Isolation of Eicosapentaenoic Acid-Producing Fungi from Soil Based on Polymerase Chain Reaction Amplification

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Z. Naturforsch. **66c**, 429–433 (2011); received August 16, 2010/January 4, 2011

A method was developed for rapid screening and isolation of eicosapentaenoic acid (EPA)-producing soil fungi through polymerase chain reaction (PCR) amplification. Genes coding for Δ6 fatty acid desaturase and Δ5 fatty acid desaturase were used as molecular markers for screening these EPA-producing fungi from soil. Three out of 65 soil fungi gave positive results through PCR amplification. Two out of these three strains were found to produce EPA when they had grown in 80 ml potato/dextrose liquid medium at (25 ± 1) °C for 144 h. The EPA yields were 215.81 mg l^{-1} and 263.80 mg l^{-1}, respectively. The other positive strain was detected to produce arachidonic acid (AA). This study indicates that molecular detection of genes encoding Δ6 and Δ5 desaturases is an efficient method for primary screening of EPA- or its related polyunsaturated fatty acids (PuFAs)-producing fungi, which can improve the screening efficiency prominently.

Key words: Eicosapentaenoic Acid, Strain Screening, Soil Fungi

Introduction

The long-chain polyunsaturated fatty acids (LC-PuFAs) have various important physiological roles in human health and have been recognized as important dietary compounds (Kris-Etherton *et al*., 2000). Eicosapentaenoic acid (EPA, 20:5$\Delta^{5Z,8Z,11Z,14Z,17Z}$), in particular, has been shown to have highly beneficial effects in lowering the incidence of cardiovascular diseases and atherosclerosis (Jacobson, 2008; Manger *et al*., 2010), and antineoplastic (Pardini, 2006) and anti-inflammatory processes (Mullen *et al*., 2010). Regular consumption and an accordingly sustainable source of EPA are highly desirable.

The primary source of EPA supplements has been marine fish oil, which has the disadvantages of contamination, odour, and instability. In addition, the world's fish stocks are declining owing to overfishing and environmental pollution. As alternative sources, microbial fermentation has attracted much interest. Some fungi, algae, and bacteria were found to produce EPA, such as *Mortierella* (Shimizu *et al*., 1988), *Pythium irregulare* (Obrien *et al*., 1993), *Phaeodactylum tricornutum* (Wen and Chen, 2003), and *Shewanella pneumatophori* (Orikasa *et al*., 2009).

A range of methods for screening EPA-producing microorganisms have been used once the strain is isolated, such as thin-layer chromatography (TLC) (Cho and Mo, 1999), gas chromatography (GC) (Obrien *et al*., 1993), and gas chromatography-mass spectrophotometry (GC-MS) (Gentile *et al*., 2003). However, and while the methods are accurate, they require significant resources and time since each strain must be treated separately. Other methods, detecting enzyme functionality and growth at low temperatures, have been used to identify PuFA contents of fungi. Arachidonic acid (AA)-producing fungi were selectively isolated from soil at a low temperature of 4 °C, and the mycelia of these isolates were stained with triphenyltetrazolium chloride (TTC); the staining degree of mycelia increased when the AA content in mycelia lipids increased (Zhu *et al*., 2004). But the approach works only with the known microbial sources of PuFAs and requires relatively long growth periods.

Quick and simple approaches are needed for primary screening of EPA-producing microor-

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ganisms. Polymerase chain reaction (PCR)-based molecular markers have been successfully used to screen taxol-producing endophytic fungi from *Taxus* (Zhou *et al.*, 2007). In the present study, genes encoding Δ6 and Δ5 desaturases were used as molecular markers for screening EPA-producing soil fungi. EPA can be synthesized through n-6 and n-3 routes, and the substrates are linoleic acid and α-linolenic acid, respectively (Wu *et al.*, 2005). Both of them are first desaturated by Δ6 desaturase. The products of this desaturation, γ-linolenic acid ($18{:}3\Delta^{6Z,9Z,12Z}$) or stearidonic acid ($18{:}4\Delta^{6Z,9Z,12Z,15Z}$), are then converted by an elongase into di-homo-γ-linolenic acid ($20{:}3\Delta^{8Z,11Z,14Z}$) or $20{:}4\Delta^{8Z,11Z,14Z,17Z}$, individually, which are converted by Δ5 desaturase into AA and EPA, respectively (Hornung *et al.*, 2005). Thereafter AA can be converted into EPA by Δ17 desaturase (Shimizu *et al.*, 1989). Whether the n-6 or n-3 route are used, Δ6 desaturase and Δ5 desaturase are necessary for EPA biosynthesis. Strains with these two genes will be found through PCR amplification, suggesting that they may have the ability to produce EPA.

Material and Methods

Isolation of soil fungi

Soil samples were collected from the Yujia Mountain of Huazhong University of Science and Technology, Wuhan, Hubei Province, central China. One gram of soil sample was mixed with 1 ml sterile distilled water and serially diluted, and appropriate dilutions (10^{-5}) were plated on potato/dextrose agar (PDA) medium supplemented with chloramphenicol (100 mg l^{-1}) in Petri dishes. The plates were incubated at (28 ± 1) °C for 72 h (Mamatha *et al.*, 2010). Each fungal colony was checked for purity and then transferred to a fresh PDA plate.

Screening of EPA-producing fungi

Samples of fungi isolated as described above were inoculated individually into 250-ml Erlenmeyer flasks containing 60 ml potato/dextrose liquid medium. Cultures were incubated at (25 ± 1) °C with shaking at 180 rpm for 72 h and harvested by centrifugation at 10,000 × *g* for 5 min. Fresh mycelia a (0.5–1 g) was ground to powder in liquid nitrogen. Genomic DNA was extracted using the sodium dodecylsulfate-cetyltrimethylammonium bromide (SDS-CTAB) method (Kim *et al.*, 1990).

Based on the conserved sequence of the Δ6 desaturase gene (GenBank No. AF419296.1), primers D6-F (5'-GGGCTCTTCTACCAGCAGTCC-3') and D6-R (5'-GCACGAGCGGGAACAGGTGATG-3') were designed and synthesized. The primers D5-F (5'-GCAGGAGGTCGCCAAGCACAAC-3') and D5-R (5'-GACGTTCGTGTAGATGTGGTGG-3') were designed and synthesized according to the conserved sequence of the Δ5 desaturase gene (GenBank No. AJ510244.1). PCR amplification was performed in a PTC-100 Peltier thermal cycler (Bio-Rad, Hercules, CA, USA).

The fungal isolates were first screened by PCR for the presence of the Δ6 desaturase gene. PCR amplification was carried out with the primers D6-F and D6-R in 20 μl reaction mixture containing 1 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania), using a program of 5 min denaturation at 95 °C, 40 s at 94 °C, 40 s at 62 °C, and 1 min at 72 °C for 30 cycles, followed by extension for 10 min at 72 °C. The amplified DNA fragments were analysed through agarose gel electrophoresis, and those fungi which were PCR positive for the Δ6 desaturase gene were then screened for the gene encoding Δ5 desaturase. PCR amplification of the Δ5 desaturase gene was carried out using the primers D5-F and D5-R in 20 μl reaction mixture containing 1 U Taq DNA polymerase (Fermentas), using a program of 5 min denaturation at 95 °C, 40 s at 94 °C, 40 s at 61 °C, 1 min at 72 °C for 30 cycles, and terminated by 10 min at 72 °C. The amplified DNA fragments were analysed through agarose gel electrophoresis. The fungi which contained the Δ5 desaturase gene as well as the Δ6 desaturase gene were selected for determination of EPA.

Determination of EPA-producing fungi

The fungi registering positive for the genes encoding Δ6 and Δ5 desaturases were inoculated into 250-ml Erlenmeyer flasks containing 80 ml potato/dextrose liquid medium. Cultures were grown at (25 ± 1) °C for 144 h at 180 rpm. Fungal cells were harvested by suction filtration, washed with distilled water, and dried at 80 °C for 6–8 h. Total fatty acids were extracted and transmethylated as previously described (Cheng *et al.*, 1999). Qualitative analysis of fatty acid methyl esters (FAMEs)

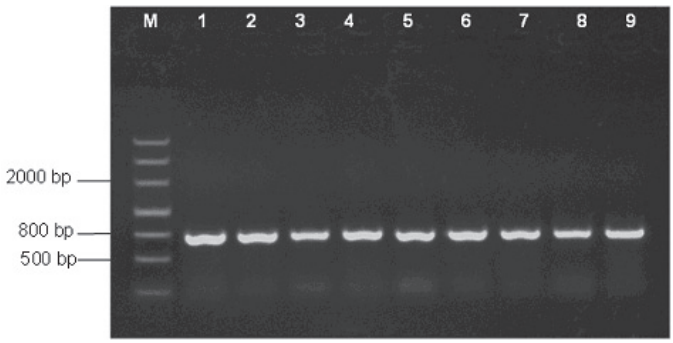

Fig. 1. PCR analysis for the presence of the Δ6 desaturase gene in fungi isolates. Lane M, DNA marker |||; lanes 1–9, PCR products of the Δ6 desaturase gene conserved sequence of 9 fungal isolates.

was conducted by GC-MS using an Agilent (Wilmington, USA) 7890A-5975C GC-MS network system. The EPA content was expressed as the weight % of total lipid. The EPA yield was calculated by measuring the dry biomass and lipid content.

Results and Discussion

A total of 65 fungal isolates separated from soil were screened for the presence of the Δ6 desaturase gene. Nine out of the 65 isolates had approximately 720-bp fragments of the conserved sequence of the Δ6 desaturase gene (Fig. 1).

The nine fungi containing the Δ6 desaturase gene were further screened for the presence of the Δ5 desaturase gene whose enzymic product catalyzes the next step for EPA biosynthesis (Hornung *et al.*, 2005). Three of the nine fungi had about 620-bp fragments of the Δ5 desaturase gene conserved sequence (Fig. 2).

The three possible EPA-producing fungi were designated RBB-2, RBB-5, and RBB-7. GC-MS analysis of mycelia lipids showed that both fungi, RBB-2 and RBB-5, could produce EPA with a content of 6.95% and 9.90% of total lipids, individually (Fig. 3). The dry biomass of RBB-2 and RBB-5 were 20.67 g l^{-1} and 20.52 g l^{-1}, and the yields of EPA were 215.81 mg l^{-1} and 263.80 mg l^{-1}, respectively. The third positive isolate RBB-7 was detected to produce AA.

Both Δ6 desaturase and Δ5 desaturase are essential for EPA biosynthesis through the n-6 or n-3 route, respectively (Hornung *et al.*, 2005). However, the fact that the isolate RBB-7, which contains the Δ6 and Δ5 desaturase genes, only produces AA, but cannot produce EPA, may be due to the low expression or low activity of Δ17 desaturase. The isolate RBB-7 cannot catalyze the conversion of AA into EPA. Another reason may be that the content of EPA was too low to be detected.

We also screened all 65 isolates for the presence of the Δ5 desaturase gene, and seven fungi, including RBB-2, RBB-5, and RBB-7, showed positive. The seven isolates were tested for EPA production, and one additional fungus, besides RBB-2 and RBB-5, was found to produce EPA. The reason for the failure of the PCR analysis to detect the Δ6 desaturase gene in this isolate may be that it has no Δ6 desaturase gene. This also indicates that some EPA-producing fungi may have pathways for EPA biosynthesis different from the usual pathway.

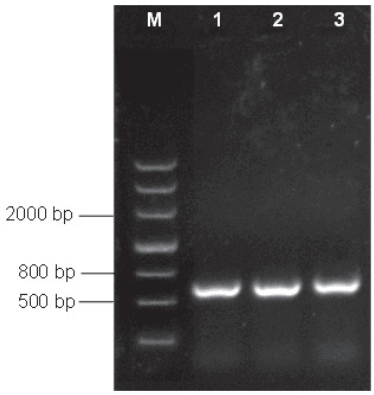

Fig. 2. PCR analysis for the presence of the Δ5 desaturase gene in fungi isolates of RBB-2, RBB-5, and RBB-7. Lane M, DNA marker |||; lanes 1–3, PCR products of the Δ5 desaturase gene conserved sequence of RBB-2, RBB-5, and RBB-7.

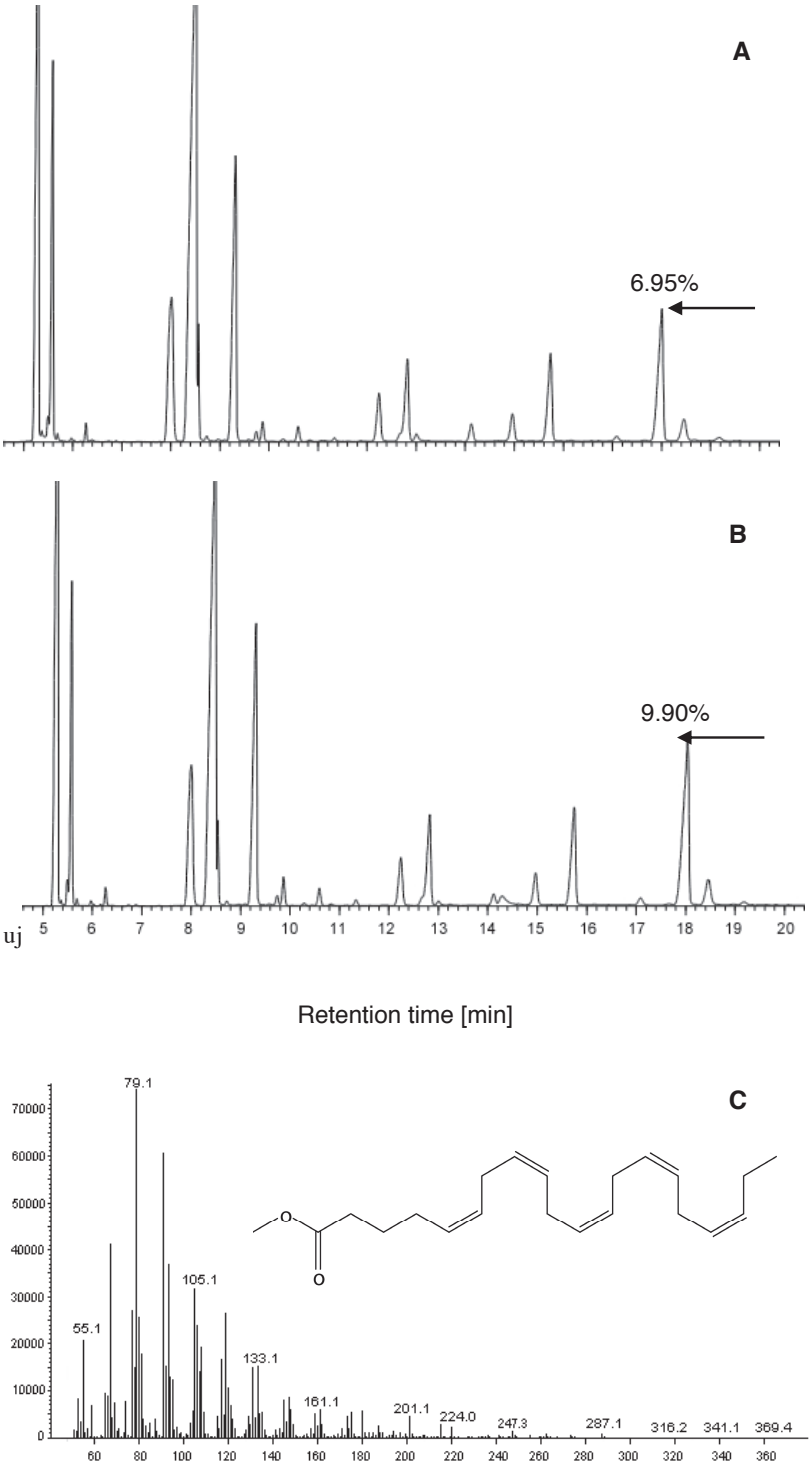

Fig. 3. GC-MS analysis of fatty acids in strains RBB-2 and RBB-5. The arrows indicate the peak of eicosapentaenoic acid (EPA). (A) EPA accounted for 6.95% of total fatty acids in strain RBB-2. (B) EPA accounted for 9.90% of total fatty acids in strain RBB-5. (C) The mass spectrum of the EPA methyl ester.

In this paper, a rapid and economic method has been developed to screen EPA- or its related PuFAs-producing fungi using the genes coding for Δ6 and Δ5 desaturases as molecular markers. This new approach significantly reduces the number of samples submitted to GC or GC-MS analysis. Therefore, it reduces the time, effort, and cost of screening EPA-producing fungi. The soil fungi RBB-2 and RBB-5 isolated here are thought to be promising resources for microbial EPA production.

Acknowledgement

This research is supported by National Natural Science Foundation (Grant No. 20776058) and National High-Tech Research and Development Plan (Grant No. 2007AA100402) of China. We thank Mrs. Xiaoman Gu and Hong Cheng of the Analysis and Testing Center of Huazhong University of Science and Technology, Wuhan, China for GC-MS analysis.

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