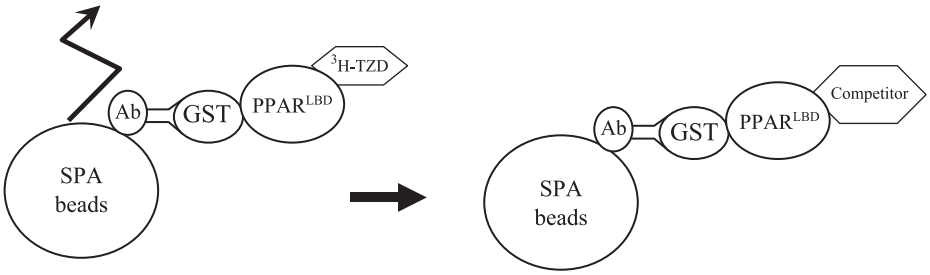

Figure 1. Scintillation proximity assay (SPA). SPA beads are impregnated with scintillate and coated with anti-GST Ab. When GST-PPAR^{LBD} is attached to the bead through antibody binding and a radio ligand (^{3}H-TZD) is bound, they are sufficiently close to allow the emission from the tritium to be absorbed by the scintillate, resulting in the emission of light. A reduction in the radioactivity is an indication that the test compound has bound to PPAR ligand binding domain, competing out the ^{3}H-TZD.

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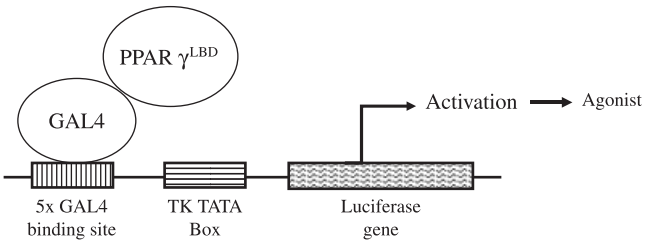

Figure 2. Transcriptional activation assay (TA) for PPARγ ligand identification. For transactivation assay, Huh-7 cells were transfected with the following plasmids: the GAL4-PPAR plasmid, pG5-TK-luc and pSV40-Ren. The GAL4-PPAR chimera genes were generated by individually fusing the ligand binding domains of PPARs (α and γ) with the GAL4-DNA binding domain. Plasmid pG5-TK-luc was employed as the reporter plasmid containing five repeats of the GAL4 response element, upstream of a minimal thymidine kinase promoter (TK TATA box) followed by the firefly luciferase gene. In addition, the renilla luciferase gene encoded in pSV40-Ren under the control of the SV40 promoter was used as an internal control for transfection efficiency. The potency of a compound is directly proportional to the normalized firefly luciferase activity.

Then 24 h after transfection, the cells were treated with fractions obtained from *C. sorokiniana* extracts for another 24 h and then harvested. Cell lysates were produced using Passive® Lysis Buffer (Promega, Madison, WI) following the manufacturer's instructions. Luciferase activity in cell extracts was determined using the Dual-Luciferase® Reporter Assay kit (Promega, Madison, WI) and counted in a SIRIUS-0 luminometer (Berthold detection systems, Pforzheim, Germany). Briefly, 50 µL of Luciferase Assay Reagent II (LARII) was added into a vial containing 5 µL of cell lysate and then the firefly luciferase activity of the mixture was measured. Fifty µL of Stop & Glo® Reagent was then added into the vial and the renilla luciferase activity of the mixture was measured. The highest DMSO concentration used in the assay was 0.1%, which was found to have no effect on transactivation activity. In all these results, activation by PPAR ligand rosiglitazone (2 µM) was used as a positive control.

Preadipocyte differentiation assay. Confluent preadipocyte 3T3-L1 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 100 units/mL penicillin G, 10 µg/mL streptomycin sulfate and 150 nM insulin, in the absence or presence of increasing concentrations of test fractions at 37 °C in 5% CO_2 for 3 days. The cells were kept under these conditions until the appearance of adipocytes (about 9 days) with the medium changed every 2 days. The cells that differentiated into adipocytes were stained with Oil Red-O (Sigma) as previously described (Trouba *et al.*, 2000). Briefly, cells were fixed in 10% formalin for at least 1 h and stained by immersion in Oil Red-O for 2 h then exhaustively rinsed with water. Samples were then dried by incubation at 32 °C.

Nuclear magnetic resonance (NMR) analysis. ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Varian Mercury-300 spectrometer operated at 300 MHz and 75 MHz, respectively, with chemical shift in parts per million (ppm) from TMS as an internal standard. Flash column chromatography was performed using silica gel (Merck Kieselgel 60, No. 9385, 230–400 mesh ASTM). Isolated fractions were analysed by thin layer chromatography (TLC) using Merck 60 F_{254} silica gel glass backed plates (5 × 10 cm). Zones were detected visually under ultraviolet irradiation (254 and 360 nm) or by spraying with 10% aqueous sulfuric acid followed by heating at 110 °C for a few minutes. All solvents were dried according to standard procedures and the reactions were carried out under an atmosphere of dry nitrogen.

Isolation and purification of *C. sorokiniana* extract. One hundred mL of *C. sorokiniana* crude extract was diluted with water to twice the original volume and extracted using EtOAc (200 mL × 4). The organic solvent in the upper layer was then evaporated under reduced pressure to obtain the EtOAc extract. The EtOAc extract was then fractionated by silica gel column chromatography and eluted with acetone gradient (30%–100%) in *n*-hexane to obtain various fractions. The active fraction obtained from these fractions was further purified using preparative TLC and developed with 33% EtOAc in *n*-hexane to obtain further sub-fractions.

GC-MS analysis. The CE 3-3 fraction (8.2 mg) was dissolved in dichloromethane (0.6 mL) and mixed with 20% boron trifluoride etherate in methanol (4 mL) under nitrogen gas. The solution was then sealed and stirred at 100 °C for 5.0 min. After cooling, the solution was neutralized by the addition of saturated aqueous sodium chloride (10 mL) and extracted with *n*-hexane (2 mL). The organic layer was dried with $MgSO_4$ and evaporated under reduced pressure, which gave rise to a methyl ester product of the CE 3-3 fraction as a yellow colored oil (CE 3-3M). The composition of FA methyl esters in CE 3-3M was analysed using a Hewlett-Packard 6890 gas chromatography system coupled with a HP 5973 mass selective detector, a HP 7673 automatic liquid sampler and an Agilent DB-5MS column (30 m × 250 µm; film thickness, 0.25 µm). Helium was used as the carrier gas at a flow rate of 1 mL/min. The inlet temperature was maintained at 250 °C. The sample (1 µL) was injected with a 1:50 split ratio. The initial oven temperature was maintained at 120 °C for 3 min and programmed to increase to 180 °C at a rate of 10 °C/min (held for 1 min) then to 210 °C at a rate of 2 °C/min (held for 5 min), with a total run time of 30 min. Mass spectra were recorded over a 50–550 amu range, with 70 eV ionization energy and 230 °C MS source temperature. Data collection and integration were performed using HP Chem Station software. The quantity of compounds was determined by integrating the peak area of the total ions current spectrograms and transformed into percentage. The individual components were identified by comparison of their retention times with commercial standard compounds and NIST (National Institute of Standards and Technology) MS Search program.

Data analysis. Data for PPARγ ligand binding activity and PPARα charcoal binding activity were expressed as % inhibition by [^{3}H] rosiglitazone and % competition by [^{3}H] L-783 483, respectively. The transactivation results were expressed as the ratio of firefly luciferase signal over the renilla luciferase signal. IC_{50} values were determined using dose response curves with 6 (PPARα

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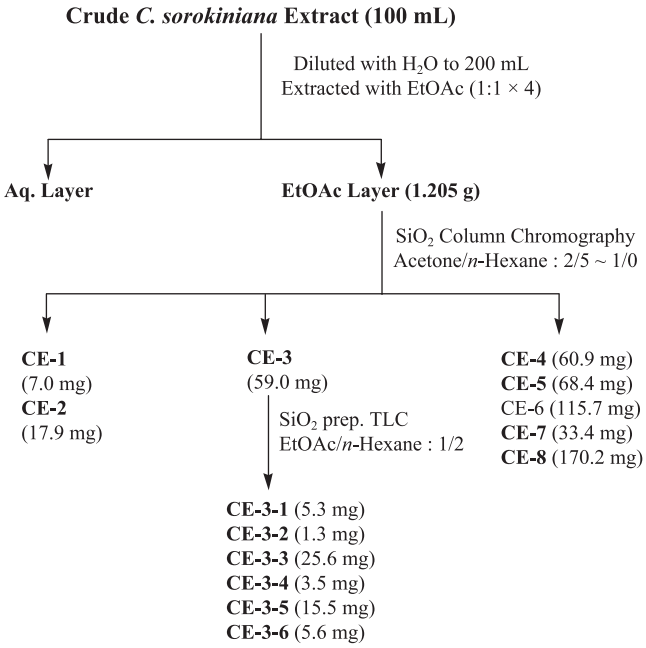
charcoal binding assay), 8 (PPARγ ligand binding assay) and 12 (PPARα transactivation assay) data points in triplicates. Data were analysed using Sigma plot 8 software.

RESULTS AND DISCUSSION

The crude lysate of *C. sorokiniana* was initially extracted with EtOAc. The EtOAc extract (1.2 g) was subsequently fractionated by chromatography on a silica gel column to give rise to eight fractions (CE 1, 7.0 mg; CE 2, 17.9 mg; CE 3, 59.0 mg; CE 4, 60.9 mg; CE 5, 68.4 mg; CE 6, 115.7 mg; CE 7, 33.4 mg and CE 8, 170.2 mg) (Scheme 1). When these eight fractions from the EtOAc extract were examined for their ligand binding activity to PPARγ using SPA, 5 μg/mL of fractions CE 2, CE 3, CE 4 and CE 5, showed more than 50% displacement of [^{3}H] rosiglitazone binding to PPARγ^{LBD} (Fig. 3a). Fraction CE 3 showed the most potent binding activity of all the fractions with >95% displacement of [^{3}H] rosiglitazone bound to PPARγ^{LBD} in the SPA.

The CE 3 fraction was further extracted with EtOAc/*n*-hexane (1:2 v/v) and separated by preparative TLC to obtain the sub-fractions CE 3-1 to CE 3-6 (CE 3-1, 5.3 mg; CE 3-2, 1.3 mg; CE 3-3, 25.6 mg; CE 3-4, 3.5 mg; CE 3-5, 15.5 mg; CE 3-6, 5.6 mg). When these six sub-fractions were analysed by SPA, fraction CE 3-3 showed the highest binding activity toward PPARγ with 99.8% displacement of [^{3}H] rosiglitazone (Fig. 3b). The IC_{50} values of fractions CE 3 and CE 3-3 for PPARγ^{LBD}, as determined by the dose-response curves, were found to be 2.7 μg/mL and 1.6 μg/mL, respectively (Fig. 3c, d). To evaluate whether the bound ligands also activate PPARγ, transactivation assays (TA) were used for



Scheme 1. The isolation and purification of *C. sorokiniana* extract.

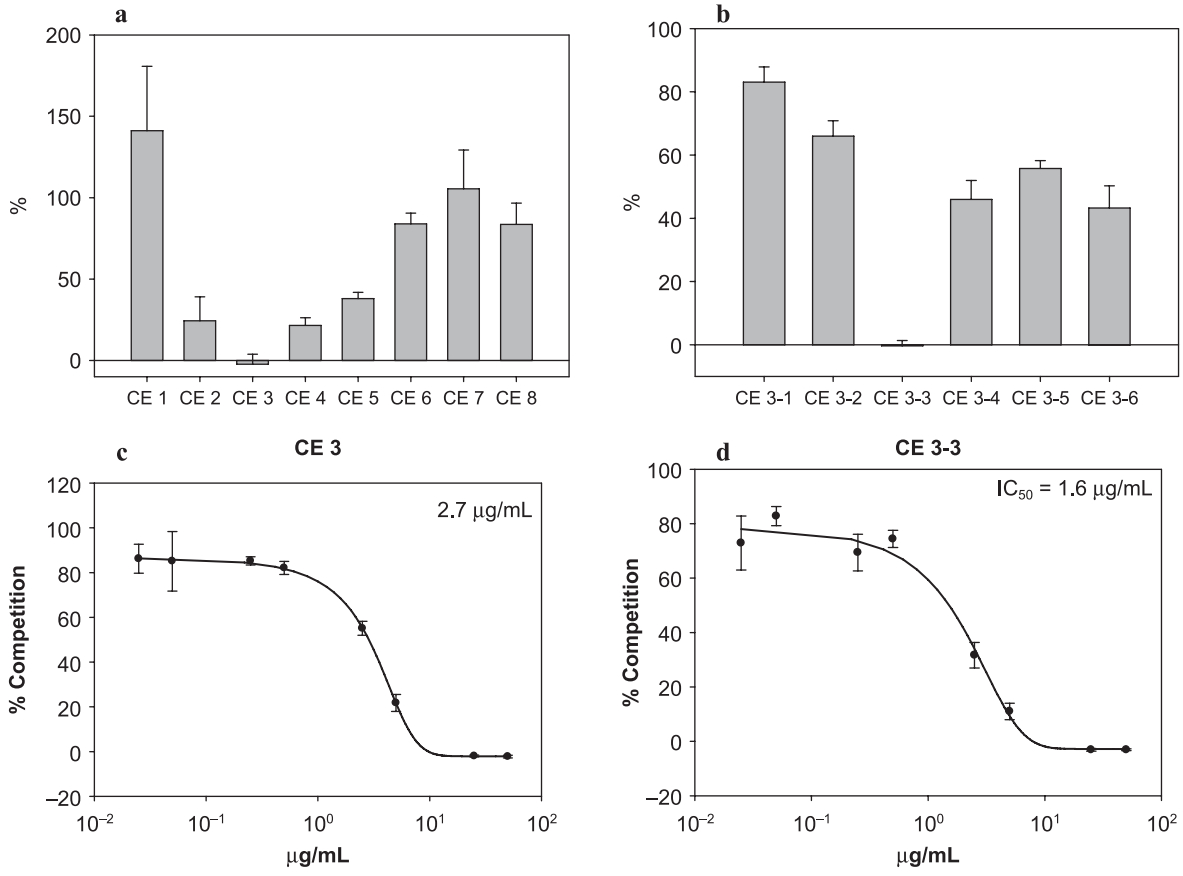


Figure 3. PPAR ligand-binding activity (SPA) of *C. sorokiniana* fractions. (a) Fraction CE 1 to CE 8. (b) Fraction CE 3-1 to CE 3-6. (c) The IC_{50} values of CE 3 and (d) CE 3-3 for PPARγ^{LBD} as determined by SPA analysis. All fractions were tested at a concentration of 5 μg/mL.

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determining the activity of PPARγ ligands in a cell-based environment. A facile TA assay employing the GAL4-PPAR chimeric receptor and a reporter gene construct was utilized in this study (Grun and Blumberg, 2003). Fraction CE 3 showed 55.6% of maximum PPARγ activation among the eight fractions (CE 1–CE 8) (Fig. 4a, b) (activation of PPARγ by rosiglitazone at 2 μM was defined as 100% of positive control). Similarly, fraction CE 3-3 achieved 63.4% of the positive control making it the most active of the six subfractions derived from the CE 3 fraction. Thus, using an *in vitro* ligand binding assay and a cell-based TA assay, the most active fraction CE 3-3 was identified.

Since fractions CE 3 and CE 3-3 showed PPARγ binding and TA activity, these two active fractions were further evaluated for their binding activity to PPARα^{LBD} using a charcoal binding assay. The IC_{50} values for CE 3 and CE 3-3 to displace the binding of [^{3}H] L-783 483 to PPARα^{LBD} were found to be 5.0 and 2.3 μg/mL, respectively (Fig. 5a, b). These results correlated well with the EC_{50} values obtained in a cell-based PPARα TA assay using the GAL4-PPARα chimeric reporter system. The EC_{50} of CE 3 and CE 3-3 to PPARα were 6.0 and 2.0 μg/mL, respectively (Fig. 5c, d). Thus, the CE 3-3 fraction was found to contain activators for both PPARγ and PPARα.

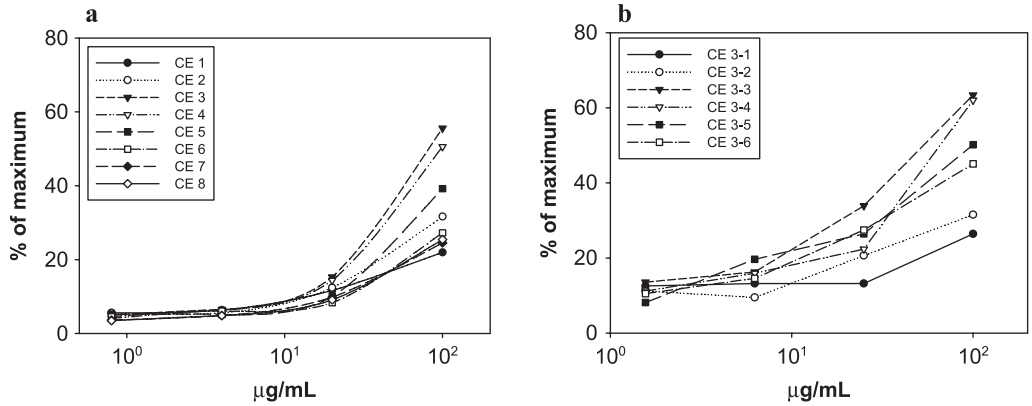

Figure 4. PPARγ transcriptional activation (TA) assay of *C. sorokiniana* fractions. (a) Fractions CE 1 to CE 8, tested at 0.8, 4, 20 and 100 μg/mL and (b) CE 3-1 to CE 3-6, tested at 1.6, 6.3, 25 and 100 μg/mL. The transcriptional effect of rosiglitazone at 2 μM was defined as the positive control.

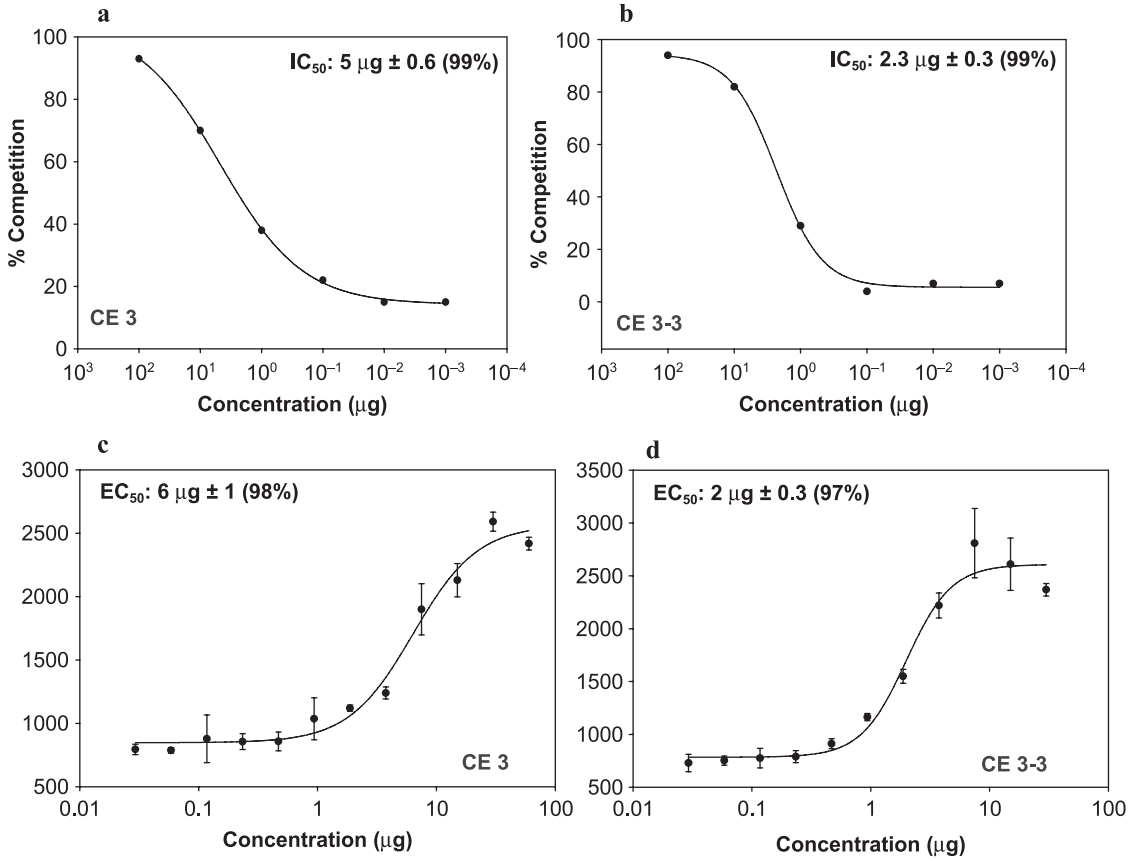

Figure 5. PPARα activities of various fractions of *C. sorokiniana*. Binding analysis of fraction CE 3 (a) and fraction CE 3-3 (b) to PPARα receptor, obtained by charcoal binding assay. EC_{50} values of the CE 3 (c) and CE 3-3 fraction (d), were obtained via TA assay using a GAL4-PPARα chimera and luciferase reporter system.

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Adipocyte differentiation is a key developmental process with important roles in energy storage and operates under tight hormonal control (Rosen, 2005). The formation of such new adipocytes from progenitor cells or resident preadipocytes requires the activation of PPARγ. It has also been noted that non-adipogenic cells could be effectively converted into mature adipocytes upon forced expression of PPAR (Tontonoz *et al.*, 1994). In our analysis, upon exposure to various fractions of the *C. sorokiniana* extract, confluent 3T3-L1 preadipocytes underwent differentiation in 6–8 days. The adipogenic differentiation was determined by the gradual accumulation of cytoplasmic fat droplets as observed by light microscopy as well as by staining of lipid droplets using Oil Red O stain (data not shown). Among the various treatments, maximum adipogenic differentiation in 3T3-L1 cells was achieved by treatment of cells with 2 μM rosiglitazone, while fractions CE 3 and CE 3-3 showed moderate adipogenic differentiation activity of 3T3-L1 preadipocytes (Fig. 6E, F). The adipogenic capacity of the crude and EtOAc extracts of *C. sorokiniana* were comparable to that of the active fractions, while the DMSO control showed no activity (Fig. 6C, D, A). The adipogenic capacity of *C. sorokiniana* extracts and the active fractions could be due to their potential PPARγ activity as shown by their PPARγ binding and TA activity. These results corroborate with the fact that drugs with antidiabetic indications have been reported to promote adipocyte differentiation of 3T3-L1 preadipocytes (Sekiya *et al.*, 2004; Xu *et al.*, 2006). Subsequently, the CE 3-3 fraction was chosen for further analysis of its chemical composition.

The CE 3-3 fraction appeared as an amorphous solid, which was visualized as a light-brown spot with a comet tail on TLC. The ^{1}H- and ^{13}C-NMR spectra of

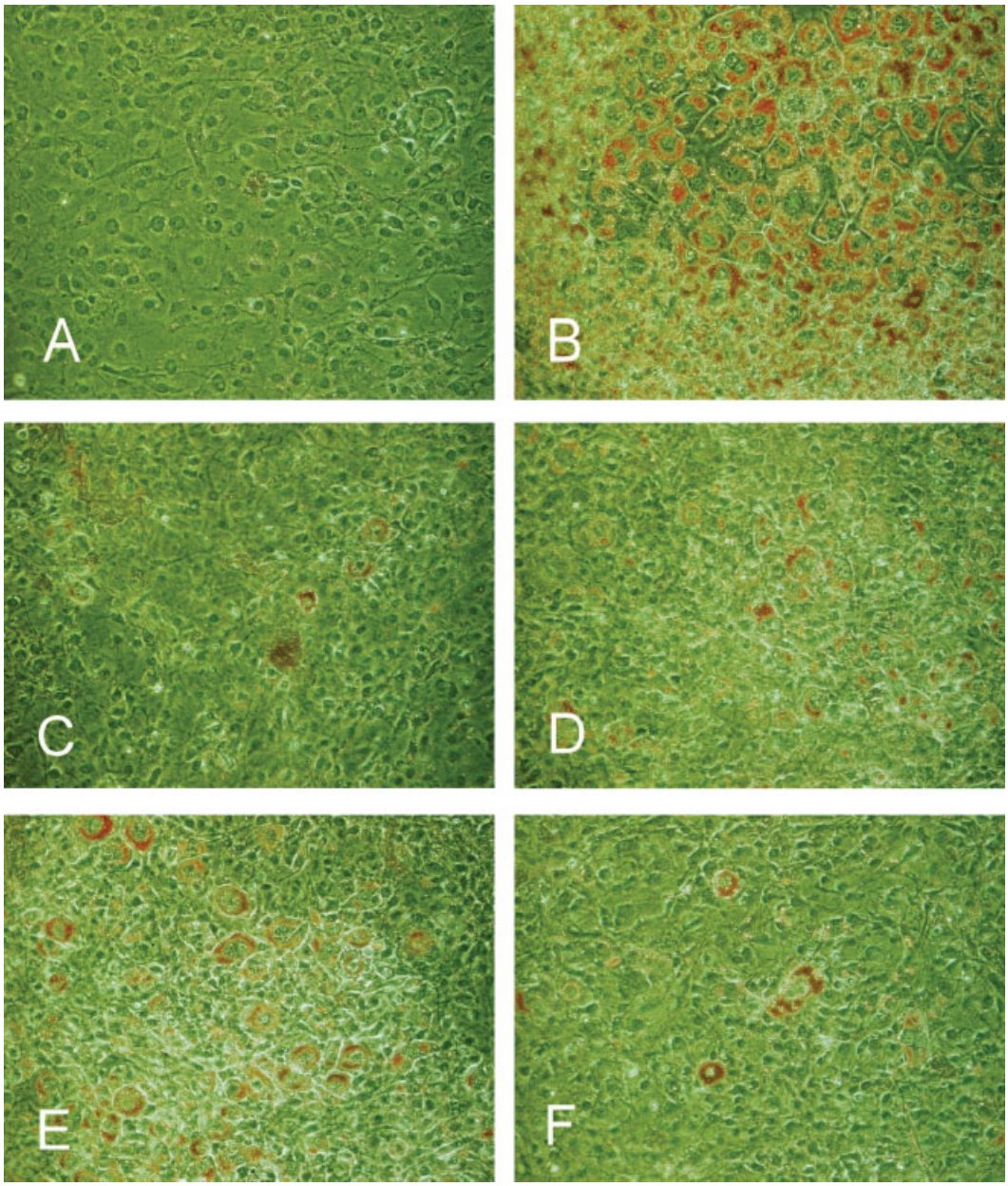

Figure 6. 3T3-L1 preadipocyte differentiation assay. 3T3-L1 preadipocytes were treated with (A) negative control, (B) 2 μM Rosiglitazone, (C) crude *C. sorokiniana* extract at 100 μg/mL, (D) EtOAc extract of *C. sorokiniana* at 100 μg/mL; (E) fraction CE 3 at 100 μg/mL and (F) fraction CE 3-3 at 100 μg/mL.

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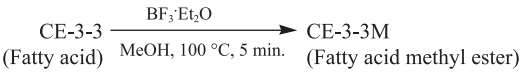
CE 3-3 showed typical signals of FAs. Since gas chromatography-mass spectrometry (GC-MS) is considered ideal for the analysis of small volatile lipophilic molecules, fraction CE 3-3 was esterified with boron trifluoride etherate ($BF_3{\cdot}Et_2O$) in methanol to be converted into the methyl ester derivative, CE 3-3M (7.0 mg) (Scheme 2). Ten components (Table 1) were observed from the GC chromatogram of CE 3-3M (Fig. 7). Seven of these components were identified by a GC-MS library search using the NIST MS Search program and by comparison with standard, commercially available reference compounds. These components were identified as palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), myristic acid ($C_{14:0}$), palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), hexadecenoic acid ($C_{16:1}$), octadecenoic acid ($C_{18:1}$), hexadecadienoic acid ($C_{16:2}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$). The relative percentage and retention time of all these FAs obtained from CE 3-3M are summarized in Table 1.

Similar to the presence of FAs in the active fraction CE 3-3 of *C. sorokiniana*, other *Chlorella* species such as *C. pyrenoidosa* and *C. vulgaris* have also been reported to be rich in polyunsaturated fatty acids (PUFAs) that are endogenous ligands for PPARs (Otles and Pire, 2001). It is well known that structurally diverse groups of FAs and their metabolites can directly and indirectly modulate signaling pathways at multiple levels by binding to PPARs (Wolfrum *et al.*, 2001). The bioactivities of each individual fatty acid identified in CE 3-3M including palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), myristic acid ($C_{14:0}$), palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were analysed by SPA and TA using PPARγ^{LBD} (Table 2). In the SPA analysis, with the exception of palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$), all the other FAs showed PPARγ binding activity. The IC_{50} values for palmitoleic acid, oleic acid, linoleic acid, linolenic acid and myristic acid were determined to be 1.6, 2.3, 0.9, 2.0 and 1.4 μg/mL, respectively.

Similarly, all these pure fatty acids were shown to be active in the PPARγ TA assay. The level of activation of PPAR-mediated reporter gene expression was 40%, 22%, 33%, 24%, 4%, 9% and 4% of the positive control when the cells were treated with 100 μg/mL of linolenic acid, myristic acid, palmitoleic acid, oleic acid, stearic acid, linoleic acid and palmitic acid, respectively. Of these, linolenic acid ($C_{18:3}$) showed the highest TA activity, which was 40% of the positive control. The essential FAs such as linoleic acid and linolenic acid observed in the CE 3-3M fraction of *C. sorokiniana* have been reported to be PPARγ ligands with binding affinities in the μM range (Kliewer *et al.*, 1997); close to their physiological concentrations (Jungling and Kammermeier, 1988). Analysis of the bioactivities of



Scheme 2. Esterification of CE 3-3.

Table 1. The constituents and contents of fatty acids from CE 3-3

No.	Compound name[a]		Retention time (min)	Relative %
1	Myristic acid [tetradecanoic acid]	C14:0	11.9	1.7
2	Hexadecadienoic acid[b]	C16:2	15.6	4.3
3	Hexadecenoic acid[b]	C16:1	15.9	6.0
4	Palmitoleic acid [(*Z*)-9-hexadecenoic acid]	C16:1	15.9	10.1
5	Palmitic acid [hexadecanoic acid]	C16:0	16.6	43.6
6	Linoleic acid [(*Z*),(*Z*)-9-12-octadecadienoic acid]	C18:2	21.5	8.8
7	Linolenic acid [(*Z*),(*Z*),(*Z*)-9-,12-,15-octadecadienoic acid]	C18:3	21.7	4.0[c]
8	Oleic acid [(*Z*)-9-octadecenoic acid]	C18:1	21.8	10.0[c]
9	Octadecenoic acid[b]	C18:1	22.0	5.9
10	Stearic acid [octadecanoic acid]	C18:0	22.7	2.6

[a] By comparison of the mass spectrum with those of the computer mass library and the retention time of pure standard compounds.
[b] Compared only by the mass spectrum of the computer mass library, since the pure standard compounds are not commercial available.
[c] Shoulder-like peak, peak area is estimated by peak height.

Table 2. Bioactivities of pure fatty acids obtained from CE 3-3 against PPARγ^{LBD}

Name		SPA[a] IC_{50} (μg/mL)	TA[b] % of maximum	Relative %
Palmitic acid	C16:0	NA[c]	4	43.6
Palmitoleic acid	C16:1	1.6	33	10.1
Oleic acid	C18:1	2.3	24	~10.0
Linoleic acid	C18:2	0.9	9	8.8
Linolenic acid	C18:3	2.0	40	~4.0
Stearic acid	C18:0	NA[c]	4	2.6
Myristic acid	C14:0	1.4	22	1.7
CE 3-3		1.6	33	100

[a] Concentration of the test compounds required to displace 50% of tritiated ligand.
[b] Tested at 100 μg/mL.
[c] No activity.

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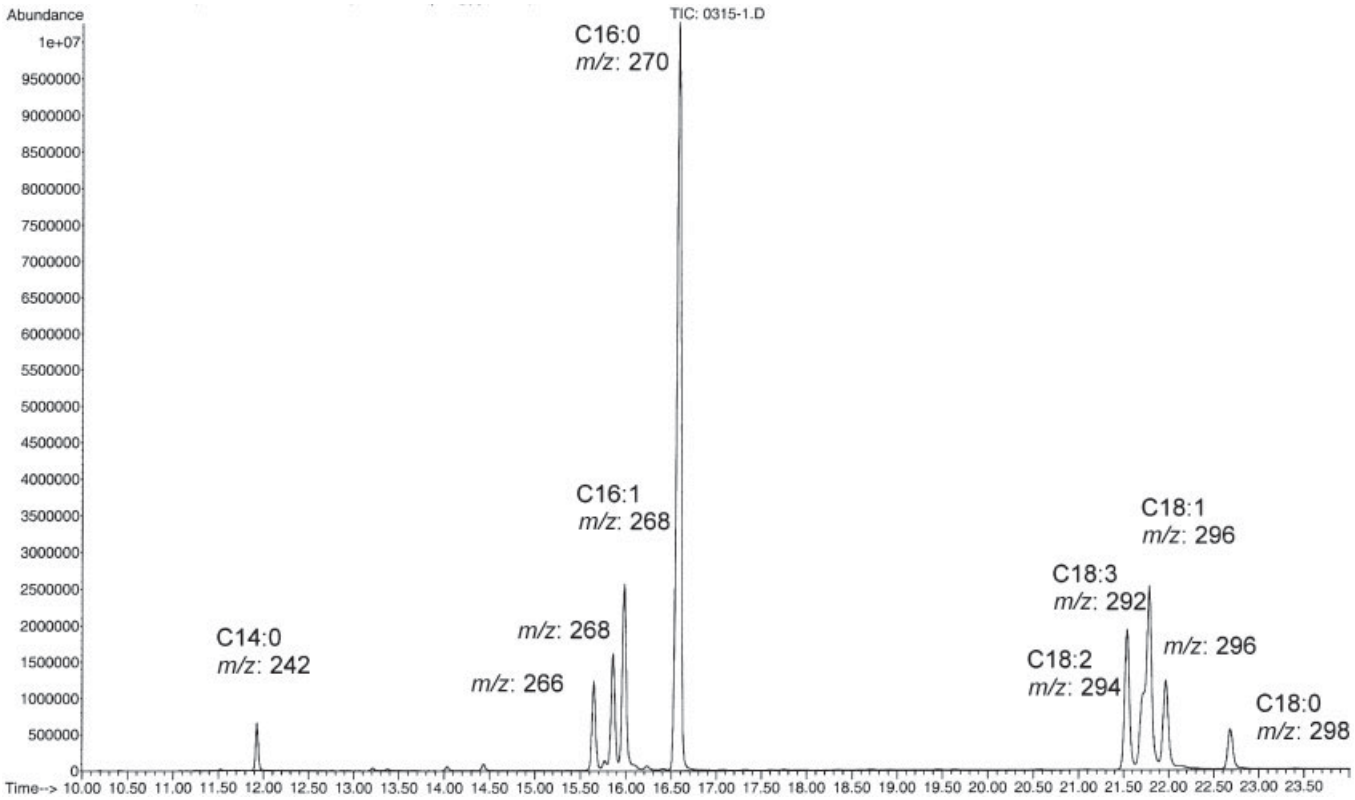

Figure 7. GC chromatogram of CE 3-3M, the methyl ester derivative of the active fraction CE 3-3.

these seven FAs suggests that linolenic acid and linoleic acid were very potent in both the TA and SPA assays. A recent report suggests that a diet balanced with both these two essential fatty acids is crucial for the prevention of many chronic diseases (Simopoulos, 2006).

Considering the importance of glucose and lipid homeostasis in diabetic patients who may suffer from both hyperglycemia and dyslipidemia, it is desirable to find ligands/natural products that can activate both PPARγ and PPARα. This study established that extracts from the alga *C. sorokiniana* contain activators for both PPARγ and PPARα. This has important implications for the mechanism-based understanding of the health benefits offered by this, as well as various other natural products. The results from this study would suggest that through appropriate dietary manipulations, lipid metabolism might be directly regulated by the fatty acid composition existing in *C. sorokiniana*. Further studies are required to evaluate whether the dual activation of PPARγ and PPARα by *C. sorokiniana* results in complementary or synergistic action on lipid metabolism and insulin sensitivity *in vivo*.

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